National Institute of Allergy and Infectious Diseases, NIH

Volume 3

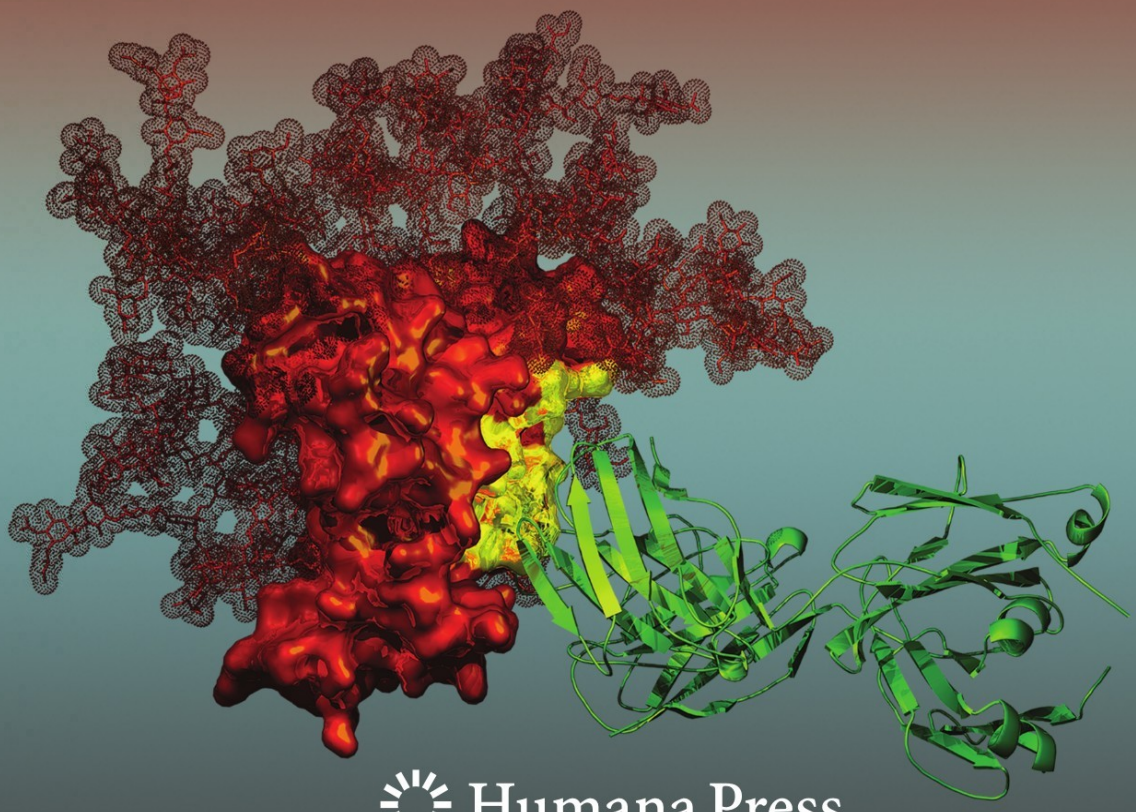
Intramural Research

Edited by

VASSIL ST. GEORGIEV, PHD

Foreword by

KATHRYN C. ZOON, PHD



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Intramural Research

Infectious Disease

Vassil St. Georgiev

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Foreword

The huge biomedical research enterprise that today is the National Institutes of Health traces its roots to 1887 and a small bacteriology lab on Staten Island. As they had been for centuries, infectious diseases were still the scourge of public health, and this lab marked the federal government's first efforts to study contagious diseases, hygiene, and public health. By the end of the 1930s, the lab had become the National Institute of Health and had relocated about 10 miles from the White House to Bethesda, Maryland. Since then, NIH has grown and been shaped by new health threats and new opportunities to address them. Today, NIH's 27 institutes, organized around threats (infections, cancer), opportunities (genomics), and anatomy (heart, lung, blood) conduct and support research in every area of biomedicine. In addition to Bethesda, many of these institutes have government laboratories in other regions of the USA and around the world.

These volumes represent the work of investigators in one of NIH's largest institutes, the National Institute of Allergy and Infectious Diseases (NIAID). As the infectious diseases institute, we claim that very first NIH lab as our own. Dr. Joseph Kinyoun directed the lab, then called the Hygienic Laboratory, from 1887 to 1899. His research in bacteriology was the corner stone for the NIAID programs today.

While the vast majority of NIAID's funding is used to support extramural research, approximately 10% of the budget supports NIAID's own scientists, the heirs of Dr. Kinyoun and so many others whose research informs our work today. Today NIAID's intramural researchers conduct basic, translational, and clinical research covering a broad spectrum of immunology, allergy, and infectious diseases. Many NIAID researchers study the causative agents, vectors, and pathogenesis of infectious diseases in human and animal hosts. Our immunologists' interests range from the basic mechanisms of immune cell signaling to antigen processing and vaccine development. NIAID clinician-researchers study AIDS, primary immune deficiencies, asthma and allergy, and many other diseases.

The accomplishments of NIAID researchers are numerous. For example, they discovered the Lyme disease bacterium, the Norwalk virus responsible for epidemic gastrointestinal disease, and the immunoregulatory cytokine, IL-4. They developed vaccines for hepatitis A and E and rotavirus, and are currently conducting more than 20 vaccine clinical trials. They defined the autoimmune lymphoproliferative syndrome and discovered its underlying genetic basis, discovered the mutations responsible for Job's syndrome, and developed therapeutic strategies sfor severe combined immunodeficiency and chronic granulomatous disease. And for 25 years, NIAID researchers have made many important discoveries that have elucidated the pathogenesis of AIDS.

Over the years, NIAID scientists have increasingly focused on research that takes advantage of the special attributes of the NIH intramural program. Most important among these is the stable, long-term funding that allows us to tackle the most difficult problems—for example, how to eradicate HIV from the body, safely deliver a therapeutic gene, or develop a malaria vaccine.

I am honored to lead the men and women whose work is represented here. And I know that the intractable problems of today will yield to their discoveries of tomorrow.

June 19, 2009

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Part I
Microbiology: Virology

Chapter 1
The Evolution of Gammaretrovirus Restriction Factors in the Mouse

Christine A. Kozak

1.1 Introduction

Mice exposed to infectious retroviruses are subject to virus-induced disease or virus-induced genetic mutations. While mice are protected by the innate and acquired immune systems, they have also evolved numerous constitutively expressed antiviral factors that target various stages of the retroviral life cycle, and these factors have been identified largely in studies with gammaretroviruses, specifically the mouse leukaemia viruses (MLVs). The factors responsible for this intrinsic immunity can block or interfere with different stages in the viral life cycle, namely virus entry, uncoating and reverse transcription, integration, assembly, or release. These host resistance factors can also select for the outgrowth of virus variants able to circumvent those blocks. These evolutionary pressures result in a ratchet-like pattern of sequential mutations in host and virus that, in the critical regions of the responsible genes, generate substantial polymorphism.

Although the inbred strains of laboratory mice have been especially useful in these studies, by providing genetically distinct but homogenous populations for testing, work in our laboratory and others over the last 40 years has identified many naturally occurring virus resistance factors that were not captured in inbred strains. Some populations of wild mice carry infectious viruses as well as germline DNA copies of viral genomes that mark past infections. For such populations, survival is dependent on the development of resistance, and novel resistance genes or novel variants of known resistance genes have been identified through analysis of many of the ~40 species of *Mus.* The characterization of such resistance factors has obvious importance for a broader understanding of how natural populations adapt to retrovirus infection, but there is also another reason for studying genetic factors that underlie resistance to mouse gammaretroviruses: these retroviruses are capable of transspecies transmission. The most well-documented example of transspecies transmission is, of course, the observation that HIV-1 is derived from simian lentivirus precursors, but there are also numerous examples of the transmission of mouse gammaretroviruses to other species. MLV-related viruses have been found in koalas and gibbon apes [1–3], and have been isolated from human with prostate cancer and chronic fatigue syndrome patients [4–6]. More examples will undoubtedly be uncovered. Examination of the co-evolution of interacting viral and host genes is thus important for an understanding of the natural history of infectious pathogenic gammaretroviruses in their murine hosts as well for providing a foundation for the study of epizoonotic infections.

Among the host cellular genes that control susceptibility to viruses, we are particularly interested in those that target the early stages in the viral replicative cycle, specifically the entry process and post-entry early events. I will discuss 3 genes whose antiviral roles have been characterized through use of wild mouse species: the *Xpr1* receptor, the *Rmcf2* interference gene, and the *Fv1* post-entry restriction factor (Fig. 1.1).

1.2 Host Factors that Suppress Retrovirus Entry

1.2.1 Receptor polymorphism: XPR1

1.2.1.1 Identification of receptor determinants in XPR1

The best-characterized mechanisms of resistance to mouse retroviruses rely on inhibition of virus entry. Chief among these mechanisms are polymorphic mutations in the cell surface receptor genes that alter the receptor-virus interaction. To date, six receptors for mouse gammaretroviruses have been identified; of the six, four are used by different host range subclasses of MLVs [7]. Two of these MLV receptors have naturally occurring variants associated with virus resistance: the CAT-1 receptor for the ecotropic MLVs (viruses that infect only mouse cells) and the XPR1 receptor for the xenotropic and polytropic MLVs (XMVs, PMVs) (viruses capable of infecting cells of non-rodent species).

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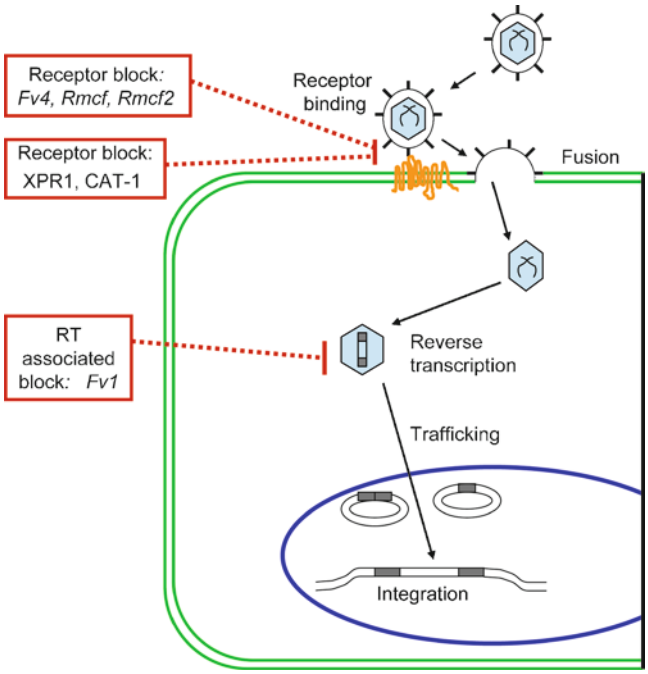
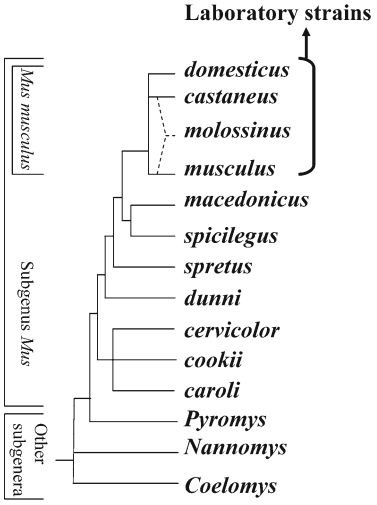


Fig. 1.1 Blocks to mouse gammaretrovirus replication by 3 types of host restriction factors in early stages of the viral lifecycle

Studies on these receptors have identified residues critical for virus entry and described 2 variants of CAT-1 and 4 of XPR1 that differ in their ability to mediate entry of different virus isolates [8, 9, 10, 12].

The XMV and PMV mouse virus subgroups were initially described as distinct host range groups based on their ability to infect mouse cells [13, 14, 15]. In the laboratory mouse genome, both PMVs and XMVs are present as endogenous retroviruses (ERVs) [16], but these provirus subtypes are generally restricted to different taxonomic groups of wild mouse species [17, 18] (Fig. 1.2). While a few XMV proviruses are nondefective and capable of producing infectious virus [22], infectious PMVs are generated only after recombination between endogenous defective PMVs and replicating mouse-tropic MLVs [23]. Also, XMVs are not known to be pathogenic in mice whereas PMVs are associated with neoplastic transformation and are cytopathic in mink lung cells [15]. The observed host range differences are due to sequence polymorphisms in both receptor and viral envelope genes.

Among mouse species, we have identified four functionally distinct allelic variants of the receptor gene *Xpr1*. These variants are found in laboratory mouse strains and European



Xpr1	X/PMV ERVs	*Fv4/Rmcf2*	*Fv1*
n	X/PMV	-/-	n,b,nr
n	PMV	-/-	nr,0,+
c	XMV	+/+	+
?	XMV	+/?	+
n,sxv	XMV	-/-	nr
?	?	?/?	+
sxv	-	-/-	0
sxv	(PMV)	-/-	0
sxv	-	-/-	0
sxv	-	-/-	+
sxv	-	-/-	+
sxv	-	-/-	0
sxv	-	-/-	0,-
sxv	-	-/-	m,s
p	-	-/-	-

Fig.1.2 Schematic representation of the evolution of *Mus*. This tree is based largely on the synthetic tree developed by others [19, 20]. The most recent node of the tree represents the house mouse *M. musculus* complex; laboratory mouse strains represent a mixture of these subspecies [21]. The indicated *Xpr1* alleles are based on observed susceptibility phenotypes. Mice that carry predominantly XMV and/or PMV ERV sequences are indicated; *M. spretus* carries a few PMV copies likely acquired through interactions with *M. m. domesticus*; laboratory strains have multiple copies of both ERV types. The *Fv4* and *Rmcf2* gene sequences are either present (+) or absent (-). For *Fv1*, multiple alleles are found in the various inbred strains or in different individuals of several taxa; some species lack the *Fv1* sequence (-), and some species have the sequence (+) but either have not been tested for restriction (*molossinus, castaneus*) or display novel *Fv1*-like restrictions (*cervicolor, cookii, domesticus*). "?" indicates no information is available.

Table 1.1 Susceptibility of mouse X/PMV gammaretroviruses on mouse cells and cells of other mammalian species

			Infectivity			
Cells	Species	Mouse *Xpr1* allele	PMV	CasE#1	Cz524	XMV
NIH 3T3	*M. m. domesticus*	$Xpr1^{n}$	++	-	-	-
M. dunni	*M. dunni*	$Xpr1^{sxv}$	++	++	++	++
M. pahari	*M. pahari*	$Xpr1^{p}$	-	++	-	++
NXPR-C	*M. m. castaneus*	$Xpr1^{c}$	-	-	-	+
293	*H. sapiens*		+	++	++	++
Mv-1-Lu	*Mustela vison* (mink)		++	++	++	++
Tu-1-Lu	*Tadarida brasiliensis* (bat)		-	++	++	++
MDCK	*Canis familiaris* (dog)		-	-	+	++
E36	*Cricetulus griseus* (Chinese hamster)		-	-	-	-

Relative infectivity was measured by focus formation on an indicator cell line, mink S+L-cells. Subconfluent cultures of each cell type were infected with virus dilutions in the presence of polybrene. Four days later, cells were irradiated and overlaid with mink S+L-cells [10, 26]. Clusters of infected cells were identified by the formation of foci. Log_{10} virus titer: +, 0-1; ++, >2.

Table 1.2 Virus susceptibility of Chinese hamster cells expressing different XPR1 receptors

			Infectivity		
Xpr1 Receptor	ECL3	ECL4	PMV	XMV	CasE#1
$Xpr1^{n}$	$Xpr1^{n}$	$Xpr1^{n}$	++	-	-
$Xpr1^{p}$	$Xpr1^{p}$	$Xpr1^{p}$	-	++	++
ECL3-1 mutant	$Xpr1^{n}$-E500K	$Xpr1^{n}$	++	++	-
ECL4-1 mutant	$Xpr1^{n}$	$Xpr1^{n}$-Δ392T	++	++	+
Pah3 chimera	$Xpr1^{p}$	$Xpr1^{n}$	-	-	-
Pah4 chimera	$Xpr1^{n}$	$Xpr1^{p}$	++	++	+
Pah3/4 chimera	$Xpr1^{p}$	$Xpr1^{p}$	-	++	++

Xpr1 receptor genes cloned from NIH 3T3 cells ($Xpr1^{n}$) and *M. pahari* cells ($Xpr1^{p}$) were expressed in hamster cells that are not infectable by X/PMV gammaretroviruses. The indicated mutations were made in $Xpr1^{n}$, and the three chimeras have the ECL3 and/or ECL4 segments of the *M. pahari* gene. Virus susceptibility was tested using LacZ pseudotypes, infectious viral particles carrying the LacZ reporter gene. Log_{10} virus titer: +, 0-1; ++, >2.

M. m. domesticus ($Xpr1^{n}$), in the Asian species *M. m. castaneus* ($Xpr1^{c}$) *(21)*, in the Asian species *M. pahari* ($Xpr1^{p}$) *(8)*, and in other Asian species ($Xpr1^{sxv}$) *(22)* (Table 1.1, Fig. 1.2). These variants are defined by their differential susceptibility to prototype XMV and PMV viruses as well as to two novel MLV isolates from wild mice, CasE#1 and Cz524, that by sequence and biological properties cannot be clearly grouped with either the XMV or PMV subtypes. These various X/PMV MLVs can also infect cells of other mammalian species, and the observed susceptibility differences suggest there are additional functional variants of the XPR1 receptor (Table 1.1) [27].

The XPR1 receptor is a transmembrane protein likely to be involved in signal transduction and phosphate transport [28, 29, 30]. XPR1 has 8 predicted transmembrane domains and shows greatest sequence divergence in its fourth extracellular loop, ECL4. Sequence comparisons and mutagenesis suggested that there are two major receptor determinants that independently mediate virus entry. These determinants are found in ECL3 and ECL4, and two critical amino acids have been identified for XMV entry, K500 in ECL3, and T582 in ECL4 [12, 31].

We generated chimeras between molecular clones of the functionally distinct $Xpr1^{n}$ *and* $Xpr1^{p}$ genes and also made specific changes in $Xpr1^{n}$ by mutagenesis [10, 11] (Table 1.2). Chinese hamster cells expressing these variants were tested for virus susceptibility. Introduction of the ECL3 substitution E500K or the ECL4 Δ582T mutation into the laboratory mouse $Xpr1^{n}$ receptor generates a functional XMV receptor. However, these two mutant receptors are not functionally equivalent; the Δ582T mutation also generates a receptor for CasE#1, but the E500K mutation does not.

Functional analysis of the XPR1 chimeras suggests that additional XPR1 residues are needed for entry of both XMV and PMV. For XMV, the involvement of other residues is suggested by the fact that the Pah3-chimera does not mediate XMV entry, despite the fact that it has K500. For PMV, infectivity is associated with the presence of ECL3 of $Xpr1^{n}$; while the critical residues have not yet been identified, PMV entry is not mediated by the K/E500 XMV determinant. The ECL3 segment of *Xpr1* carrying the PMV entry determinant is large (88 amino acids) with significant sequence variation at both ends, particularly near the XMV receptor determinant K500. Ongoing mutagenesis studies are focused on these sites, particularly substitutions that introduce or eliminate sites for N-linked glycosylation, as glycosylation sites are frequently in close proximity to virus receptor sites and can have a regulatory role in receptor function [32].

This [specify what analysis] analysis also suggests that ECL3 and ECL4 may both contribute to a single virus binding site rather than specifying separate entry determinants.

The Pah4 chimera with the *Xpr1*^{p} ECL4 is an efficient receptor for XMV but is less efficient for CasE#1 (Table 1.2). The presence of ECL3 together with ECL4 in the Pah3/4 chimera generates a receptor that is very efficient for both viruses. All of the known gammaretrovirus receptors have multiple transmembrane domains, and the suggestion that residues in two XPR1 loops may be needed for receptor function is consistent with the observation that many other receptors require multiple domains for receptor function [33].

1.2.1.2 Co-evolution of the XPR1 receptor and X/PMV Env variants

Viruses have evolved mechanisms to evade entry restrictions due to receptor variation. The major determinant of receptor recognition (and thus virus host range) in MLVs is within the variable receptor binding domain of the surface (SU) subunit of the Env protein [34]. Mutations within this region can produce retroviruses able to use alternative receptors, multiple receptors, or even multiple receptor determinants on the same protein; this ability to alter receptor usage contributes to virus survival by limiting the impact of host escape mutations.

The 4 functionally distinct mouse XPR1 receptors were identified in specific taxonomic groups of wild mice species (Fig. 1.2). To shed light on the adaptive co-evolution of receptor and virus, the distribution of these 4 receptor phenotypes can be examined in relation to the appearance and spread of the X/PMV viruses in these populations as documented by the presence of related ERVs [16, 17, 32]. The XPR1 variant with the broadest susceptibility phenotype is widely distributed among the older Asian *Mus* species, species that lack X/PMV ERVs (Fig. 1.2) [25]. This broad distribution of *Xpr1*^{sxv} suggests a long period of stasis that ended with the appearance of the house mouse (*M. musculus*) about 1 MYA at which time mice were exposed to XMVs, and began to acquire copies of XMV ERVs (Fig. 1.2). This period is also coincident with the appearance of restrictive *Xpr1* variants in *M. m. castaneus* (*Xpr1*^{c}) and *M. m. domesticus* (*Xpr1*^{n}). It is likely that the mutations restricting *Xpr1* receptor function in these mice initially provided a survival advantage in the face of endemic gammaretrovirus infection but may have also resulted in the selection of viral variants with altered receptor specificities. For the European *M. m. domesticus* mice, like the laboratory strains they gave rise to [21], the *Xpr1*^{n} mutation may have driven the evolution of the PMV host range variant that targets this novel XPR1. The appearance of this *Xpr1*^{n} variant [specify which variant] also accounts for the multiple PMV ERVs found in these mice [25]. For the X/PMV gammaretroviruses, the co-evolution of virus and its cellular receptor has clearly resulted in exceptional sequence diversity and functional plasticity.

1.2.2 Interference Mechanisms: Rmcf2

A second group of receptor-mediated MLV resistance genes functions through an interference mechanism. These genes represent ERVs that encode Env glycoproteins that have been co-opted by the host to block exogenous MLV infection (Fig. 1.3). The best-characterized of these genes is *Fv4*. The *Fv4* gene produces an ecotropic Env glycoprotein that is thought to interfere with MLV binding to the CAT-1 receptor [37]. This proviral gene ["this" has an unclear reference, specify what "this" refers to] also has a mutation in the fusion peptide of its transmembrane domain; virions that are produced in *Fv4*^{r} cells incorporate this glycoprotein and are fusion defective [38]. Studies on another gene, the *Rmcf* resistance locus of DBA/2 mice [39], suggest that an analogous interference mechanism may be responsible for *Rmcf*-mediated resistance to PMVs. This suggestion was based on the observation that cells of these mice express a unique cell surface PMV Env glycoprotein [40] and are resistant to PMV-induced disease [41, 42]. We identified a provirus linked to the *Rmcf* resistance gene and confirmed its association with resistance [36].

Among wild mouse populations, the Asian mouse species *M. m. castaneus* is of particular interest because it carries infectious MLVs of ecotropic and xenotropic host range. Examination of laboratory colonies of these mice indicates that these animals are protected from the deleterious effects of MLV infection. In addition to carrying the *Fv4* ecotropic

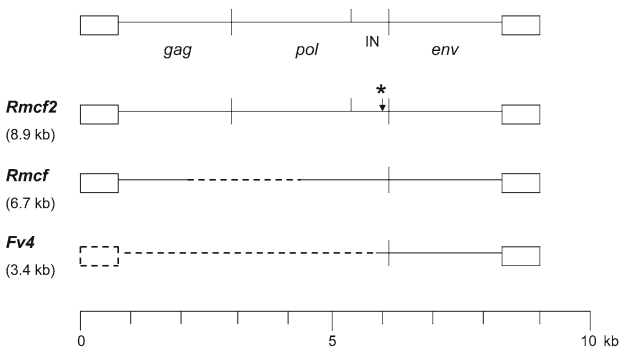


Fig. 1.3 Structures of three gammaretrovirus ERVs that are associated with virus resistance. At the top is shown the general structure of a mouse gammaretrovirus with relative positions for the long terminal repeats (boxes), and the *gag, pol* and *env* genes along with the *pol* IN gene. All three interfering ERVs have different defects preventing production of infectious virus, but all have intact *env* genes. *Rmcf2* contains a termination codon (*) in IN [35]. *Rmcf* has a deletion spanning *gag* and *pol* [36]. *Fv4* is a truncated provirus that relies on a cellular promoter [37]

MLV resistance gene and an XPR1 receptor that blocks infection of the more pathogenic X/PMV subtypes, this mouse contains an additional factor responsible for resistance to PMVs [43]. This resistance correlates with the expression of cell surface nonecotropic Env glycoproteins [43], suggesting the presence of an interfering *Fv4*-like resistance gene. This resistance to PMV infection was shown to be controlled by a single gene, unlinked to *Xpr1*. The locus for this gene, *Rmcf2*, contains an endogenous XMV with a functional *env* gene [35] (Fig. 1.3).

The identification of three mouse genes that function through interference (*Fv4*, *Rmcf*, *Rmcf2*) (Fig. 1.3) suggests that co-opting proviral *env* genes may actually be a relatively common defense strategy in populations exposed to endemic infections. Such genes have long been recognized in chickens [44] and cats [45] and have now been identified in sheep [46]. This type of resistance gene is rare in laboratory mouse strains, but it is instructive that *Rmcf2* was found along with *Fv4* in *M. m. castaneus,* the only wild mouse species known to harbor infectious ecotropic and xenotropic MLVs [22, 47] and clearly in need of such survival strategies to mitigate the consequences of infection. This mouse is protected from the disease inducing properties of the viruses it carries on 3 fronts: *Fv4* protects it from ecotropic MLVs and probably originated in this species [17, 48, 49]; protection against nonecotropic MLVs is provided by its variant *Xpr1* receptor and by *Rmcf2*.

It is not clear whether all 3 of the protective antiviral genes (*Fv4, Xpr1*^{c}*, Rmcf2*) found in *M. m. castaneus* are widespread among other Asian wild mouse populations. *M. m. castaneus* is a SE Asian subspecies of house (commensal) mice and is interfertile with other species of commensal mice that are found in western Europe (*domesticus*), eastern Europe and northern Asia (*musculus*), and Japan (*molossinus*). These species undergo genetic exchanges at the borders of their geographic ranges. The *Fv4* gene of *M. castaneus* is fairly widely dispersed among Asian commensal mice; *Fv4* has been found in all *M. m. castaneus* mice tested as well as in mice from hybrid zones in Japan and Korea and in mice identified as *M. m. bactrianus* trapped in Pakistan [48]. It is not known if the two genes conferring resistance to nonecotropic MLVs, *Rmcf2 and Xpr1*^{c}, are also found in other Asian commensal mice. However, infectious XMVs are readily isolated from *M. m. castaneus* and *M. m. molossinus* mice [47], and large numbers of XMV ERVs are found in *M.m. castaneus* and in *M. m. musculus* trapped at the western end of its range in central Europe (Fig. 1.2) [17]. For mice such as these that are exposed to endemic infection and/or contact with virus infected populations, survival is dependent on the development of resistance, and studies on the *Fv1* restriction gene suggests that such strongly selected genes are likely to be more widely dispersed than predicted by the observable but limited genetic exchanges [50].

1.3 Host Factors that Restrict Early Post-Entry Stages of Replication: *Fv1*

1.3.1 *Novel Fv1-like restrictions in Mus Species*

Host genes identified in multiple mammalian species can interfere with early post-entry and pre-integration stages of the retrovirus life cycle. These genes include *Fv1*, APOBEC3, and TRIM5α. *Fv1* was the first of these genes to be identified and is found only in mice. Alleles at the *Fv1* locus, control the relative sensitivities of mouse cells to different subgroups of MLVs [51]. Four allelic variants of *Fv1* have been described in studies on laboratory mice, 3 of which produce different patterns of resistance to the N-, B-, or NR-tropic subgroups of mouse-tropic viruses. This resistance produces a 100-1000 fold reduction in virus titer [52]. The N-tropic viruses replicate best in cells with the *Fv1*^{n} allele, B-tropic viruses replicate best in *Fv1*^{b} cells, and NB-tropic viruses grow equally well in both cell types. A third restriction allele, termed *Fv1*^{nr}, restricts susceptibility to B-tropic viruses as well as to some but not all N-tropic viruses [53, 54]. A null, non-restrictive allele, *Fv1*^{o}, has been identified in cell lines derived from wild mouse species [53, 55, 56]. There is also evidence for an additional *Fv1* variant in DBA/2 strain mice that has less dramatic effects on virus replication [57].

The sequence of the *Fv1* gene is related to the *gag* gene of the MuERV-L family of ERVs [58]. The mechanism of restriction is unknown, but *Fv1* generally blocks virus replication at or just after reverse transcription to limit proviral integrations (Fig. 1.1) [59]. *Fv1* targets the viral capsid (CA); the major determinant that distinguishes N-and B-tropic viruses is at CA position 110 [57], but other targets in this same CA region have been identified in studies on NR-and NB-tropism [54, 60]. Interestingly, the primate HIV-1 restriction gene, TRIM5α, also restricts MLVs by targeting amino acids at two of the CA sites also targeted by *Fv1*, 110 and 117 [61]. TRIM5α may thus function to limit transpecies transmission by the broadly infectious subgroups of MLVs. The evolution of two polymorphic host genes (*Fv1* and TRIM5α) targeting the same hypervariable capsid segment also suggests that, like the viral Env and host receptor genes, virus capsid and host genes that target capsid represent a major battleground in the co-evolution of virus and host.

While various strains of inbred laboratory mice were found to carry different *Fv1* restriction alleles, two cell lines developed from wild-derived mice showed no *Fv1*-type restriction at all: *M. dunni* cells from the Asian mouse species *M. terricolor*, and the SC-1 cell line developed from a mouse trapped in California [55, 56]. To screen for additional variation in wild mouse species, I tested cells of 13 species and subspecies of wild-derived mice for

susceptibility to a panel of viruses that define known *Fv1* alleles [53]. Surprisingly, none of the species tested showed the $Fv1^n$ or $Fv1^b$ patterns of restriction that are most common among inbred strains of mice. However, some *M. musculus* mice from Europe and the U.S. showed an $Fv1^{nr}$-like restriction phenotype. Most mice tested, especially species outside the *M. musculus* house mouse group (Fig. 1.2), were generally equally susceptible to all of the viruses, and linkage tests confirmed that this null allele mapped at or near *Fv1* [53].

This early study also hinted at the existence of other *Fv1*-like phenotypes in some *Mus* species and provided the impetus to survey additional species from all 4 subgenera of *Mus*, namely *Coelomys, Nannomys, Pyromys,* and *Mus* [62]. Cells from two species of African pygmy mice of the subgenus *Nannomys*, showed unusual patterns of MLV resistance. These cells, from *M. minutoides* and *M. setulosus*, were susceptible to laboratory-derived Moloney ecotropic MLVs but were resistant to the AKV-type MLVs found in many laboratory mice. The block to replication in these cells, like that of *Fv1*, occurs post-entry and before integration, and infection of these cells with viral chimeras constructed between AKV and MoMLV showed that this restriction targets a segment of the virus CA that also contains the *Fv1* target sequence [62].

In order to determine if this restriction was a novel allelic variant of *Fv1*, we cloned *Fv1* from the two *Nannomys* species [63]. The genes were about 87% identical to the laboratory mouse *Fv1* genes. Interestingly, these sequence differences included substitutions at positions 352, 358, 399, the three sites in the *Fv1* ORF that distinguish the *n, b*, and *nr* alleles of *Fv1* and have been shown to mediate restriction [60, 64].

Expression of the *M. minutoides Fv1* allele, $Fv1^m$, *in* $Fv1^0$ *M. dunni* cells reproduced the *M. minutoides* pattern of MLV restriction [63]. The observation that the *Fv1* genes of two species of *Nannomys* have novel *Fv1*-mediated antiviral phenotypes suggests that *Fv1* may have had antiviral function 7 MYA, when the 4 *Mus* subgenera diverged.

1.3.2 Positive Darwinian Selection of Fv1 in Species of Mus

Analyses based on evolutionary genomics have been useful in the characterization of genes involved in host-pathogen interactions. Genes involved in such antagonistic interactions with pathogens can be identified by sequence comparisons that reveal evidence of positive selection as shown by the rapid fixation of amino acid replacements. For this type of analysis, comparisons are made between the rate of fixation of nonsynonymous mutations (those that change the amino acid) and synonymous mutations (those that do not change the amino acid). For most genes, the number of synonymous substitutions exceeds the number of nonsynonymous substitutions, since the latter can be deleterious and are removed by purifying selection. Describing the phylogenetic history of such replacements can identify lineages marked by these genetic conflicts, and can also define the protein regions involved. This pattern of positive selection has been observed for genes where co-evolving pathogens select for repeated changes in the residues involved in host-pathogen interactions.

To determine if *Fv1* has had an antiviral role throughout *Mus* evolution, and to identify possible sites of virus interaction, we analyzed 22 sequenced *Fv1* genes from various *Mus* species [63]. The 22 *Fv1* genes were aligned and used to construct a maximum parsimony tree. Using the suite of programs in PAML, we then determined the ratio of nonsynonymous to synonymous substitutions along each branch of the tree [65]. Likelihood ratio tests showed that *Fv1* has a significant probability of having experienced positive selection. The Bayes empirical Bayes calculation of posterior probabilities [66] identified 6 specific *Fv1* codon positions as having significant probability of positive selection with posterior probability greater than 0.99 (Fig. 1.4), the generally accepted criterion for strong positive selection.

The 6 codons identified as being under positive selection were grouped in 2 sets of 3 codons. The first set of 3 codons, at positions 261, 265, and 270, clustered at the 5′ end of the major homology region (MHR), a highly conserved region known to be involved in CA interactions [67, 68, 69, 70, 71]. Mutations of codons in and around the MHR regions of various retroviruses disrupt virus assembly, maturation, and infectivity [69, 70, 71].

Mutational analysis in and around the MHR of Moloney MLV and HIV-1 determined that the segment identified in our analysis is critical for formation of the high-affinity capsid interface and that this segment determines the specificity of heterodimeric interactions [67, 69]. These interactions are important for virus assembly, but they are important in the *Fv1* sensitive early stages of replication, as the majority of replication defective MoMLV CA mutants are blocked before reverse transcription [68]. The mechanism of *Fv1* restriction has not been elucidated, but our finding that the CA-like MHR region of *Fv1* involved in dimerization is under positive selection suggests that *Fv1* may bind capsids of exogenous virus and interfere with capsid disassembly and reverse transcription. While studies on *Fv1* have not produced direct evidence for Fv1-capsid binding, functional analysis of mutants within this MHR associated region of *Fv1* demonstrate this region is critical for *Fv1* restriction [64].

The second group of three codons identified as being under positive selection is in the *C*-terminal *Fv1* region at positions 352, 399, and 401. F/S352 has been shown to be responsible for the $Fv1^n$ and $Fv1^{nr}$ restriction types [60],

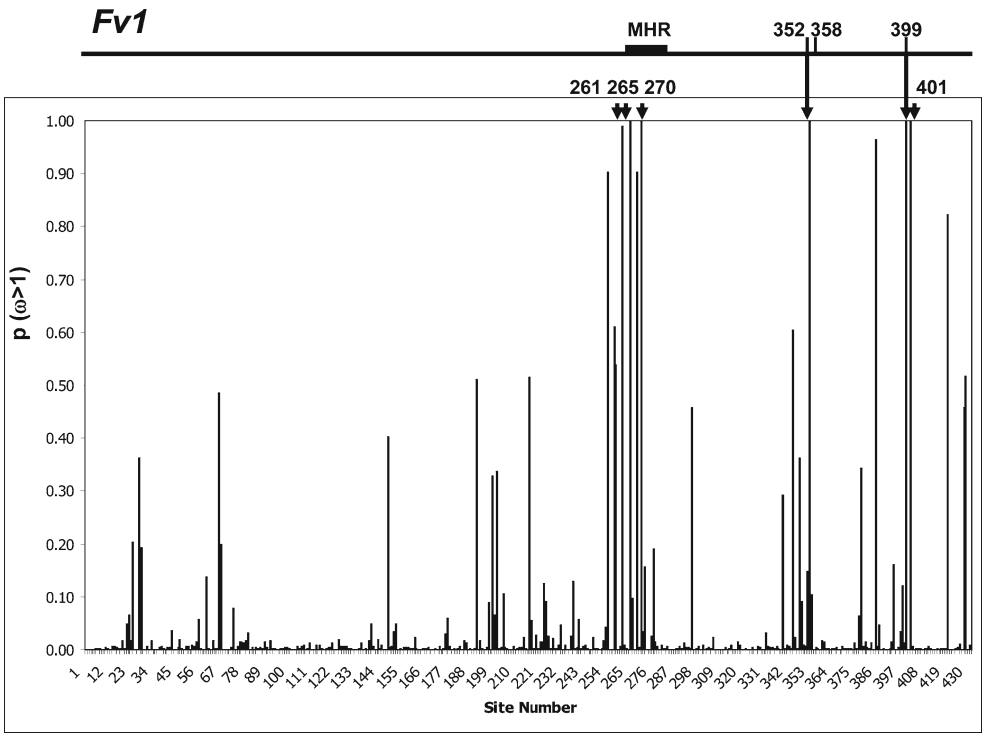


Fig. 1.4 *Fv1* sites that have been subject to positive selection. At the top is a schematic representation of the *Fv1* gene that indicates the location of the MHR (black box) and the three codon sites (352, 358, 399) that distinguish the laboratory mouse restrictive alleles $Fv1^n$, $Fv1^b$ and $Fv1^{nr}$; all three of these codons have been associated with restriction *(57,63)*. The graph indicates the posterior probability of positive selection at each of the codon sites in the *Fv1* gene; at the top, arrows indicate the 6 codons under strong positive selection (p>0.99)

and substitutions at codon 399 alter restriction specificity [64]. Our analysis also identified positive selection at a position in this region not previously known to affect restriction, 401; its role in restriction may be related to its proximity to 399, and this role should be defined by mutational analysis. Substitutions at a fourth codon in this region of the *C*-terminal domain, 358, distinguish $Fv1^b$ from $Fv1^n$ and $Fv1^{nr}$ and have been implicated in restriction [64]; however, this codon was not identified as being under positive selection. This site, in fact, was highly invariant; all *Fv1* genes except for $Fv1^b$ have K at this site suggesting that the laboratory mouse $Fv1^b$ K358E variant arose fairly recently in *Mus* evolution. Lack of sequence variation at this site suggests that the only known substitution at the 358 codon identified to date, in the $Fv1^b$ laboratory mouse strains, likely emerged very recently in *Mus* evolution. This variant may have appeared in response to the emergence of the N-tropic MLV it restricts.

These results indicate that the *Fv1* gene has been subjected to positive selection over nearly 7 MY of *Mus* evolution. This selection involves codons known to be responsible for restriction of MLVs in laboratory mice, suggesting that these same codons may mediate antiviral activity in more evolutionarily divergent species and subgenera. This antiviral role for *Fv1* is supported by the additional finding described above, which indicates that the *Fv1* genes of two species of *Nannomys* are responsible for novel antiviral phenotypes.

The exposure of mice to retroviral infection is marked by the generation of related ERVs, but the evidence for an antiviral evolutionary history of *Fv1* in *Mus* significantly predates the acquisition of ERVs of MLVs, the only viruses known to be restricted by *Fv1* (Fig. 1.2). As noted earlier, ERVs related to the MLVs known to be restricted by *Fv1* appeared in the *Mus* germline only about 1 MYA, with the appearance of house mouse *M. musculus* complex. X/PMV ERVs are found in all laboratory strains and are segregated in *M. musculus* subspecies, while endogenous ecotropic MLVs are only found in the Asian subspecies [17] (Fig. 1.2). The fact that our evolutionary and functional analysis of *Fv1* in the various species and subgenera of *Mus* uncovered evidence of antiviral activity in MLV-free species suggests that *Fv1* may have broader antiretroviral activity than previously appreciated or that these mice may harbour novel MLV-like virus variants.

1.4 Conclusions

Studies on the various *Mus* species have provided a window into the natural history of infectious MLV gammaretroviruses and their mouse hosts. In particular, analysis of evolutionarily divergent *Mus* species, especially Asian species that harbour

infectious viruses, which have lead to the identification of several novel genes and several novel variants of known genes that provide protective antiviral functions for their host. Two observations emerge from this work: First, co-evolutionary pressures at the points of interaction between host and virus have forced the rapid modification of these interacting genes, as illustrated here for the Fv1-capsid interactions and for the host cell surface receptor-viral Env interactions. Detection of this positive selection can identify genes with antiviral function, identify the points of virus-host interaction, and help to elucidate the mechanisms responsible for restriction. Second, ERVs derived from past infections and fixed in the *Mus* germline can contribute novel protein coding sequences to the host genome that can then be co-opted for cellular functions. Such genes have played an important role in shaping host-virus interactions, and, in fact, the most easily recognized of these domesticated ERVs are those, like *Rmcf2* and *Fv1*, that have antiviral functions. Further work on these elements should produce additional insights into their evolutionary past and refine our understanding of the host-virus interaction during infection, as well as provide useful information for dealing with emerging transspecies transmissions.

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Chapter 2
Herpesvirus Research at the National Institute of Allergy and Infectious Diseases: Thirty Years of Progress

Jeffrey I. Cohen

The year 2009 marks the 30th year anniversary of Dr. Stephen Straus's arrival to head the Medical Virology Section of the Laboratory of Clinical Investigation at the National Institute of Allergy and Infectious Diseases. Dr. Straus initiated basic laboratory and clinical studies on herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus. The Medical Virology Section (currently in the Laboratory of Clinical Infectious Diseases) continues to conduct studies of human herpesviruses, including bench-to-bedside and bedside-to-bench translational research involving these viruses. This chapter provides an overview of studies from the Medical Virology Section during the past 30 years.

2.1 Studies of Herpes Simplex Virus

Herpes simplex virus 1 (HSV-1) and HSV-2 cause several diseases. In otherwise healthy individuals, HSV-1 causes oral lesions (gingivostomatitis), ocular disease (keratoconjunctivitis), skin lesions (cutaneous herpes), and less commonly genital herpes, neonatal herpes, or herpes encephalitis [1]. HSV-2 causes genital herpes and neonatal herpes; less commonly HSV-2 causes aseptic meningitis, oral, or ocular disease.

Immunocompromised persons can develop visceral disease due to HSV-1 or HSV-2 involving the gastrointestinal tract (esophagitis, hepatitis), eye (retinitis), or the lungs (pneumonitis). Approximately 50% to 70% of adults in the United States are infected with HSV-1, and about 20% to 50% of adults are infected with HSV-2. HSV-1 and HSV-2 establish latency in cranial and sensory nerve ganglia. After primary infection, HSV-1 and HSV-2 can recur resulting in frequent episodes of cold sores or genital herpes, respectively.

2.1.1 Entry of HSV into Cells

Previous studies have shown that HSV enters cells by fusion of the viral envelope with the host cell cytoplasmic membrane at the surface of the cell. Nicola and colleagues [2] first showed that HSV can enter cells by endocytosis and exposure to low pH. HSV was found to enter certain cells using the endocytosis pathway, while the virus entered other cells by fusion at the plasma membrane on the surface of the cell.

In a follow-up study, Nicola et al., [3] found that HSV enters keratinocytes, the cells on the surface of the skin and mucosa in which the virus replicates, by endocytosis. In contrast, the virus enters neurons, the site of latent infection, by a pathway that does not involve endocytosis. Thus, HSV uses different pathways during infection of humans.

2.1.2 HSV Latency

Initial studies in mice showed that latently infected neurons express a single transcript. Croen and colleagues [4] showed that human trigeminal ganglia express the same transcript detected in mice referred to as LAT (Fig. 2.1).

LAT, which overlaps another HSV gene (ICP0) in the viral genome, was detected in 67% of human trigeminal ganglia. In contrast, the ICP0 gene was not expressed in any of the latently infected ganglia. The function of the LAT gene has been unclear. Krause and colleagues [5] constructed a mutant HSV-2, which was unable to express LAT. The HSV-2 mutant replicated in cell culture, caused acute genital herpes, and established latency in guinea pig ganglia at similar levels as wild-type virus; however, the mutant was impaired for reactivation in guinea pigs (Fig. 2.2).

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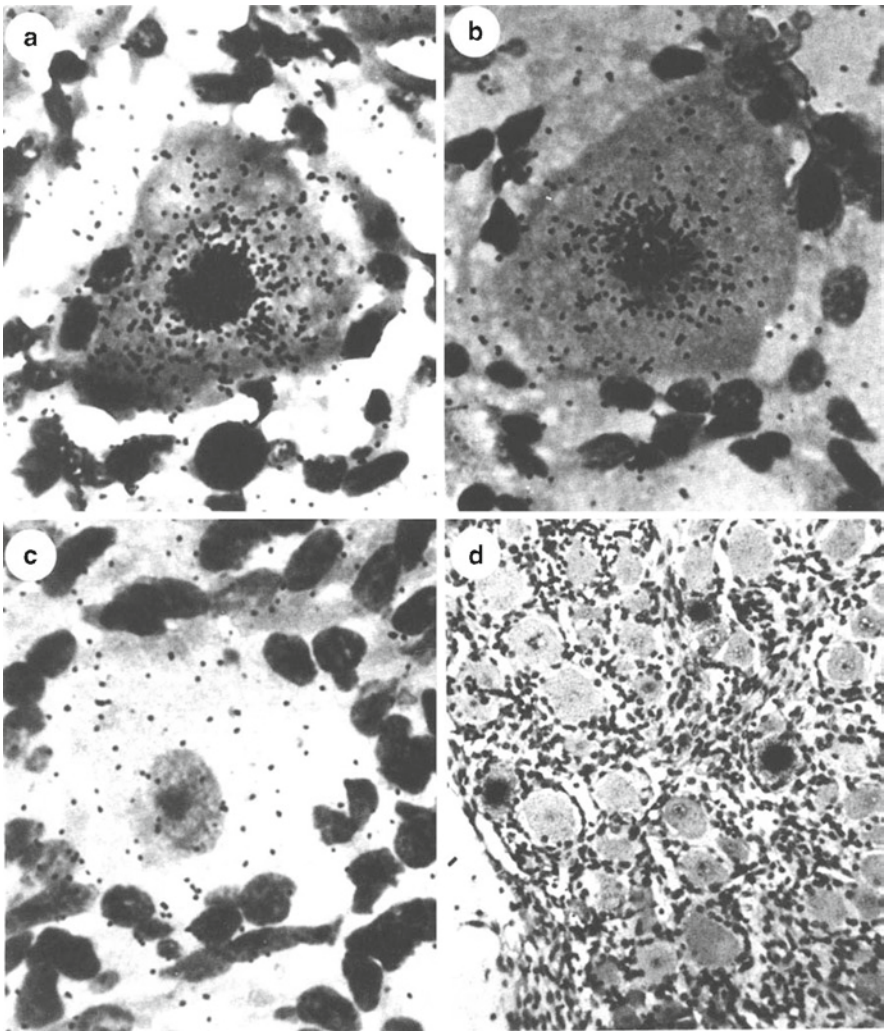

Fig. 2.1 In situ hybridization shows that HSV-1 LAT is expressed in human trigeminal ganglia (**a**,**b**,**d**), but HSV-1 ICP27 is not expressed in ganglia (**c**). (Reproduced with permission from *The New England Journal of Medicine*)

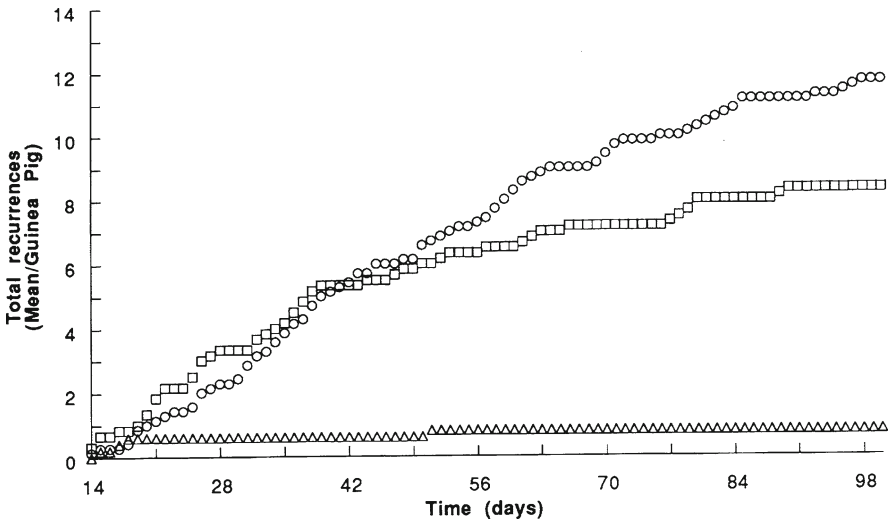

Fig. 2.2 Recurrences of genital herpes in guinea pigs infected with wild type HSV-2 (open circles), HSV-2 unable to express LAT (open triangles), and virus in which the LAT mutation has been repaired (open squares). (Reproduced with permission from *The Journal of Experimental Medicine*)

These experiments indicate that HSV-2 LAT has an important role in the ability of the virus to cause recurrent lesions.

The cell type in which HSV-1 is latent and the number of copies of latent HSV in human ganglia have been controversial. Wang et al., [6] used laser capture microdissection to remove individual cells from ganglia on microscope slides and performed PCR to determine the number of copies of HSV DNA in each cell. A median of 11 copies of HSV-1 DNA were present in virus-positive neurons and viral DNA was very rarely detected in non-neuronal cells. From 2% to 11% of neurons contained HSV-1 DNA. Most virus-infected neurons did not express the HSV-1 LAT. These results indicate that HSV-1 is latent exclusively in neurons but that not all latently infected neurons express LAT.

Hoshino and colleagues [7] studied the immune response to HSV and showed that reactivation of HSV-2 in mice correlates directly with the amount of viral DNA in latently infected ganglia. HSV-2 specific $CD8^+$ T-cells were detected in latently infected ganglia. When $CD8^+$ T-cells were removed from the ganglia and added back to virus-infected ganglia cells *in vitro,* the T cells were able to reduce reactivation of virus compared with virus-infected ganglia cells alone. Thus, $CD8^+$ T-cells have an important role in reducing reactivation of HSV-2.

2.1.3 Symptomatic Versus Asymptomatic Infection with HSV

Some patients infected with HSV are asymptomatic and never know that they have been infected, while others have frequent recurrences with lesions. Rooney and colleagues [8] showed that genital herpes can be transmitted by virus shedding from an asymptomatic partner to their susceptible partner. Viruses from both partners were shown to be identical by restriction endonuclease fragment lengths. The only sexual contact that occurred shortly before infection of the susceptible partner had occurred between the two partners at a time when the infected partner was asymptomatic.

Langenberg and colleagues [9], working with investigators in the Medical Virology Section, studied the natural history of HSV-2. They found that 63% of new HSV-2 infections were asymptomatic and that 15% of these patients subsequently developed genital lesions during follow-up. As expected, women were more likely to become infected and to have symptomatic HSV-2 disease than were men. Individuals who had been infected with HSV-1 at the time of HSV-2 infection had a 2.6-fold higher likelihood of having asymptomatic HSV-2 infection than those who were HSV-1 negative. However, persons who were previously infected with HSV-1 did not have a lower rate of primary HSV-2 infection than those who had not been infected with HSV-1.

Lekstrom-Himes et al., [10] studied genetic factors that correlate with symptomatic HSV-2 infection. They found that specific human leukocyte antigen (HLA) types, HLA-B27 and HLA-Cw2 correlated with symptomatic HSV-2 disease. Another HLA type, HLA-Cw4, significantly correlated with HSV-2 infection. This study identified the first common genetic factors that are associated with HSV infection and symptoms.

2.1.4 Studies of Ultraviolet (UV) Light-Induced Reactivation of HSV

Sunlight exposure, which contains UV light, is known to induce recurrences of HSV cold sores in humans. Rooney and colleagues [11] showed that application of sunscreen to the lips before exposure to UV light prevented recurrence of HSV-1 lesions at the site. In patients who received placebo, 71% of UV exposures resulted in lesions and 66% caused virus shedding. In contrast, in patients who were given sunscreen before UV light exposure, 0% of UV exposures resulted in lesions and 3% caused virus shedding.

In a related study, Rooney and colleagues [12] tested the effectiveness of acyclovir to prevent reactivation of perigenital HSV-2 that was induced by UV light. In a double-blind placebo controlled study, oral acyclovir significantly reduced the rate of UV light induced herpetic lesions compared with placebo. These two studies indicate that sunscreen or acyclovir is effective for reducing HSV lesions in persons exposed to UV light.

2.1.5 Antiviral Therapy for HSV

Straus and colleagues [13] found that while vidarabine slowed the progression of HSV in immunodeficient patients, it did not cure infections. Subsequently, these investigators used intravenous or oral acyclovir in five immunodeficient patients and found that the antiviral resulted in complete healing of lesions. Continued treatment with oral acyclovir suppressed HSV recurrences for up to 2 months. A follow-up study by Straus and colleagues showed that suppressive oral acyclovir therapy markedly reduced the rate of recurrences in four immunodeficient patients [14]. Suppressive therapy was used for up to 6 months and remained highly effective. These studies were among the first to show the effectiveness of acyclovir in immunodeficient patients.

Subsequently, Straus and his colleagues performed a double-blind placebo controlled study using oral acyclovir to suppress recurrences of genital herpes.

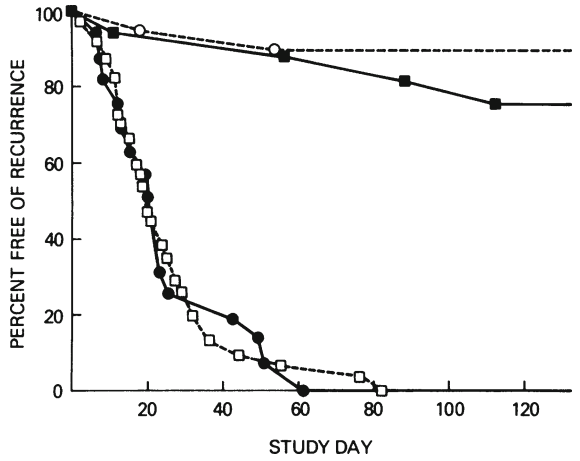

Fig. 2.3 Acyclovir suppressive therapy reduces the rate of recurrences in patients with genital herpes. Solid squares represent patients receiving acyclovir, and solid circles indicate those receiving placebo during the double-blind phase of the study. Open circles represent patients receiving acyclovir during the open phase of the study, while open squares indicate patients who were off study and not receiving acyclovir. (Reproduced with permission from *The New England Journal of Medicine*)

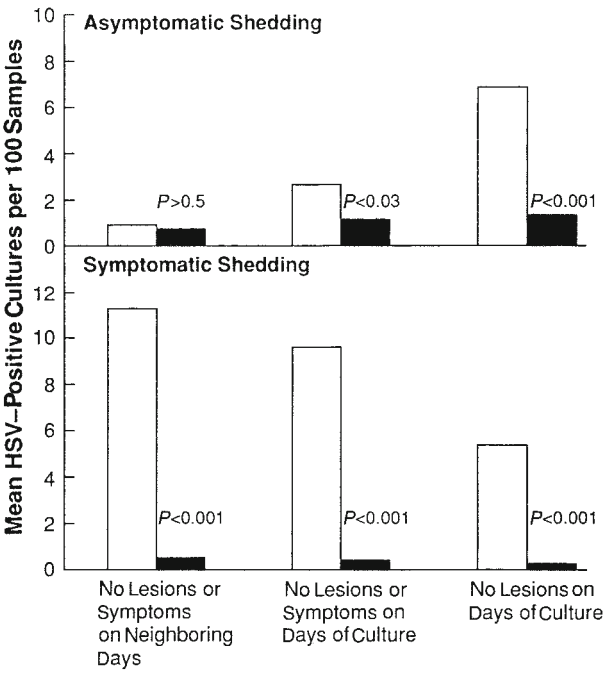

Fig. 2.4 Oral acyclovir reduces both asymptomatic (upper panel) and symptomatic (lower panel) shedding. Open bars indicate time when acyclovir was not used, and closed bars indicate time when acyclovir was used. (Reproduced with permission from *The Annals of Internal Medicine*)

When compared with placebo (Fig. 2.3), acyclovir significantly reduced the recurrence rate of genital herpes [15]. While some patients had acyclovir-resistant viruses during treatment with the drug, acyclovir-sensitive viruses were detected during recurrences when the drug had been discontinued. This study, along with a subsequent larger study, led to the licensure of acyclovir for suppression of genital herpes.

Further studies by Straus and colleagues of acyclovir for genital herpes showed that prolonged suppressive therapy for up to 1 year was effective, and that the dose of acyclovir needed to be increased further for some patients with "breakthrough" infections [16]. While recurrences occurred when suppressive therapy was discontinued, the frequency of recurrences tended to diminish over time. Thus, the importance of reassessing the need for continued suppressive therapy over time was demonstrated.

In addition to suppressing symptomatic genital herpes, Straus and colleagues showed that acyclovir suppresses subclinical shedding of the virus (Fig. 2.4) [17]. The observation that antiviral therapy suppresses shedding led to studies demonstrating that suppressive valacyclovir (which is converted to acyclovir in the body) is effective in reducing the rate of transmission of genital herpes to uninfected partners.

Rooney and colleagues [18] performed a double-blind placebo controlled trial, which showed that oral acyclovir reduces the frequency of recurrent herpes involving the lips (herpes labialis). Acyclovir also reduced the time to first recurrence, and the number of both clinical and virological confirmed herpetic lesions when compared with placebo.

While acyclovir-resistant herpes simplex had been reported to cause disease in immunocompromised patients, Kost and colleagues [19] reported the first non-immunocompromised person who developed clinically significant recurrent acyclovir-resistant genital herpes. This patient had recurrences that were not suppressed with high dose oral acyclovir and his virus had mutations in the viral thymidine kinase. HSV thymidine kinase phosphorylates acyclovir to convert the antiviral drug to its active form, which inhibits viral replication. One of the patient's HSV mutations caused a change in the putative acyclovir binding site in the viral thymidine kinase.

Wang and colleagues [20] examined individual neurons from an immunocompromised woman who was infected with acyclovir-resistant HSV-1. A variety of HSV-1 genomes were detected with different mutations localized to one region of the HSV-1 thymidine kinase gene. Each neuron contained a specific population of HSV-1 thymidine kinase mutants. Most neurons were latently infected with a mixture of viruses that had both wild-type and mutant thymidine kinase genes; others were infected with only wild-type virus or with only thymidine kinase mutant viruses. These results indicate that HSV-1 with mutations in thymidine kinase can establish latency in the absence of detectable wild-type virus and that the mutants likely arise independently of each other in the presence of acyclovir.

2.1.6 HSV Vaccines

Studies by Straus and colleagues [21] showed that inoculation of HSV-2 seronegative persons with purified recombinant glycoprotein D from HSV-2 in alum induced HSV-2 neutralizing antibody responses that were comparable to, or great than, those seen in persons previously infected with the virus. Inoculation of HSV-2 seropositive persons previously infected with HSV-2 resulted in a persistent increase in HSV-2 neutralizing antibody titers that were 2 to 4 fold higher than the titers before vaccination. The vaccine also induced cellular immune responses in 63% of HSV-2 seronegative persons; a similar percentage of HSV-2 seropositive persons had cellular responses to the vaccine.

Based on these results, two trials were performed to determine if vaccination of HSV-2 seropositive persons with frequent recurrences of genital herpes might result in fewer recurrences. In the first trial, Straus and colleagues [22] vaccinated 98 persons who had frequently recurrent genital herpes with two doses of either HSV-2 glycoprotein D in alum or alum alone in a double-blind study. Subjects who received the vaccine had fewer recurrences during the first year after vaccination (Fig. 2.5).

In the second study, Straus and colleagues vaccinated 202 subjects, who had frequently recurring genital herpes, with either HSV-2 glycoprotein B and glycoprotein D in MF59 adjuvant or MF59 alone [23]. The rate of HSV-2 recurrences was not reduced; however, the severity of the first recurrence after vaccination was reduced. While it is uncertain why these results differed from the prior study, possible explanations are the difference in adjuvant, the difference in dose of HSV-2 glycoprotein D (10-fold higher in the first study), the lower levels of antibody to glycoprotein D induced by vaccination in the second study compared with the first study, or a possible difference in the cellular immune response to the two vaccines.

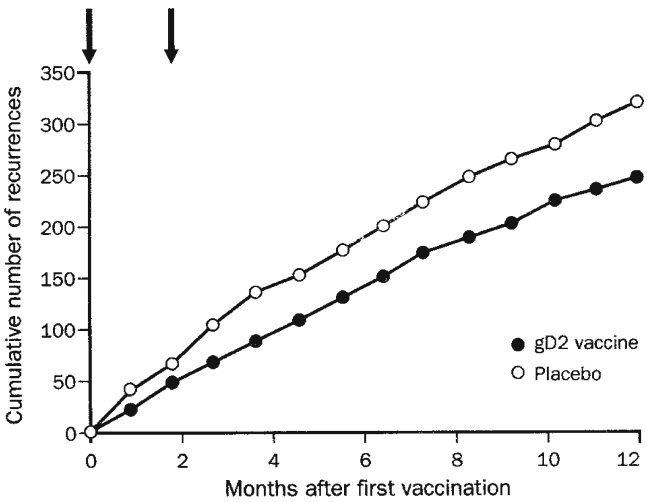

Fig. 2.5 Inoculation with recombinant HSV-2 glycoprotein D vaccine reduces the frequency of recurrences of genital herpes in persons previously infected with the virus. Vaccination times are indicated with arrows. (Reproduced with permission from *The Lancet* (Straus et al., 1994) with permission from Elsevier)

Two additional randomized, double-blind placebo-controlled trials were performed by Corey and colleagues [24] in which 2,268 subjects were vaccinated with HSV-2 glycoprotein B and glycoprotein D in MF59 adjuvant or MF59 alone. In one study, HSV-2 seronegative partners of HSV-2 infected persons received vaccine or placebo, while the other study vaccinated persons who were attending sexual transmitted disease clinics. Of subjects receiving the vaccine, the rate of new HSV-2 infections was 4.2 cases per 100 person-years, while those receiving the placebo had a rate of 4.6 cases per 100 person-years. Thus, the overall efficacy of the vaccine was 9%. The effectiveness of the vaccine was most prominent in the first 5 months after vaccination, especially in women and persons who were seropositive for HSV-1 (Fig. 2.6). The vaccine had no effect on the number of subsequent recurrences of HSV-2 in persons who became infected with HSV-2 after vaccination. Since the vaccine elicted high titers of neutralizing antibody to HSV-2, the study indicated that antibody to HSV-2 glycoproteins B and D alone does not protect against HSV-2.

Since the HSV-2 glycoprotein B and D vaccine was ineffective in preventing HSV-2 infection, preclinical studies in the Medical Virology Section have focused on other vaccines. Heineman and colleagues [25] inserted glycoprotein D of HSV-2 into the varicella-zoster virus genome and showed that the recombinant varicella-zoster virus expressed HSV-2 glycoprotein D on the surface of the virus and on virus-infected cells. Immunization of guinea pigs with varicella-zoster virus expressing HSV-2 glycoprotein D induced neutralizing antibodies to HSV-2. When the immunized animals were challenged with wild-type HSV-2, they showed reduced severity of HSV-2 genital lesions and less hindlimb paralysis than unimmunized animals. Since the strain of varicella-zoster virus used for these studies was the licensed Oka vaccine strain, this study demonstrated that the Oka vaccine strain might be used to deliver foreign proteins for new candidate vaccines.

Hoshino and colleagues [26] studied a replication defective virus termed HSV-2 *dl*5-29, developed by David Knipe at Harvard University, as a vaccine for HSV-2. HSV-2 *dl*5-29 is deleted for two essential HSV-2 proteins (UL5, UL29) and can grow only in cells that express the two viral proteins. This virus can infect non-complementing cells and express nearly all of the HSV-2 proteins in the context of major histocompatibility complex (MHC) I molecules, but cannot replicate in the cells. Thus, HSV-2 *dl*5-29 should induce both antibodies and cellular immunity to HSV-2. Hoshino and

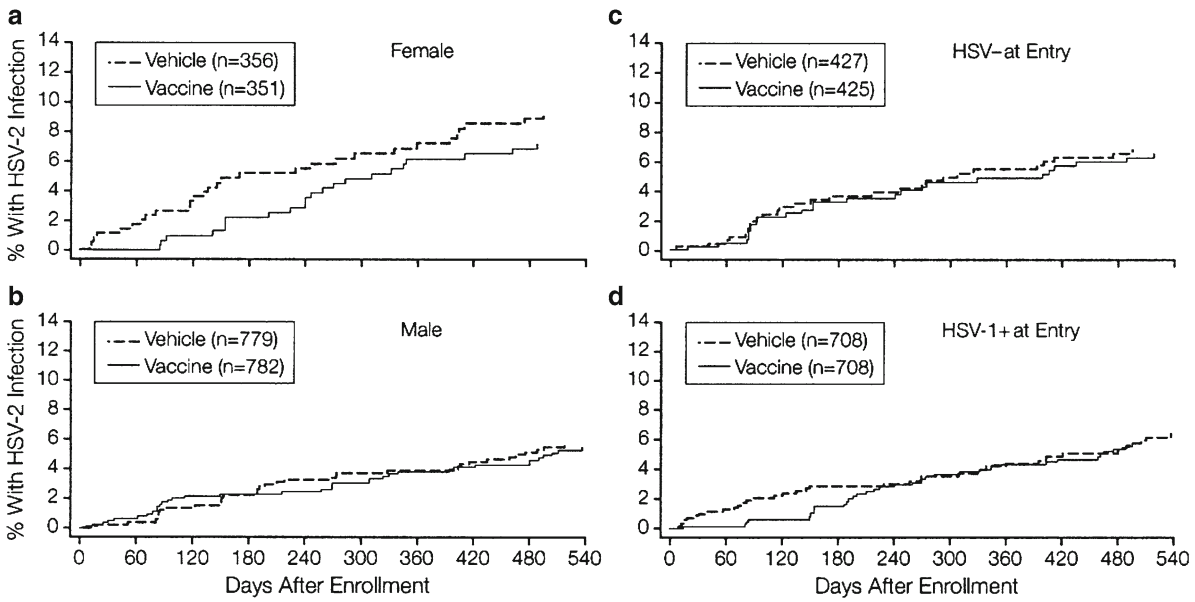

Fig. 2.6 Effectiveness of combined recombinant HSV-2 glycoprotein B and D vaccine to prevent genital herpes in women (**a**), men (**b**), HSV-1 seronegative persons (**c**), and HSV-1 seropositive persons (**d**). (Reproduced with permission from the *J. Am. Med. Assoc.*)

colleagues compared the efficacy of HSV-2 glycoprotein D, HSV-2 *dl*5-29, and plasmid DNA that expresses glycoprotein D as vaccines in guinea pigs and mice. They found that HSV-2 glycoprotein D and HSV-2 *dl*5-29 were both effective in reducing severity of acute genital herpes and recurrent disease in guinea pigs after challenge with wild-type virus. HSV-2 *dl*5-29 induced higher levels of neutralizing antibody to HSV-2 than glycoprotein D in guinea pigs. Furthermore, HSV-2 *dl*5-29 induced a more rapid cellular immune response to the virus in ganglia of mice than glycoprotein D after challenge with wild-type virus. Therefore, HSV-2 *dl*5-29 may have advantages over glycoprotein D as a candidate vaccine. HSV-2 *dl*5-29 is currently being tested in other models in the Medical Virology Section.

2.2 Varicella-Zoster Virus

Primary infection with varicella-zoster virus (VZV) causes chickenpox or varicella [1]. The virus remains latent in sensory and cranial nerve ganglia for life and can reactivate later in life to cause shingles or zoster. While zoster is usually limited to a single area of the skin innervated by a sensory nerve (dermatome), the disease can involve the eye (zoster ophthalmicus) and can cause postherpetic neuralgia (severe pain that persists long after the rash has resolved). Infection of pregnant women with VZV can cause congenital varicella. Immunocompromised persons can develop disseminated varicella or zoster with visceral disease involving the gastrointestinal tract (esophagitis, hepatitis), eye (retinitis), lungs (pneumonitis), or central nervous system (encephalitis, myelitis). Approximately 85% to 95% of adults in the United States have been infected with VZV.

2.2.1 Cloning and Genetics of VZV

Straus and colleagues were the first to fully clone and map the VZV genome [27]. The viral genome was cloned into bacteriophage lambda and a restriction map of the genome was generated. VZV DNA was shown to exist in two isomeric forms depending on the orientation of the unique short region of the genome. Electron microscopy of VZV DNA showed that the short region of the genome could invert. These studies enabled the development of "molecular fingerprinting" in which sizes of restriction endonuclease fragments were used distinguish different strains of VZV. An early application of this technology was used by Straus and colleagues [28] to conclusively demonstrate that the same molecular clone of virus that causes chickenpox subsequently reactivates to cause shingles. Analysis of VZV DNA obtained from lesions of a child with chickenpox and later from the same child after an episode of zoster showed identical patterns of restriction endonuclease fragments.

A versatile method was developed by investigators in the Medical Virology Section to rapidly engineer mutations into the VZV genome [29]. The entire VZV genome was cloned into a set of four large circular DNA molecules termed cosmids. When these cosmids were transfected into cells they reassembled to produce full length viral DNA and infectious virus. A mutation was then engineered into one of the cosmids which inactivated the viral thymidylate synthetase protein. Transfection of cells with the mutant cos-

mid along with the three other cosmids resulted in a virus that grew well in cell culture but did not express the thymidylate synthetase protein. Subsequently, investigators in the Medical Virology Section have shown that four viral genes are essential for replication of VZV in cell culture (ORF4 [30], ORF 21 [31], ORF29 [32], and ORF68 [33], while many viral genes others are dispensable for growth in vitro [31–34].

In collaboration with a group at Stanford University, investigators in the Medical Virology Section showed that the VZV ORF47 and ORF66 proteins are important for virus growth in human T cells and skin [35]. VZV ORF47 and ORF66 encode viral protein kinases that phosphorylate cellular and viral proteins. VZV deleted for ORF47 and ORF66 grows to titers similar to wild-type virus in melanoma cells and human fibroblasts. In contrast, VZV deleted for ORF47 was unable to grow in human skin and human T cells that had been implanted into severe combined immunodeficiency (SCID) mice. While VZV ORF66 protein was important for growth in human T cells, it was not important in human skin in SCID mice. These findings show that specific viral genes are required for growth in specialized cells.

2.2.2 Entry of VZV into Cells

Li et al., [36] identified the first cellular receptor for VZV. Insulin degrading enzyme (IDE) was found to bind to the extracellular domain of VZV glycoprotein E which is present on the surface of the virus. Incubation of cells with antibody to IDE, siRNA to IDE that inhibits expression of the protein (Fig. 2.7) or bacitracin that reduces IDE activity, each inhibited VZV infection in vitro and cell-to-cell spread of the virus. Conversely, overexpression of IDE in cells impaired for VZV infection enhanced virus infection and entry with cell-free and cell-associated virus. A recombinant soluble form of IDE, produced in baculovirus, enhanced infectivity of VZV *in vitro*. IDE is present both inside cells within endosomes and on the surface of cells. Since VZV can enter cells by endocytosis, the presence of IDE in endosomes may allow the virus to enter the cytoplasm by interacting with IDE in endosomes. These studies show that IDE functions as a receptor for both cell-free and cell-associated VZV.

2.2.3 Latency of VZV

The mechanism of how VZV maintains latency is important for understanding virus reactivation and shingles. Croen and colleagues [37] showed that, unlike HSV the other human alphaherpesvirus, VZV expresses multiple viral genes during latency in trigeminal ganglia. These studies used in situ hybridization, in which viral RNA was demonstrated directly in microscopic sections of latently infected ganglia. Additional studies by Meier et al., [38] extended these findings and confirmed that two VZV gene products, ORF29 and ORF62, are expressed in ganglia based on Northern blotting, while several other genes are not expressed during latency.

Using the cosmid mutagenesis system described above, investigators in the Medical Virology Section showed that certain VZV genes [ORF4 [30], ORF29 [32], ORF63 [39]] are essential for latency in a rodent model of VZV infection, while many other viral genes are not required for latent infection.

VZV has been reported to be latent in both neurons and non-neuronal cells and the number of copies of VZV DNA per latently infected cell has been controversial. Wang and colleagues [6] used laser capture microdissection to show that VZV is latent in 1 to 7% of neurons in human trigeminal

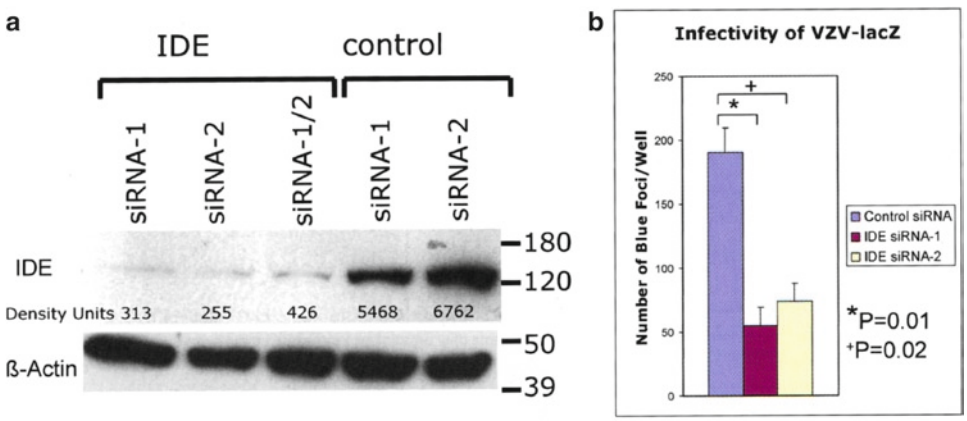

Fig. 2.7 Knock down of insulin degrading enzyme expression inhibits infectivity of VZV. (**a**) Immunoblot shows reduced IDE protein after transfection of cells with siRNA specific for IDE, but not with control siRNA. Density units below IDE indicate the intensity of the protein bands. (**b**) Reduction in the number of VZV (blue) foci in cells transfected with siRNA specific for IDE, but not with control siRNA (Reproduced from *Cell* (Li et al., 2006) with permission from Elsevier)

ganglia and is rarely, if at all, present in non-neuronal cells. A median of 7 VZV genome copies were present in virus-infected cells. This study indicates that VZV is latent predominantly in neurons within ganglia.

2.2.4 Immune Modulation by VZV

Many herpesviruses have evolved methods to avoid destruction by the immune system. VZV-specific cytotoxic T cells recognize virus-infected cells by detecting viral proteins complexed with MHC class I molecules. Investigators in the Medical Virology Section [40] showed that VZV infection reduces expression of MHC proteins on the surface of virus-infected cells. This allows the virus to limit presentation of viral proteins on the surface of cells and thereby should reduce detection of VZV-infected cells by the immune system.

Ambagala and colleagues [41] identified a VZV protein that inhibits the activity of interferon-alpha that has potent antiviral activity. VZV ORF63, which is the most abundantly expressed viral gene during latency, was found to be required for the virus to replicate efficiently in the presence of interferon-alpha. VZV ORF63 inhibited phosphorylation of eIF-2alpha, which is normally phosphorylated by proteins that are activated by interferon-alpha and inhibits protein synthesis. Since VZV ORF63 is expressed during virus latency, the ability of ORF63 to inhibit the activity of interferon-alpha may allow the virus to escape destruction by the immune system during latency.

2.2.5 VZV Antivirals and Vaccines

While several antivirals are available to treat varicella and zoster- acyclovir, valacyclovir, famciclovir, and foscarnet-, all of these compounds work by inhibiting the viral DNA polymerase. Working with investigators at Wyeth Vaccines, members of the Medical Virology Section identified a small molecule inhibitor, a thiourea analogue, which has potent activity against VZV *in vitro* [42]. The antiviral compound was found to impair formation of viral nucleocapsids and to interfere with the activity of the VZV ORF54 protein. The compound, which has a different mechanism of action than all of the licensed drugs used to treat VZV, was shown to inhibit growth of acyclovir-resistant VZV in vitro.

As part of the Shingles Prevention Study Group, the Medical Virology Section enrolled over 1,700 patients in a double blind vaccine trial comparing the ability of one dose of the live attenuated Oka VZV vaccine with placebo to prevent herpes zoster and postherpetic neuralgia [43]. The zoster vaccine was found to reduce the incidence of shingles by 51% and the rate of postherpetic neuralgia by 67% in persons 60 years of age or older (Fig. 2.8). This study was responsible for licensure of the zoster vaccine.

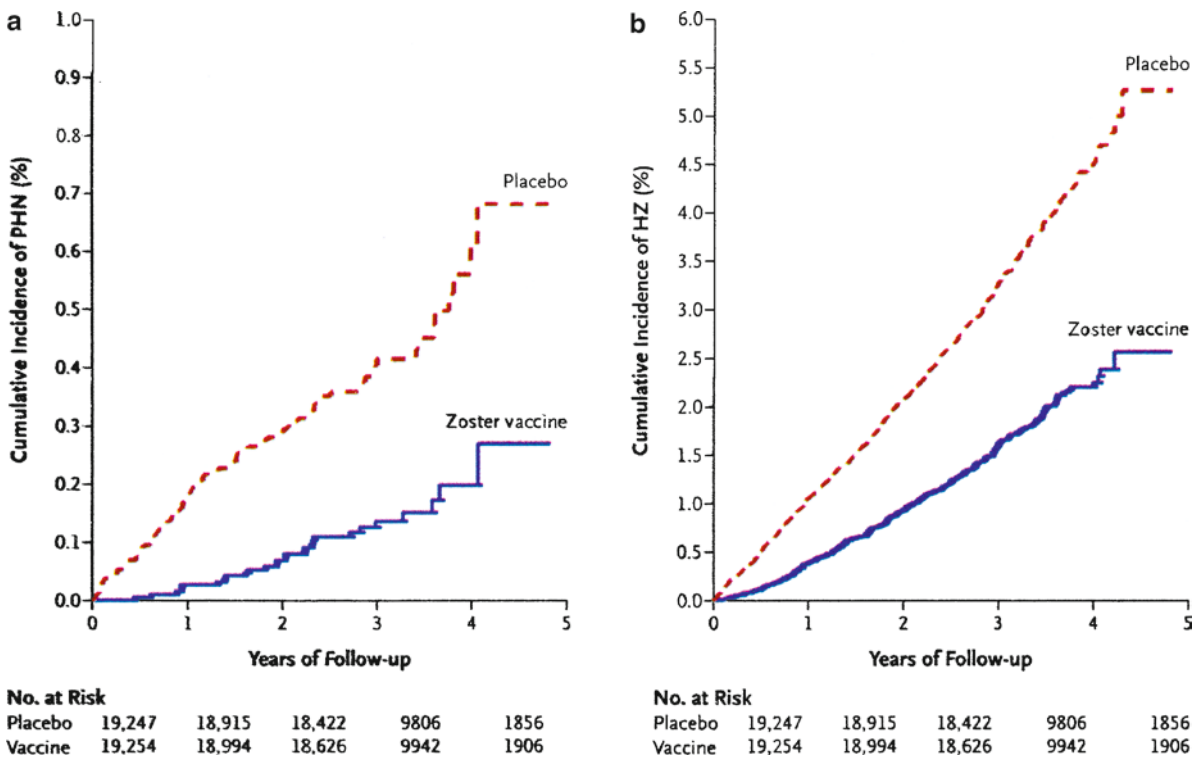

Fig. 2.8 Vaccination with the zoster vaccine reduces the rate of postherpetic neuralgia (PHN, **a**) and herpes zoster (HZ, **b**). (Reproduced with permission from *The New England Journal of Medicine*)

While vaccines are available for varicella and zoster, they are not approved for highly immunocompromised patients. Alternative approaches that would result in safer vaccines are needed for these persons. Investigators in the Medical Virology Section constructed Oka VZV vaccine mutant viruses lacking essential viral genes that could only be propagated in cells expressing the missing viral gene [30, 31, 32, 33]. Such viruses might be safer vaccines candidates as they can infect human cells and present viral proteins with MHC class I molecules, but they cannot replicate in the cells or spread to adjuvant cells. Concerns with using replication-defective viruses as vaccines include whether such vaccine candidates can be grown in sufficient titers to produce adequate quantities of vaccine virus and if such vaccines would be sufficiently immunogenic.

As noted above, VZV deleted for the ORF66 protein is impaired for growth in human T cells but not in human skin [35]. Therefore, this mutant virus should be able to replicate at the site of subcutaneous inoculation and induce a good immune response but not replicate in T cells, and, therefore, it should be impaired for dissemination in the body. Thus, VZV deleted for the ORF66 protein might be a promising vaccine candidate. An alternative approach is the development of a VZV vaccine that can replicate but not reactivate from latency. Substitution of the natural promoter for the ORF29 gene, which is expressed during latency, with a promoter from cytomegalovirus resulted in a virus that was unimpaired for replication in cell culture but was significantly impaired for latent infection in animals (Fig. 2.9) [32]. The VZV mutant with ORF29 driven by the cytomegalovirus promoter should be less likely to reactivate from latency and cause shingles.

2.3 Epstein-Barr Virus

Infection of infants and children with Epstein-Barr virus (EBV) is usually asymptomatic or results in nonspecific symptoms, while infection of adolescents and young adults can result in infectious mononucleosis [44]. Approximately 80% to 95% of adults in the United States have been infected with EBV. The virus establishes latency in memory B cells. EBV is associated with several malignancies in otherwise healthy persons including Hodgkin's disease, nasopharyngeal carcinoma, Burkitt lymphoma, and nonHodgkin's lymphoma. Immunocompromised persons such as transplant recipients or patients with AIDS are at increased risk for developing EBV-associated lymphomas and can develop oral hairy leukoplakia with white plaques on the tongue or cheek. Very rarely, individuals infected with EBV can develop chronic active EBV infection, which presents with fever, enlarged lymph nodes, splenomegaly, and infiltration of multiple organs with EBV. These patients often develop EBV lymphomas that are refractory to chemotherapy.

2.3.1 EBV Latency Genes

EBV encodes a number of proteins that cause B cell proliferation and that are important for immortalization of the cells by the virus. One of these proteins, EBV nuclear antigen 2 (EBNA-2) is essential for EBV immortalization of B cells. Investigators in the Medical Virology Section identified a region of EBV EBNA-2 that is required for immortalization by the virus and showed that substitution of this region with

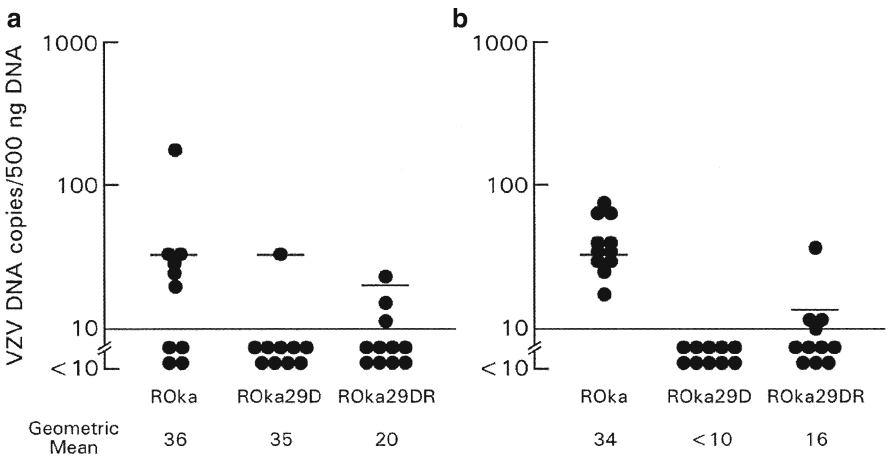

Fig. 2.9 VZV with a cytomegalovirus promoter driving ORF29 is impaired for latency. Number of copies of VZV genomes is shown in animals latently infected with parental VZV (ROka), virus deleted for ORF29 (ROka29D), or virus with a foreign promoter driving ORF29 (ROka29DR). The horizontal line indicates the lower limit of detection (10 copies) and the geometric mean number of copies of VZV DNA per 500 ng of DNA for VZV-positive ganglia is at the bottom. Two independent experiments are shown. (Reproduced from *The Journal of Virology* (Cohen et al., 2007) with permission from American Society for Microbiology)

a portion of an HSV gene could restore the immortalization function of EBNA-2 [45]. Mutation of a single amino acid in this region of EBV EBNA-2 completely prevented the ability of EBNA-2 to immortalize B cells.

Inoculation of EBV-immortalized B cells into SCID mice results in the formation of B cell lymphomas. Studies by investigators in the Medical Virology Section showed that EBV transformed B cells with mutations in EBNA-2 that impaired growth of the cells in vitro, also impaired formation of B cell lymphomas in SCID mice [46]. These studies indicate the importance of EBNA-2 in oncogenesis.

2.3.2 Immune Modulation by EBV

EBV encodes several proteins that modulate the immune response to the virus, including a viral homolog of interleukin 10 and three viral proteins that inhibit programmed cell death. Spriggs and colleagues [47], working with members of the Medical Virology Section, identified an EBV protein (BZLF2) that binds to the HLA-DR beta chain of MHC class II molecules. EBV BZLF2 inhibited production of cytotoxic T cells in a mixed lymphocyte reaction, and the protein prevented antigen-induced proliferation of peripheral blood mononuclear cells. Thus, EBV BZLF2 might allow the virus to inhibit cellular immunity during EBV infection.

Working with investigators in the Medical Virology Section, Strockbine and colleagues [48] identified an EBV protein (BARF1) that binds to colony stimulating factor 1 (CSF-1). EBV BARF1 is secreted from virus-infected B cells and inhibits the ability of CSF-1 to induce macrophage proliferation. Additional experiments showed that recombinant EBV BARF1 inhibits secretion of interferon-alpha from human mononuclear cells [49]. Furthermore, B cells infected with an EBV mutant deleted for BARF1 showed a reduced ability to inhibit interferon-alpha secretion by mononuclear cells compared with B cells infected with wild-type EBV. Since interferon-alpha is known to inhibit EBV transformation and enhances cellular immunity, EBV BARF1 may help virus-infected cells to escape attack by the immune system.

2.3.3 Studies of Patients Infected with EBV

Investigators in the Medical Virology Section participated in a study showing that patients with chronic active EBV had abnormal antibody responses to the virus, and often lack antibodies to the EBV nuclear antigen 1 protein [50]. While the cause of most cases of chronic active EBV are unknown,

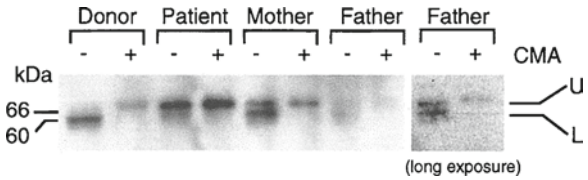

Fig. 2.10 Immature perforin in a patient with chronic active EBV infection. Peripheral blood mononuclear cells were incubated with (+) or without (–) concanamycin A (CMA) which impairs maturation of perforin, and cell lysates were immunoblotted with an antibody to perforin. Cells from a blood bank donor express the mature (60 kDa) form of perforin (lane 1), but only the immature (66kDa) form of perforin is detected after treatment with CMA. Cells from the patient with chronic active EBV express only the immature (66kDa) form of perforin. The patient's mother and father are heterozygous for the perforin gene and their cells express both mature and immature forms of perforin. U indicates upper band (immature perforin), and L indicates lower band (mature perforin). (This research was originally published in *Blood* (Katano et al., 2004) © the American Society of Hematology)

Katano and colleagues [51] showed that one patient with chronic active EBV had a mutation in both alleles of his perforin gene. Perforin is produced by cytotoxic T cells and is important for killing virus-infected cells. Katano et al., showed that the patient produced only an immature form of perforin (Fig. 2.10) and that the patient's cells could not kill target cells effectively.

EBV is associated with lymphomas in patients with rheumatoid arthritis or polymyositis who are treated with methotrexate. In collaboration with a group at the University of North Carolina, investigators in the Medical Virology Section showed that methotrexate induces reactivation of EBV from latently infected B cells *in vitro* [52]. Patients with rheumatoid arthritis or polymyositis who were treated with methotrexate had higher levels of EBV DNA in their peripheral blood than those receiving other immunosuppressive regimens that did not contain methotrexate. In contrast, patients receiving methotrexate for other diseases did not have higher levels of EBV in the blood than those who were receiving other immunosuppressive regimens. Therefore, methotrexate induced reactivation of EBV might contribute to development of lymphomas in patients with rheumatoid arthritis and polymyositis.

EBV is known to infect epithelial cells and B cells in the oropharynx and spread through the body in B cells. However, it is not known whether these B cells are required for persistent shedding of the virus from the oropharynx. Hoover and colleagues [53] studied lymphoma patients receiving a monoclonal antibody to B cells (rituximab) that depletes B cells from the blood and reduces the number of B cells in tissues. Patients who received rituximab in whom neither B cells nor EBV DNA could be detected in the peripheral blood still shed EBV from the oropharynx. These observations

indicate that B cells in the blood are not required for virus to persist in the oropharynx and to be shed from the throat.

2.3.4 Identification of Candidate Drugs to Treat EBV Lymphomas

EBV is a cause of B cell lymphomas in transplant recipients. These patients have tumors that express each of the EBV latency proteins. These same latency proteins are expressed in B cells immortalized by EBV in cell culture and in EBV tumors in SCID mice. Katano et al., [54] showed that simvastatin, a drug used to treat high cholesterol, kills EBV immortalized B cells in culture by inducing apoptosis (programmed cell death). EBV immortalized B cells grow in tight clumps and adhesion molecules, including LFA-1 and ICAM-1, are important for formation of these clumps. Simvastatin inhibits the interaction of LFA-1 with ICAM-1 and inhibits clump formation. EBV latent membrane protein 1 (LMP1) is located in lipid rafts on the surface of EBV-infected B cells and oligomerizes to activate NF-κB and other signaling molecules, resulting in B cell proliferation. Katano et al showed that simvastatin displaces EBV LMP1 from lipid rafts and inhibits activation of NF-κB. Treatment of SCID mice with simvastatin followed by injection of the animals with EBV-infected B cells resulted in delayed development of B cell lymphomas and prolonged survival of the animals compared with animals that did not receive simvastatin. While the dose of simvastatin used in SCID mice was much higher than that used to treat elevated cholesterol levels in humans, peak serum levels of simvastatin achieved in humans trials of the drug for solid tumors were similar to the levels of simvastatin that killed EBV immortalized B cells in culture. These experiments suggest that other statins or statin-like molecules might be used to treat or prevent EBV associated B cell lymphomas in the future.

Bortezomib is approved for the treatment of multiple myeloma and inhibits the proteosome in the cell. Zou and colleagues [55] showed that bortezomib induces apoptosis of EBV immortalized B cells by activating the cleavage of caspases 8 and 9 in the cell. Treatment of EBV immortalized B cells with bortezomib resulted in lower levels of several components of the NF-κB pathway, including p50, p52, and p65, which are normally induced by EBV LMP1 and are important for immortalization of B cells. Bortezomib also reduced levels of three proteins, cIAP-1, cIAP-2, and XIAP, which normally inhibit apoptosis in cells. Treatment of SCID mice with bortezomib resulted in delayed development of lymphomas and prolonged survival of the mice (Fig. 2.11). Clinical trials are currently underway at outside institutions using bortezomib in the treatment of EBV lymphomas.

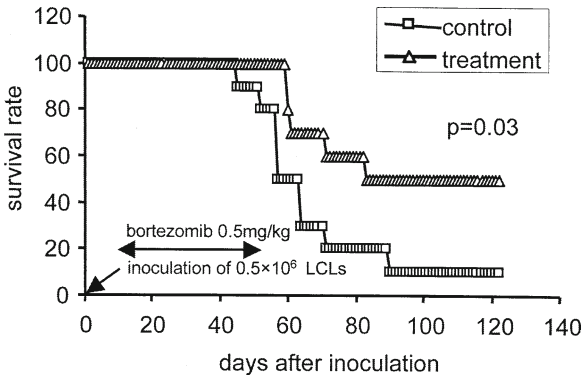

Fig. 2.11 Bortezomib delays development of lymphomas and prolongs survival of SCID mice inoculated with EBV-transformed B cells. After inoculation with EBV-immortalized B cells, mice were treated with bortezomib (triangles) or PBS (squares) for 6 weeks and then observed for development of lymphomas. (Reproduced from *The Journal of Virology* (Zou et al., 2007) with permission from American Society for Microbiology)

2.4 Other Herpesviruses

Many viruses have developed mechanisms to avoid their destruction by the immune system. Bertin and colleagues [56] discovered a new class of viral proteins that contain death effector domains and bind to corresponding death effector domains in cellular proteins that mediate apoptosis. The initial report identified proteins encoded by equine herpesvirus 2 and molluscum contagiosum virus (a human poxvirus) that bind to caspase-8 and FADD, respectively. Expression of either of the viral proteins in cells protected the cells from Fas and TNFR1 induced apoptosis. Wang et al., [57] subsequently showed that other herpesviruses (Kaposi's sarcoma-associated herpesvirus, bovine herpesvirus 4, and herpesvirus saimri) encode proteins that also contain death effector domains. Viral proteins that encode these death effector domains have now been named vFLIPs (viral FLICE inhibitory proteins), and these studies led to the discovery by other investigators of a cellular protein called c-FLIP, which is thought to be the origin of the viral proteins.

Herpes B virus (Cercopithecine herpesvirus 1) establishes a latent infection in macaques and can reactivate in these animals to cause lesions that resemble HSV in humans. Humans who are bitten or scratched by macaques can develop fatal encephalitis due to herpes B virus. Freifeld and colleagues [58] performed a seroprevalence survey of primate workers and found that while many of them had been injured by macaques, none who were asymptomatic had evidence of herpes B virus infection (as defined by antibody to the virus). This study showed that asymptomatic infections with herpes B virus occur very rarely, if ever, in humans. Subsequently members of the Medical Virology Section [59] helped to

write the current guidelines for postexposure prophylaxis of humans after exposure to herpes B virus and for treatment of those infected with the virus.

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Chapter 3
Why Study Mouse Retroviruses?

Kim J. Hasenkrug

3.1 Introduction

Retroviruses are a unique group of viruses that insert their genetic material into the host genome and cause a variety of diseases ranging from AIDS in humans infected with human immunodeficiency virus (HIV) to leukemia in humans infected with human T cell lymphotropic virus-1 (HTLV-1). Before retroviruses were discovered in humans, they had been studied for many years in mice and other species, mainly because of their interesting ability to cause cancer. As an immunologist, I became interested in studying mouse retroviruses because of intriguing work by Bruce Chesebro who showed that numerous immunological host genes controlled the ability of mouse retroviruses to cause disease [1]. While the immune systems of mice and humans are not identical, they are remarkably similar, and discoveries in mouse models have led to the development of diverse medical advances such as successful transplantation surgery, cancer therapies, antiviral drugs, and vaccines.

In scientific research, the mouse is such a powerful subject, due to our remarkable ability to precisely control and manipulate mouse genetics. Since the early 1900s, mouse breeding for scientific research has been going on and has led to the development of strains of mice that were genetically identical. During the mid-1900s, George Snell developed a technique to breed mice that were genetically identical except for a single genetic region, a region that controlled the ability to accept or reject transplanted tissues. That work directly led to the ability to successfully transplant tissues in humans, and in 1980, he was awarded a share of the Nobel Prize for Physiology or Medicine. The genetic region that George Snell was studying (the major histocompatibility complex or MHC) turned out to be critical for many immune responses and was later found to correlate with different levels of resistance to infections with HIV [2, 3, 4] and HTLV-1 [5, 6, 7]. Further genetic developments that have kept mice in the forefront of medical research were the discovery of a way to introduce foreign genes into the mouse genome (transgenic mice) by Richard Palmiter and Ralph Brinster, and the development by Mario Capecchi, Oliver Smithies, and Martin Evans of a way to inactivate specific genes in the mouse (knockout mice). The latter group was awarded the 2007 Nobel Prize for Physiology or Medicine. Adding to the value of the mouse in medical research, is a multitudinous array of tools such as monoclonal antibodies that specifically recognize almost every protein in the animal.

3.2 Genetic Resistance to Retroviral Infection

In the late 1970s, Bruce Chesebro and colleagues were studying mice infected with a retrovirus called Friend virus, named for Charlotte Friend, the scientist who discovered it in 1957 [8]. The Chesebro group discovered that a single gene (**R**esistance to **F**riend **v**irus gene **3** or Rfv3) controlled the ability of the mice to mount antibody responses against the virus [9, 10, 11]. Mice that had the right genetic type mounted good neutralizing antibody responses and controlled the infection while mice with the wrong genetic type developed leukemia and died. I was very interested in determining what the Rfv3 gene was because it was known that people infected with HIV mounted very poor virus-neutralizing antibody responses; discovering how Rfv3 worked in mice might give us clues about antibody responses in humans. We used microsatellite mapping to locate the Rfv3 gene on mouse chromosome 15 [12, 13] in a region syntenic with human chromosome 22. Interestingly, an HIV resistance gene was also mapped to this syntenic region [14]. One gene of high interest in this region of chromosome 15 was **m**ouse ***A**pobec**3*** (*mA3*), which encoded a protein with known anti-retroviral activity. In a collaboration with Mario Santiago and Warner Greene's laboratory at the Gladstone

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Institute for Virology and Immunology, the *mA3* gene was knocked out, and extensive testing showed it to be the gene responsible for the Rfv3 phenotype [15]. The mechanisms through which mA3 influences the antibody response are still under investigation, but the results suggest that blocking HIV Vif, a protein that the virus has evolved to counteract the effects of Apobec, could not only improve the direct antiviral effects of Apobec but might also enhance virus-neutralizing antibody responses.

3.3 Acute and Chronic Infections

A strong virus-neutralizing antibody response is a critical component of the immune system, but by itself it is not sufficient to prevent Friend virus-induced leukemia [16, 17]. In addition to antibodies the control of acute infection of Friend virus requires multiple components of the immune system including both $CD4^+$ and $CD8^+$ T-cell responses [18, 19]. $CD4^+$ T-cells are generally known as "helper" cells because they provide stimuli to help other immune cells: B cells that make antibodies and $CD8^+$ T-cells that kill infected cells. Most strains of laboratory mice lack optimal responses in one or another immune component and die from acute Friend virus-induced leukemia. However, some mouse strains have a very resistant genetic makeup and are able to recover from acute infection. The differing resistance and susceptibility profiles in various mouse strains have allowed identification of the components of the host response involved in natural recovery from infection [1]. Interestingly, even the most resistant mouse strains that recover from acute infection develop chronic infections and remain chronically infected for life [18, 20]. Chronic infections with Friend virus do not usually cause significant clinical signs, but about 5% of chronically infected mice will relapse and die of leukemia [18, 20]. The long-term control of chronic infections is mediated in large part by Th1 $CD4^+$ T-cells [21, 22] and by IFN-γ [23]. In striking contrast to the requirement for $CD8^+$ T-cells during acute infections, $CD8^+$ T-cells are not involved in control during the chronic phase [21]. We were interested in how chronic Friend virus avoided $CD8^+$ T-cell immunity and found that the virus-specific $CD8^+$ T-cells had lost their effector function [24] due to suppression by $CD4^+$*regulatory T cells (Tregs)* [25]. Tregs are a specialized subpopulation of $CD4^+$ T-cells that were known at the time only to be involved in suppressing autoimmune responses [26]. In response to FV infection, the Tregs became activated and suppressed virus-specific $CD8^+$ T-cell responses both *in vivo* and *in vitro* [27]. Studies soon followed showing suppression of $CD8^+$ T-cells by Tregs from HIV patients [28, 29, 30].

3.4 Immunotherapy to Treat Chronic Infection

The finding that chronic Friend virus infections and virus escape were associated with loss of $CD8^+$ T-cell function due to Treg-mediated suppression suggested possible immunotherapies to prevent or counteract suppression. To determine if immunotherapy during acute infection could prevent Treg-mediated suppression and the establishment of chronic infection, we first investigated stimulation of the glucocorticoid-induced tumor *necrosis factor receptor (GITR)*. GITR stimulation had been shown to eliminate suppressive function in Treg's [31, 32] and acted as a costimulatory molecule for effector T cells [33, 34]. During acute Friend virus infection treatment of mice with an agonistic anti-GITR antibody significantly reduced virus loads during the chronic phase [35]. A more difficult challenge was the reduction of virus loads in mice with already established chronic infections; however, we found that treatment with an agonistic antibody that triggered CD137 (CD137 is a costimulatory molecule expressed on activated T cells) was highly effective at reducing chronic virus loads when used in combination with adoptive transfers of virus-specific $CD8^+$ T-cells. Treatment with anti-CD137 rendered the adoptively transferred cells resistant to Treg-mediated suppression and stimulated dramatic reductions in chronic virus loads [36]. Current studies are focused on immunotherapies that work without a requirement for adoptive T cell transfers, as this is a more practical therapeutic approach.

3.5 Vaccine Studies

It would be most desirable to develop a vaccine that completely protected against both acute disease and chronic retroviral infections. Studies in the SIV model have shown live attenuated viruses to be the most effective vaccines against retroviral infection. However, live attenuated viruses are not safe for use in humans, and it was not known how or why live attenuated viruses provided protection [37]. Thus, we decided to use live attenuated Friend virus vaccination to approach the problem of determining what types of immune mediators were involved in protection. Experiments in which single, double, or triple combinations of immune cell subsets were adoptively transferred from vaccinated mice to unvaccinated mice revealed a number of interesting findings [38]. By themselves, immune $CD8^+$ T-cells reduced acute virus loads by an order of magnitude, but the mice still became chronically infected. Adding immune $CD4^+$ T-cells or B-cells did not significantly improve protection by $CD8^+$ T-cells. In fact, no two subsets of immune lymphocytes protected the mice

from chronic infection. However, any combination of two subsets of immune lymphocytes protected the mice from lethal disease. Most interestingly, adoptive transfer of all three immune subsets afforded complete protection from both clinical disease and the establishment of chronic infection. Subsequent studies showed that the primary role of immune B cells was the production of virus-specific antibodies [17]. The most effective antibodies in protection were neutralizing antibodies; however, non-neutralizing antibodies also contributed to protection. Full protection against infection was afforded only when antibodies were combined with vaccine-induced T-cell responses. The requirement for complex immune responses against Friend virus, a rather simple retrovirus, suggests that immune responses of equal or greater complexity will be required for protection against HIV.

These selected studies briefly illustrate how research in the mouse model can be used to discover basic scientific principles that govern susceptibility or resistance to retroviral infection and how these discoveries can lead to therapies and vaccines to treat or prevent diseases.

In summary, the Retroviral Immunology Section of the Laboratory of Persistent Viral Diseases does basic research in a mouse model to discover how immunological responses deal with natural retroviral infections, how the immune responses can be improved to prevent or cure retroviral diseases, and how vaccines can be used to prevent infections. This review highlights some the basic research findings by this section over the last decade that have relevance to human diseases and medicine. Included are three related categories of study: genetic resistance to retroviral infection, immunological control of acute and chronic infection, and vaccine development and function.

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Chapter 4
Functions of the Rotavirus RNA Polymerase in Virus Replication

Kristen M. Guglielmi and John T. Patton

Abbreviations

–	negative-sense
+	positive-sense
3′CS-	3′ CS of –RNA, 5′-$(A/U)_7$GCC-3′
3′CS+	3′ CS of +RNA, 5′-UGUGACC-3′
CS	consensus sequence
DLP	double-layered particle
ds	double-stranded
N	incoming nucleotide
NSP	viral nonstructural protein
NTP	nucleoside triphosphate
P	priming
r	recombinant
RdRP	RNA-dependent RNA polymerase
RI	replication intermediate
RV	rotavirus
TLP	triple-layered particle
UTR	untranslated region
VP	viral structural protein

4.1 Introduction

To achieve productive infection of a target cell, a virus must express viral proteins, replicate its genome, and assemble infectious particles. For many viruses with RNA genomes, all stages of RNA synthesis are mediated by a virus-encoded RNA-dependent RNA polymerase (RdRP). Viruses with a segmented, double-stranded (ds) RNA genome rely on the encoded RdRP to perform both transcription (dsRNA → +RNA) and replication (+RNA → dsRNA). For dsRNA viruses, the RdRP also plays an important role in recognizing and packaging the correct number and constellation of viral RNAs into progeny particles.

Rotaviruses (RVs) are segmented, dsRNA viruses of the family *Reoviridae*. Owing to the medical importance of these viruses as the primary cause of acute, dehydrating diarrhea in infants and young children [1, 2, 3], RV pathology and biology have remained active areas of research for several years. Based on serological and phylogenetic analyses, human RVs are classified into one of four groups: A, B, C, or non-A/B/C [4, 5, 6]. Group A includes the most medically important RVs, causing approximately 500,000 deaths each year, worldwide [3]. In addition to being medically important viruses, RVs provide an intriguing system for study of the coordinated mechanisms by which dsRNA viruses achieve genome replication, packaging, and transcription. Like other dsRNA viruses, RV synthesizes RNA inside of subviral particles [7]. RV also encapsidates a genome composed of 11 distinct dsRNA segments. Precisely how these processes are coordinated remains an area of active interest and inquiry.

4.1.1 Rotavirus Structure

RV virions are non-enveloped, icosahedral particles composed of three concentric layers of protein [8, 9] (Fig. 4.1a). The core of the virion is formed from the viral genome and viral proteins VP1, VP2, and VP3 [10]. Sixty asymmetric dimers of VP2 comprise the core shell, an icosahedral structure with pseudo T = 1 symmetry and the innermost layer of the RV triple-layered particle (TLP) [8, 11]. VP1 is the viral RdRP [12, 13, 14, 15]. VP3, the viral capping enzyme, exhibits guanylyltransferase and methyltransferase activities [13, 16, 18]. The precise locations and orientations of VP1 and VP3 in the virion are not known. However, a single copy each of VP1 and VP3 is thought to reside at each of the icosahedral five-fold vertices of the core, where they are anchored to the inner surface of the VP2 shell [15, 18, 19] (Fig. 4.1b). The middle layer of the RV virion is composed of 260 trimers of VP6 organized with T=13 symmetry, which surrounds the core and forms a double-layered particle (DLP) [8, 9, 20] (Fig. 4.1a). The outermost layer of the RV virion also has T = 13 symmetry and is comprised of 260 trimers of VP7, the viral glycoprotein, and 60 trimers of VP4, the trypsin-sensitive viral hemagglutinin [21, 22, 23, 24, 25].

RVs encapsidate a genome of 11 segments of dsRNA, which typically encode a total of six viral structural proteins

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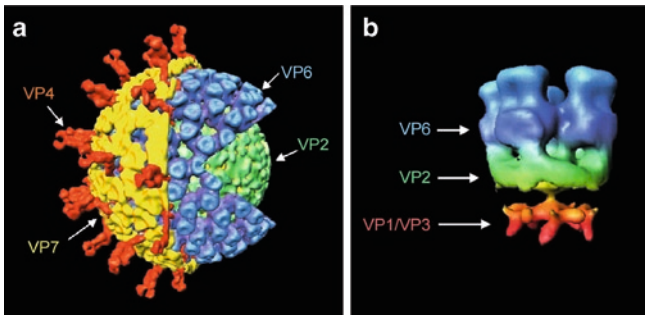

Fig. 4.1 Structure of the RV virion. (**a**) Cryoelectron micrograph image reconstruction of the RV TLP, revealing surface regions of the DLP (VP6) and core (VP2). (**b**) Cryoelectron micrograph image reconstruction showing protein organization at the five-fold axis of a DLP. Images shown with permission from B. V. V. Prasad

(VPs) and six nonstructural proteins (NSPs) [10]. The 5′ and 3′ untranslated regions (UTRs) of each gene segment vary in length and sequence, but are conserved among homologous gene segments of viruses classified within the same group [14]. The extreme 5′ and 3′ termini of the + and – strands include short sequences that are conserved among all RV gene segments [26]. These consensus sequences (CSs) contain *cis*-acting signals important for RNA recognition by the RV RdRP and for genome replication and transcription [27, 28, 29, 30].

4.1.2 Overview of the Rotavirus Life Cycle

During entry into the host cell, the outer layer of the RV virion is removed, and transcriptionally-active DLPs are released into the cytoplasm [10]. Using the dsRNA genome as template, VP1 generates positive-sense, capped, non-polyadenylated transcripts [31, 32]. These +RNAs serve dual functions, as templates for translation by host ribosomes and for – strand synthesis during replication to make the dsRNA genome [33, 34]. Through channels located at the icosahedral vertices, the +RNAs are extruded from the RV DLP into the cytoplasm [8]. Following translation, the viral structural proteins VP1, VP2, VP3, and VP6, the nonstructural proteins NSP2 and NSP5, and +RNAs accumulate in viral inclusion bodies in the cytoplasm termed viroplasms [10, 35]. It is at these sites that the genome is packaged and replicated and cores and DLPs are assembled. NSP2 and NSP5 mediate formation of viroplasms and recruit core viral structural proteins [36, 37, 38, 39]. With packaging of the 11 distinct viral +RNAs into cores, replication to generate the dsRNA genome occurs concurrently [40]. Following association of VP6 with cores, newly-formed DLPs migrate from the viroplasm to the endoplasmic reticulum where they acquire the VP4-VP7 outer capsid shell during budding [41, 42, 43].

For RV, transcription, packaging, and genome replication are highly coordinated processes mediated by viral proteins and signals within viral RNAs. Although the mechanisms governing all of these processes are not yet fully understood, considerable progress has been made in several areas, including identification of (i) viral proteins necessary for replication and transcription, (ii) signals within viral +RNA that permit recognition and replication by VP1, and (iii) structural features of VP1 that permit this RdRP to perform its many functions. These studies have enhanced our understanding of the most critical processes RV must complete to achieve productive infection.

4.2 Genome Replication

4.2.1 Cell-Free dsRNA Synthesis

During assembly, RV +RNAs are packaged and replicated to form the dsRNA genome. Cell-free systems have aided studies of RV replication, making it one of the most well understood processes in the RV life cycle. The first of these systems was created when it was observed that subviral particles isolated from RV-infected cells are capable of generating genomic dsRNA from endogenous viral +RNA *in vitro* when incubated with nucleoside triphosphates (NTPs) and Mg^{2+} [44, 45]. Analysis of the subviral particles allowed the isolation and characterization of RV replication intermediates (RIs), which led to the identification of proteins involved in genome replication. A second-generation cell-free system, developed by Chen et al., [29], allows synthesis of dsRNA from an exogenously-added RNA templates. This system utilizes disrupted (open) RV cores, generated from purified RV virions, as a source of active RdRP [29]. Native cores can be prepared by treating RV virions with EDTA to remove outer capsid proteins VP4 and VP7 and $CaCl_2$ to remove VP6. These native cores contain VP1, VP2, VP3, and genomic dsRNA. When dialyzed in low-salt buffer, native cores release genomic RNA, due to disruption of the VP2 shell. Upon addition of Mg^{2+}, NTPs, and exogenous viral +RNA templates, open cores can synthesize dsRNAs. This assay system has been applied extensively to mapping and characterizing *cis*-acting signals in +RNAs that promote dsRNA synthesis [27, 30, 46, 47, 48]. A third-generation system for RV replication utilizes co-expression of recombinant (r) viral proteins in insect cells [29]. In this system, insect cells are co-infected with baculoviruses expressing rVP1, rVP2, rVP3, and rVP6. The co-expressed viral proteins form RV-like rDLPs, which can be purified from insect cell lysates. Similar to native DLPs, when rDLPs are treated with $CaCl_2$ to remove VP6, dialyzed in low-salt buffer, and

incubated with Mg^{2+} and NTPs, they are capable of synthesizing dsRNA from exogenous viral +RNA templates. This system has been utilized to define protein-protein interactions and individual viral proteins contributing to replication [49]. A fourth strategy for cell-free RV replication has been developed from the expression and purification of rVP1 and rVP2 separately [49]. Co-incubation of purified rVP1 and rVP2, Mg^{2+}, NTPs, and +RNA templates in low-salt buffer is sufficient for replicase activity *in vitro*. This system has been used to define the minimal viral protein components necessary for replicase activity and to identify, via mutagenesis, critical residues and domains of VP1 and VP2 required for replication [28, 50, 51]. Thus, each of the cell-free systems has been an invaluable tool for molecular dissection of RV genome replication.

4.2.2 Roles of VP1 and VP2 in Genome Replication

VP1 and VP2 play critical roles in RV genome replication. A requirement for VP2, but not VP6, for genome replication was initially demonstrated using viruses with temperature-sensitive lesions mapping to the genes for VP2 or VP6 [52, 53]. Confirmation of this observation came from the finding that cores, which lack VP6, are capable of catalyzing dsRNA synthesis [29]. Subsequent studies using baculovirus-expressed proteins have shown that VP1 and VP2 are essential for replication whereas VP3 and VP6 are dispensable [30, 48]. Interestingly, replication is most efficient when the molar ratio of VP1:VP2 is around 1:10 [28]. This ratio approximates the molar ratio of VP1:VP2 present at each icosahedral vertex of a native core. Taken together, these findings demonstrate that the minimal viral protein components of the RV replicase are VP1 and VP2 and suggest that replication occurs in association with partially or fully assembled core-like structures.

4.2.2.1 VP1

Results from several biochemical studies have identified VP1 as the RV RdRP. These findings include the observation that VP1 contains amino acid motifs, including the GDD active-site sequence, typical of viral RdRPs [12, 13, 14]. Also, VP1 can bind NTPs, and RNA replication is inhibited by cross-linking photoreactable nucleotide analogs to VP1 [15]. Furthermore, of the components of RV open cores, only VP1 binds specifically to the 3′ end of viral +RNAs [15, 50, 54]. Finally, addition of VP1 to VP2-containing fractions from open cores stimulates replication in a dose-dependent fashion [54].

More recently, the X-ray crystal structure of VP1 has confirmed its identity as the RV RdRP and highlighted some of its distinguishing features [50]. VP1 is composed of three domains: a protruding *N*-terminal domain, a polymerase domain, and a *C*-terminal "bracelet" domain (Fig. 4.2). The polymerase domain is formed by right-handed palm, fingers, and thumb subdomains and includes the six canonical motifs (A-F) common to RdRPs [55, 56, 57, 58]. The palm, which is formed by a four-stranded β-sheet and three α-helices, contains the four conserved aspartic acid residues of the catalytic site [59, 60] and the putative priming loop. The fingers subdomain contains a β-strand followed by three α-helices [50]. A loop of the thumb encloses the catalytic core of the polymerase through its interaction with the fingers. The *N*- and *C*-terminal domains imperfectly sandwich the polymerase domain. Four tunnels extend from the surface into the catalytic core of the molecule (Fig. 4.2). This arrangement creates a hollow cage-like structure similar to the structure of λ3, the RdRP of reovirus, another *Reoviridae* family member [61]. The *N*-terminal domain of VP1 is situated such that it covers the thumb subdomain on one side [50]. On the opposite side, the *C*-terminal bracelet creates a rather flat face contacting all three polymerase subdomains and encircling the common tunnel used for the exit of dsRNA and –RNA.

The overall structural similarity of VP1 to that of reovirus λ3 [61] permitted assignment of functions for the four tunnels leading into and away from the catalytic core [50] (Fig. 4.2). Template RNA enters through a tunnel at the 'top' of the polymerase, between the fingers and thumb subdomains, and NTPs enter the catalytic core via a tunnel in the 'back' of the polymerase situated just above the palm subdomain. During transcription, the entering template is –RNA from a melted dsRNA gene segment. Nascent +RNA transcripts exit through a tunnel at the 'bottom' of the polymerase, whereas the –RNA template exits via the 'front' tunnel that is encircled by the *C*-terminal bracelet. The nascent +RNAs are then capped by VP3 and shunted out of the core into the cytoplasm of the infected cell [10]. The –RNA is used for multiple rounds of transcription, and thus by an incompletely understood mechanism must re-associate with the template entry tunnel. In contrast, VP1 supports only a single round of genome replication, and the template for this is +RNA. From the +RNA template, a complementary negative-sense strand of RNA is synthesized, and the dsRNA product exits through the same tunnel that is used for –RNA exit during transcription. Newly-formed dsRNA genome segments remain in the core of assembling virions.

A short α-helix formed by the *C*-terminus of VP1 extends into the tunnel used for exit of dsRNA and –RNA from the catalytic core, forming a "plug" [50] (Fig. 4.2). In the absence of the plug, this tunnel would be sufficiently wide to permit dsRNA to exit. However, in its presence, the plug decreases

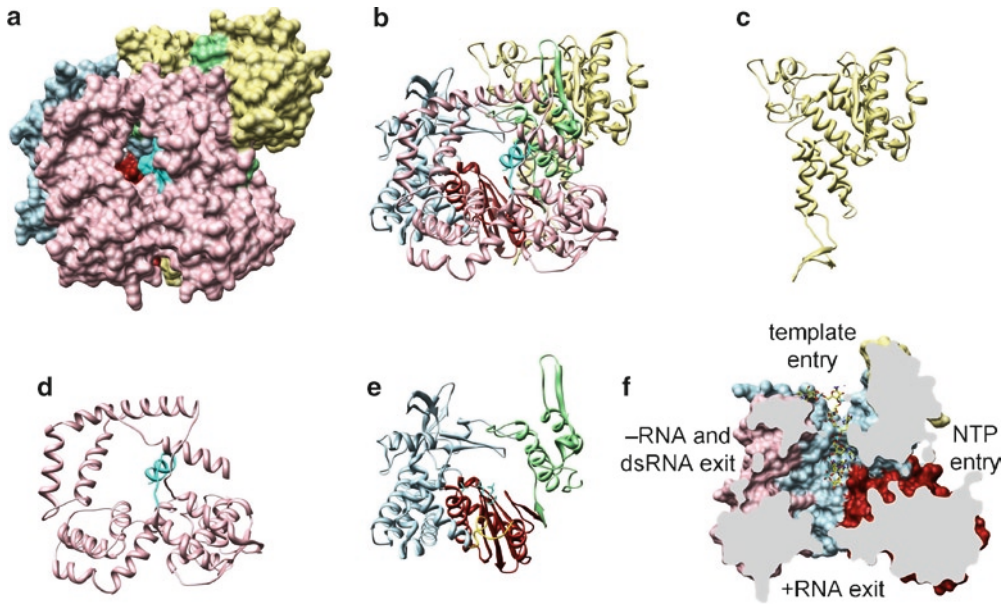

Fig. 4.2 Structure and domain organization of VP1. Surface representation (**a**) and ribbon drawing (**b**) of the full-length protein and ribbon drawings of the *N*-terminal (**c**), polymerase (**d**), and *C*-terminal (**e**) domains of VP1. The *N*-terminal domain is colored yellow, the *C*-terminal (bracelet) domain is pink, and the *C*-terminal plug is cyan. Subdomains of the polymerase domain are colored light blue (fingers), red (palm), and green (thumb). The priming loop of the palm subdomain is colored gold. Side chains of catalytic aspartic acid residues are shown in cyan in (**e**). (**f**) Sagittal cutaway of (**a**) following a 90° rotation to the left. Tunnels are labeled according to putative function. A bound RNA oligonucleotide (3′CS+) in the template entry tunnel is colored by element

the diameter of the exit tunnel to such a degree that dsRNA egress would be inhibited. Thus, it is hypothesized that the plug must move during replication. In summary, VP1 is a compact molecule whose structure is specifically tailored to channel RNA templates and the products of transcription and genome replication to appropriate sites. Nonetheless, purified VP1 alone does not replicate +RNA, but requires the presence of VP2 [54].

4.2.2.2 VP2

VP2 is an ~100 kDa protein that self-assembles into multimers and forms the RV core shell [10, 62]. VP2 monomers are composed of a conserved *C*-terminal shell domain and a variable *N*-terminal domain [63]. In RV virions, the VP2 lattice is comprised of 60 asymmetric dimers each containing VP2 monomers in two slightly different conformations [20] (Fig. 4.3). Thus, each vertex of the icosahedral VP2 shell is formed by a decamer. While the shell domain of VP2 can be visualized in cryo-electron micrograph image reconstructions, very little structural information exists for the *N*-terminal domain of VP2 (residues ~1-100). However, the ten *N*-terminal domains associated with each vertex of the core are believed to interact, producing an inner turret that extends away from the inner face of the core. Evidence that the inner turrets serve as points of contacts between VP2 and VP1, VP3, and/or RNA is limited [19, 20, 51]. The bulk comes from the analysis of DLPs and rDLPs by cryo-electron microscopy, which indicates the presence of VP1, VP3, and genomic dsRNA at the inner five-fold axes of the core in close proximety to the putative inner turrets [19].

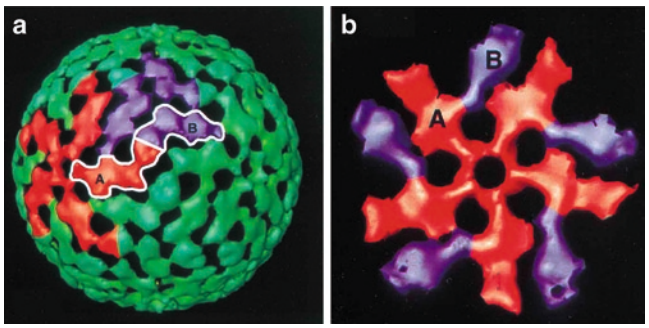

Fig. 4.3 Structural organization of the VP2 shell. (**a**) Cryoelectron micrograph image reconstruction showing the surface of the VP2 shell. A dimer of quasi-equivalent VP2 molecules is outlined in white, with type A and B monomers labeled. Monomers of type A VP2 at a five-fold axis and type B VP2 at a three-fold axis are colored red and purple, respectively. (**b**) Pentamer of asymmetric VP2 dimers, centered around the five-fold icosahedral vertex and colored by type as in (**a**). Images shown with permission from B. V. V. Prasad

In addition, examination of rDLPs formed with mutant VP2 bearing an *N*-terminal truncation has shown that these particles cannot encapsidate VP1 and VP3, suggesting that

the *N*-terminal domains may also direct recruitment of VP1 and VP3 into the core [51]. Studies with isolated VP2 have shown that the protein binds strongly but not specifically to +RNA and that this activity requires the *N*-terminus of the protein [11, 30]. Along with abrogating its RNA binding activity, deletion of the *N*-terminal domain also hinders the ability of VP2 to support the RNA polymerase activity of VP1 in cell-free replication assays [30]. While the impairment of dsRNA synthesis may stem simply from the poor RNA binding activity of the mutated VP2, it may also result from the inability of VP1 to interact with VP2 through its *N*-terminal domain. However, specific binding of VP1 by VP2 has yet to be experimentally observed. Although necessary for dsRNA synthesis, VP2 alone cannot catalyze replication [30, 49] and does not possess any obvious polymerase or helicase sequence motifs that might be suggestive of a specific function. Thus, while VP2 is an essential cofactor for VP1 function, its role in RNA synthesis remains unclear.

4.2.3 RNA Requirements for VP1 Recognition and Genome Replication

RV +RNAs are generally monocistronic, with 5′ and 3′ UTRs that are conserved among homologous genes and short 5′ and 3′ CSs that are conserved among all genes [14, 26, 31, 32] (Fig. 4.4). During assembly of progeny virions, VP1 must recognize and replicate +RNA templates. Several *cis*-acting signals that play roles in RV replication have been mapped to specific regions of +RNAs using *in vitro* replicase assays.

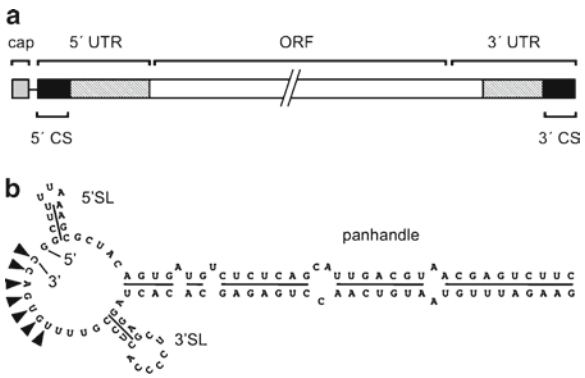

Fig. 4.4 Organization and predicted structure of RV +RNA. (**a**) Linear schematic showing relative locations of the 5′ cap, 5′ and 3′ UTRs, 5′ and 3′ CSs, and open reading frame (ORF) of an RV +RNA. (**b**) Interaction and predicted structure (mfold) of the 5′ and 3′ termini of group A RV gene 11 +RNA. A portion of the full-length folded molecule that includes the termini is shown. Arrowheads indicate the 3′CS+. The termini and 5′ and 3′ stem-loop (SL) structures are indicated. Modified from [46]

The most critical signal to replication is formed by the 3′ CS of the +RNA (3′CS+, 5′-UGUGACC-3′) [14, 26, 27, 30]. This signal is not only necessary for replication but also contains sufficient information to direct the replication of nonviral RNAs, albeit at reduced levels [27]. These characteristics led to the identification of the 3′CS+ as the minimal essential promoter for replication [30, 47, 48]. Sequences that enhance replication can be found in the 5′ UTR and in the 3′ UTR upstream of the 3′CS [27].

Sequences in the 3′CS+ are important for recognition by VP1 as well as replication [28]. VP1 contains a single binding site for the 3′-end of RNAs; this site is one and the same as the template entry tunnel. The binding site interacts with the 3′CS+ both specifically and nonspecifically. Specific recognition is mediated by base-specific interactions between the polymerase and the UGUG portion of the 3′CS+. Thus, the two extreme 3′ nucleotides (CC) of the 3′CS+ are not required for recognition by VP1 [28]. However, these two terminal residues are critical for replication, which suggests recognition and replication signals in the 3′ terminus of RV +RNAs are distinct. Additional VP1 recognition signals have been mapped in RV +RNAs to regions of the 3′ UTR upstream of the 3′CS+ [28].

The identification of important replication signals at both ends of RV +RNAs led to the suggestion that the ends might interact in *cis*. Indeed, the termini are predicted to stably interact to form panhandle structures, with the 3’CS+ extending as a single-stranded tail [46, 64, 65] (Fig. 4.4). Associated with the panhandles are stem-loop structures that are unique to each of the 11 different RV +RNAs and suspected to have roles in gene-specific packaging. Deletions of the 5′ terminus of group A +RNA diminish replication, possibly due to loss of panhandle interactions or stem-loop structures [46]. The 5′CS+ may also be involved in the recognition of +RNAs by the viral RdRP, although these sequences are less critical for recognition than are the residues of the 3′CS+ [46]. The extreme 3′ termini of group A +RNAs are predicted to remain unpaired in panhandle structures [66]. Mutations in the 5′ end of +RNA that increase complementarity between the 3′ and 5′ ends significantly decrease the efficiency of minus strand synthesis [66]. Compensatory mutations in the 3′ end of the mutant RNAs that reduce base-pairing are capable of restoring replicase activity. Thus, structural elements as well as sequence may contribute to recognition and replication of +RNAs by VP1. Regardless of the apparent requirement for an unpaired 3′CS+ for replication, mutant +RNAs with fully complementary 3′ and 5′ termini can compete well with wild-type +RNAs for VP1 binding, although the mutant +RNAs are extremely poor templates for replication [66]. This finding suggests that recognition of the 3′CS+ by VP1 is not the sole determinant of efficient RV RNA replication.

4.2.4 Replication Initiation

Formation of RV initiation complexes is a salt-sensitive process that requires GTP, Mg^{2+}, +RNA, VP1, and VP2 [67]. Once formed, initiation complexes are salt-stable and able to catalyze –RNA elongation in the presence of > 1 M NaCl. RV initiates replication following VP1 recognition of the minimal promoter region of the 3′CS+ [68, 69]. VP1 binding of +RNA alone is insufficient for initiation complex formation, as deletion of portions of the 3′CS+ (3′CC) results in +RNAs that are efficiently recognized by VP1, but poorly replicated [28]. This finding suggests that, at least in some cases, *cis*-acting signals involved in recognition by VP1 are distinct from those involved in initiation complex formation.

RV replication is inhibited in the presence of salt [29]. However, this inhibition can be overcome by the pre-incubation of open cores with +RNA, ATP, GTP, CTP, and Mg^{2+} prior to addition of salt [67]. Thus, RV replication can be separated into salt-sensitive initiation and salt-insensitive elongation phases [67]. Incubation of open cores with +RNA and GTP or UTP, prior to the addition of salt and the other NTPs, also supports replication [67]. Since the +RNA template ends with two cytosines, this finding is not surprising in the case of GTP. However, the mechanism by which UTP could substitute for the function of GTP is less clear. One possibility is that UTP could support formation of an initiation complex by basepairing with the third residue from the 3′ terminus (A3) of the 3′CS+ (5′-UGUGACC-3′). If so, this suggests some freedom of movement for the 3'CS+ within the catalytic core, to allow alignment of the A3 residue and UTP at the incoming nucleotide (N) or priming (P) site.

Initiation of dsRNA synthesis for group A RVs may also involve the template-independent synthesis of (p)pGpG moieties that, through basepairing with the two 3′-terminal cytosines of the +RNA template, prime – strand synthesis [67]. The identity of the core protein responsible for synthesis of (p)pGpG remains unresolved [67].

Similarly, the basis for the essential need of VP2 in the formation of salt-stable initiation complexes is not known but is surprising given that VP1 alone can specifically bind to recognition signals in +RNA [28].

4.2.5 Priming

The primer-independent mechanism used by RV VP1 and reovirus λ3 to initiate RNA synthesis requires alignment of the two terminal residues of the template strand with nucleotides in the P and N sites [50, 61, 70]. For λ3, the priming loop, a flexible element of the palm subdomain, forms a molecular platform that helps support the positioning of an NTP in the P site [61] (Fig. 4.5). Other residues are also involved in stabilizing NTPs in the N and P sites; these include arginine residues of motif F of the fingers subdomain and the catalytic aspartates of the palm subdomain. The λ3 priming loop moves away from the catalytic core during RNA synthesis, permitting RNA translocation. In the case of VP1, a similar mechanism of initiation is predicted to occur [50]. For example, motif F of VP1, like that of λ3, includes three similarly-situated arginine residues that project toward the N and P sites (Fig. 4.5). VP1 also contains an element of the palm subdomain that is thought to serve as a priming loop. However, unlike that of the λ3 structure, the putative VP1 priming loop is bent by 90°, such that it is incapable of supporting a nucleotide in the P site [50, 61]. The retracted conformation of its priming loop may explain why VP1, by itself, lacks polymerase activity. The interaction of VP2 with VP1 may cause the priming loop to close such that it can stabilize an NTP in the P site. The retracted conformation of the priming loop may represent the position adopted during elongation [50].

Soaks of crystallized VP1 with an oligonucleotide representing the 3′CS+ (5′-UGUGACC-3′) have revealed the atomic basis for the specific recognition of this sequence and provided additional insight into RNA initiation mechanisms [50] (Fig. 4.5). The 3′ CS+ is held in position in the VP1 tem-

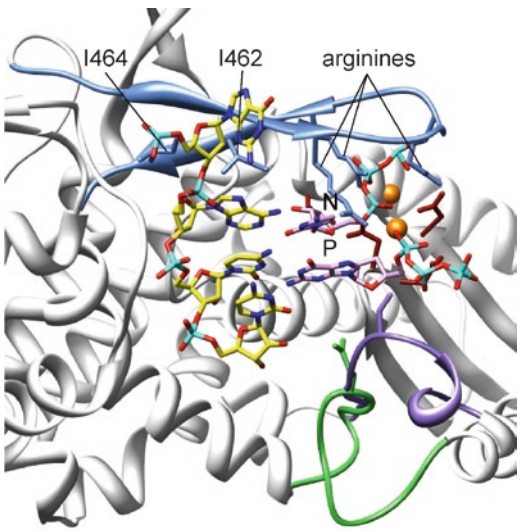

Fig. 4.5 Features of the RV RdRP catalytic site. Ribbon representation of a portion of VP1 in complex with the four terminal residues of the 3′CS+ (GACC; sticks with yellow bases, cyan backbone). NTPs (sticks with pink bases, cyan backbone) in the N and P sites and Mg2+ ions (orange spheres) are modeled from the λ3 structure. Motif F of the fingers domain is colored blue. Side chains of residues in motif F referred to in the text are labeled and shown as stick representations. Side chains of catalytic aspartates are shown as maroon sticks. The priming loops of VP1 and λ3 are colored green and purple, respectively. Residues (sticks) in the λ3 (S561) and VP1 (E492) priming loops thought to be involved in supporting an NTP in the P site are shown

plate entry tunnel through ten hydrogen bonds with phosphates and ribose rings of the RNA backbone and eight hydrogen bonds with the UGUG bases of the 3′CS+, contributed by residues of the fingers and thumb subdomains and the *N*-terminal domain. Specific interactions with bases in the template entry tunnel of an RdRP have rarely been observed and may explain the high affinity of VP1 for the 3′CS+. Interestingly, the 3′ terminal C of the 3′CS+ (C1) overshoots the register for initiation by a single nucleotide, in comparison to similar crystal soaks performed with λ3 [50, 61] (Fig. 4.5). Thus, the C2 and A3 residues of the 3′CS+, rather than C1 and C2, are aligned with the P and N sites, respectively [50]. In this position, the C1, C2, and A3 bases stack against each other. A3 is situated below the hydrophobic side chains of Ile462 and Ile464 of the F motif, which pushes the downstream template away from the catalytic core and towards the finger wall. An RNA template containing an inserted A3 (5′-UGUGAACC-3′) overshoots the register by two nucleotides, whereas deletion of A3 (5′-UGUGCC-3′) results in a template that is in register in the P and N sites. In all of the VP1 crystal soaks to date, the priming loop has remained in its retracted position. These observations confirm that base-specific interactions with the UGUG of the 3′CS+ help to define the template position in the entry tunnel and catalytic core. Despite the effect that insertion of A3 (5′-UGUGAACC-3′) or deletion of A3 (5′-UGUGCC-3′) has on the register of +RNAs in the catalytic core, the RNAs remain functional as templates for dsRNA synthesis. Moreover, replication of the RNAs remains dependent on VP2. Even though deletion of A3 brings the 3′ terminal C of the RNA template into register with the P site, this is not sufficient to allow the polymerase to synthesize RNA in the absence of VP2. Thus, the role of VP2 in VP1 activation must extend beyond simply triggering an in-register realignment of the 3′ terminus of the +RNA relative to the P and N sites. As discussed above, an additional VP2-dependent requirement may be to induce movement of the priming loop [50].

4.3 Packaging and Assembly

Three distinct types of RNA-containing RIs referred to as precore, core, and double-layered RIs have been isolated from RV-infected cells [71]. The RV structural proteins comprising precore RIs are VP1 and VP3. Core RIs contain VP1, VP2, and VP3, and double-layered RIs are formed from VP1, VP2, VP3, and VP6. Pulse-labeling experiments and differences in composition and diameter among the three types of particles suggest that assembly occurs sequentially in the infected cell, with VP2 added to precore RIs to form core RIs, followed by addition of VP6 to form double-layered RIs [71]. The association of NSP2 and NSP5 with RIs suggests that these nonstructural proteins may play important roles in the early stages of RV capsid assembly [71].

Each vertex of an RV core contains a single copy of VP1 [15] and VP3 [18]. VP1 (or a VP1-VP3 complex) specifically recognizes the 3′CS+ of viral +RNAs [50]. This recognition may serve as the first step in assembly, followed closely by interactions with (VP3 and) VP2 (Fig. 4.6). VP2 forms the core shell and is thought to anchor VP1, VP3, and the genome at the inner surface of the VP2 lattice, just below the five-fold icosahedral vertices [19]. Specific multimeric intermediates of the VP2 lattice such as decamers have not been identified,

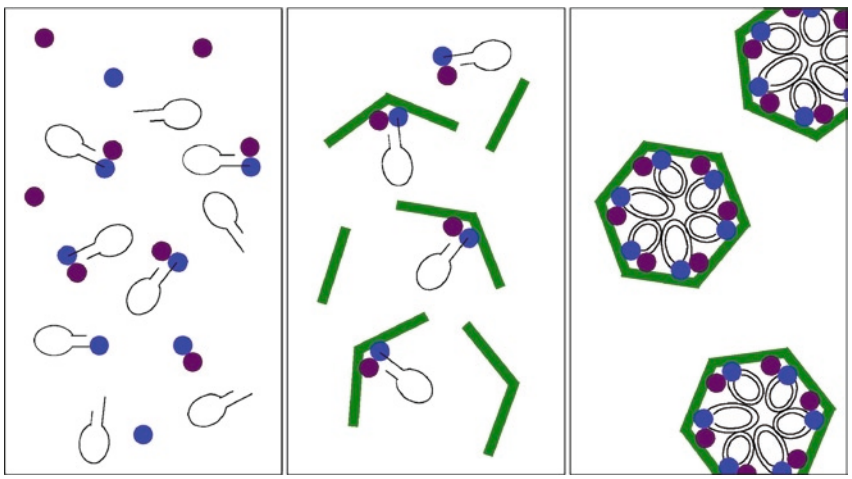

Fig. 4.6 Model of packaging and assembly. Sequential steps in RV genome packaging and assembly are represented in panels proceeding from left to right. (*left*) +RNA (black loops with panhandles), VP1 (blue), and VP3 (purple) associate, as in precore RIs. (*middle*) Complexes of RNA, VP1, and VP3 interact with VP2 (green), as it begins to self-assemble to form the core shell. (*right*) Genome replication, yielding dsRNA, accompanies the formation of intact cores

but they may exist in the infected cell and form assembly subunits together with single copies of VP1, VP3, and a +RNA. RNase sensitivity assays suggest that core formation and genome replication are temporally linked [40]. It is likely that +RNAs contain gene-specific signals that direct packaging, as the assembling particles must incorporate 11 different template +RNAs [72]. RNA-RNA interactions may provide the specificity needed to assemble the appropriate constellation of +RNAs in a single particle. However, in the absence of a reverse-genetics system for RV, this assembly model has been difficult to test.

4.4 Transcription

In comparison to numerous studies detailing the process of RV replication, there are few studies addressing RV transcription. However, transcription can be expected to be a more complicated process, given its requirement for a helicase-like activity to unwind the dsRNA template for +RNA synthesis, its involvement of all four tunnels of the viral RdRP, and its interplay with the VP3 capping enzyme [10, 50] (Fig. 4.7).

The differences between the processes account, at least in part, for the differences seen in the array of viral proteins necessary for transcription and replication [48, 53, 73]. Unlike replicase activity, which solely requires VP1 and VP2 [48], transcriptase activity has been detected only for particles that contain VP1, VP2, VP3, and VP6 [74]. The dependency on VP6 for transcription has been demonstrated by removal of the VP6 shell from DLPs using chaotropic agents,

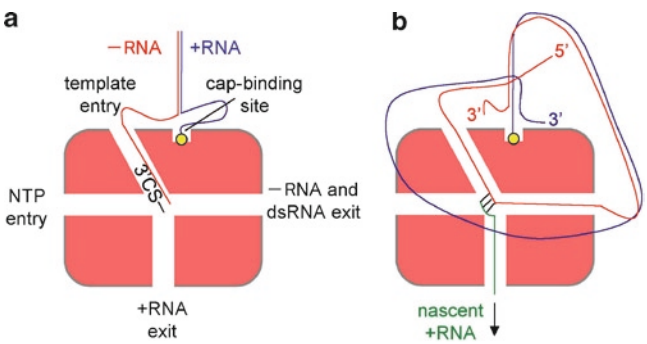

Fig. 4.7 Models of events in RV transcription. (**a**) Schematic representation of VP1 (coral) and RNA during transcription initiation. Functions of the four tunnels in VP1 are indicated. The + strand of dsRNA is represented by a blue line, and the – strand is red. The 5′ cap on the + strand is represented as a yellow circle in the cap-binding site. The 3′ terminus of the – strand is depicted within the template entry tunnel. (**b**) Schematic representation of VP1 and RNA during active transcription, colored as in (**a**). Annealing of the exiting – strand to the 5′ anchored + strand of the dsRNA template may guide the 3′CS- back to the template entry channel, supporting a subsequent round of transcription

which abolishes transcriptional activity, and the addition of purified VP6 to cores, which can restore this activity [75]. It is possible that VP6 either provides structural support for the channels through which transcripts exit the DLP or that VP6 imposes changes on VP1, VP2, or VP3 that permit transcription to occur. A functional *in vitro* transcription system using recombinant proteins has not yet been reported.

As noted above, VP1 binds specifically to the 3′CS+, which is critical for efficient replication [28, 50]. Transcription is initiated from the 3’ terminus of RV –RNA, which for group A RVs has the 3′CS, 5′-$(A/U)_7$GCC-3′ (3′CS-) [76]. In contrast to the base-specific recognition observed for the 3′CS+, VP1 exhibits only nonspecific recognition of the 3′CS- [50]. Base-specific recognition of the 3′CS- may not be as important as it is for the 3′CS+, since initiation of transcription occurs within the core, leaving the RdRP with no option other than using a viral RNA as a template. In contrast, base-specific recognition of the 3′CS+ by the RdRP provides an avenue by which the viral +RNA can be recognized within the viroplasmic milieu and form a stable complex sufficient to support RNA packaging into an assembling core. In the course of its life cycle, VP1 switches modes from directing genome replication to transcription. Transcription requires the 3’ end of the –RNA of a genome segment to enter the template entry tunnel of VP1 and move to the catalytic core where the 3′CS- can support +RNA initiation (Fig. 4.7). Since VP1 lacks specificity for the 3′CS-, an alternative mechanism may be in place that helps guide the 3′ end of the –RNA to the entry tunnel. Possibly this mechanism involves the action of a cap-binding site predicted to exist on the surface of VP1 immediately adjacent to the template entry tunnel. Through interaction with the 5′-cap of the +RNA of a genome segment, the cap binding site may indirectly recruit the 3′ end of the annealed –RNA to the template entry tunnel. Evidence supporting the existence of such a cap-binding site comes from soaks of VP1 crystals with GTP, which have revealed a binding site for the nucleotide adjoining the template entry tunnel of VP1 [50]. An architecturally similar cap-binding site has been identified at a corresponding position on λ3 [61].

Unlike a +RNA, which is used but once by VP1 in templating RNA synthesis, the –RNA of a genome segment may be used repeatedly, thereby templating multiple rounds of transcription. RV utilizes a conservative mechanism of transcription, with the parental + and – strands of a genome segment reannealing as the – strand exits the polymerase. Given the assumption that the 5′-end of the parental + strand remains anchored to the cap-binding site throughout transcription, annealing of the exiting – strand template to the parental + strand could form the basis by which the 3′-end of the –RNA could be redirected back to the template entry tunnel, in support of another round of transcription.

4.5 Conclusions

RV genome replication, transcription, and packaging all represent multifaceted highly coordinated processes that require intermolecular communication among numerous viral RNAs and proteins. A central player in each of these processes is viral RdRP VP1. While many mysteries surrounding mechanisms of VP1 function have been solved, numerous questions remain to be explored. A primary focus of future studies no doubt will be on understanding the mechanisms that govern packaging and assembly in infected cells and elucidating the signals that switch VP1 from an inactive enzyme to an active polymerase driving transcription and replication.

In summary, some of the specific functions that must be achieved by an RNA virus are replication of the viral genome, packaging of the genome into progeny particles, and generation of positive-sense transcripts. RVs are TLPs that encapsidate a genome composed of eleven segments of dsRNA. RV genome replication occurs concurrently with assembly and is one of the most well-studied processes in the infectious cycle. The viral RNA-dependent RNA polymerase, VP1, plays a prominent role in genome replication, packaging, and transcription. Biochemical studies have revealed that rotavirus genome replication is a highly coordinated process involving signals from both viral RNA and proteins. Structural studies of VP1 indicate that it is a compact molecule with architectural features specifically tailored toward performing its multiple functions. Taken together, these studies provide insights into the mechanisms of rotavirus genome replication, assembly, and transcription.

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Chapter 5
Human T-Cell Leukemia Virus Type 1, Cellular Transformation, and Adult T-Cell Leukemia

Junichiro Yasunaga and Kuan-Teh Jeang

5.1 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) belongs to the delta type retroviruses, which also include bovine leukemia virus (BLV), human T-cell leukemia virus type 2 (HTLV-2), and simian T-cell leukemia virus (STLV) [1]. HTLV-1 is the first retrovirus that was identified as a causative agent of several human diseases, including adult T-cell leukemia (ATL), HTLV-1 associated myelopathy (HAM)/tropical spastic paraparesis (TSP), and HTLV-1 associated uveitis [2, 3]. ATL is a neoplastic disease of CD4-positive T lymphocytes, which is characterized by pleomorphic tumor cells with hypersegmented nuclei, called "flower cells." HTLV-1 encodes in its pX regaion a potent oncoprotein, Tax. Tax is a transcriptional activator protein that has been reported to activate and inactivate the transcription of many cellular genes. Ectopic over expression of the HTLV-1 Tax has been found to transforms rodent cells and immortalizes human cells *ex vivo* [4, 5, 6, 7, 8]. Moreover, transgenic mice engineered to express Tax also form tumors readily [9, 10, 11, 12]. Taken together, these findings strongly support a physiological role for Tax in ATL leukemogenesis. Nevertheless, there are reports that *tax* transcripts are detected in only ~40% of transformed ATL cells and that only a small proportion of HTLV-1 carriers (6.6% for males and 2.1% for females in Japan) develop ATL after a long latency period (about 60 years in Japan, and 40 years in Jamaica) from the initial infection [1]. These observations argue that besides Tax, other factors such as additional viral genes, host cell, and immune factors may also contribute to the development of ATL. Compatible with the above thinking, it was recently discovered that the HTLV-1 bZIP factor (HBZ), which is encoded by the minus strand of the provirus, is ubiquitously expressed in all ATL cells and possesses cell proliferative function in T cells [13]. HBZ may also contribute to leukemogenesis.

5.2 HTLV-1 Provirus and Infection of Cells

5.2.1 Structure of HTLV-1 Provirus

The HTLV-1 proviral genome has *gag*, *pol* and *env* genes, flanked by long terminal repeat (LTR) sequences at both ends (Fig. 5.1). The 5′ LTR serves as the virus' transcriptional promoter; Pol provides reverse transcription and integration functions to copy viral RNA into DNA and to integrate the DNA into the host cell's chromosomes; Gag and Env are structural proteins for the HTLV-1 virion. In the HTLV-1 genome, a unique region is located between *env* and the 3′-LTR; this region is named pX. The plus strand of the pX region encodes viral regulatory proteins Tax, Rex, p12, p13, p30, and p21, which are implicated in viral infectivity and the proliferation of infected cells. The HBZ protein is encoded by the minus strand of pX [1]. There are spliced and unspliced forms of HBZ [13, 15].

5.2.2 Clonal Proliferation of HTLV-1-Infected Cells

HTLV-1 integrates randomly into the host's chromosomes [16]. Sequential analyses of integration sites in HTLV-1-infected subjects verify that the proliferation of HTLV-1-infected cells is clonal and persistent [17, 18, 19]. Experiments using a mouse model of initial HTLV-1 infection indicate that clonal expansion of primary infected cells predominate over secondary *de novo* infection of previously uninfected cells [20]. HTLV-1 induces the clonal proliferation of infected cells. These clonal cells survive durably; and over time, it is envisioned that one of these clones become fully transformed and proliferate as ATL.

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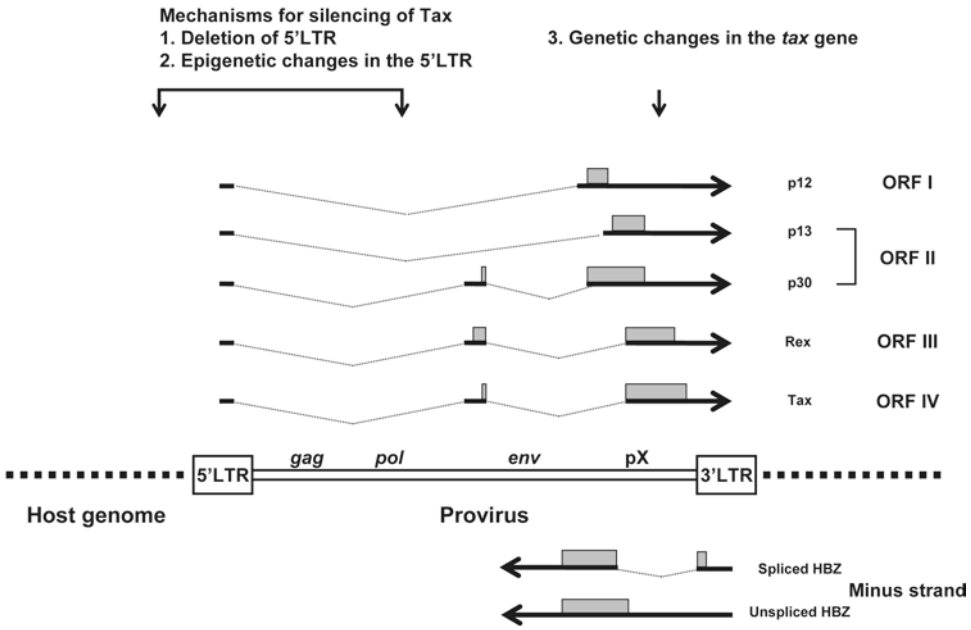

Fig. 5.1 Structure of the HTLV-1 provirus. The *gag*, *pol*, and *env* structural genes are flanked by 5′ and 3′ long terminal repeats (LTRs). Regulatory genes [p12(I), p13(II), p30(II), Rex(III), Tax(IV)] are encoded by the plus strand of the pX region in four different open reading frames (ORFs). The HTLV-1 basic leucine zipper factor (HBZ) gene is located on the minus strand of the provirus. Tax is frequently inactivated in ATL cells by (i) deletion; (ii) epigenetic changes in the 5′LTR; and (iii) genetic alteration of the *tax* gene. Roman numerals indicate the respective ORF used for the translation of the indicated protein

5.3 The Oncogenic Functions of Tax

In order to transform cells, HTLV-1 must overcome the tendency of virus-infected cells to undergo apoptosis and/or senescence. The virus must also defeat the cellular checkpoints that censor genetic damage, and the virus must stimulate proliferative factors that induce cell-cycle progression and cellular division. Tax is a central player in cellular transformation with pleiotropic functions used to conquer those cellular machineries (Fig. 5.2).

5.3.1 Cell Survival

Tax activates two major cellular pathways for quelling apoptosis; the first is the Akt pathway, and the second is the NF-κB pathway. Recent findings suggest that HTLV infection may also upregulate cellular microRNAs, which could function to prevent cellular apoptosis.

5.3.1.1 Activation of the Akt Pathway

Akt is a serine/threonine kinase that influences cell survival and proliferation and is regulated by phosphatidylinositol 3-kinase (PI3K) through site-specific phosphorylation, primarily on Ser473 [21]. Activated Akt signals through downstream transcription factors such as activator protein 1 (AP1), which is highly expressed in many invasive human cancers, including ATL [22]. Tax promotes Akt phosphorylation by directly binding PI3K, and, consequently, the survival and proliferation of virus-infected cells are increased [23].

5.3.1.2 Activation of the NF-κ B Pathway

NF-κB is a second major survival pathway engaged by HTLV-1. Many human cancers have activated NF-κB; although NF-κB is tightly regulated in normal T cells, this pathway is constitutively active in HTLV-1-infected cells [24]. Activation of NF-κB by Tax occurs predominantly in the cytoplasm. Cytoplasmic Tax was shown to bind IKKγ. This binding triggers the phosphorylation of IKKα and IKKβ, which form a complex with IKKγ. Subsequently, this activated IKK complex phosphorylates IκBα, leading to its proteasome-mediated degradation, which frees IκBα-sequestered cytoplasmic NF-κB to migrate into the nucleus where it activates the transcription of NF-κB -responsive genes [25]. Tax can also stimulate a non-canonical NFκB pathway through the IKKα-dependent processing of the NF-κB p100 precursor protein to its active p52 form. This alternative pathway is

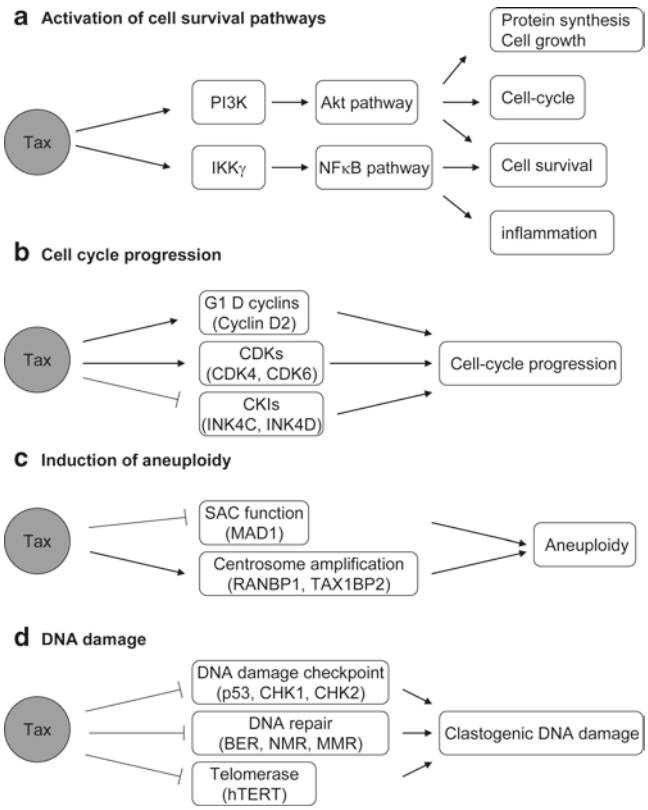

Fig. 5.2 Tax functions that affect cellular metabolism. Tax has multiple functions inside cells. Tax inhibits apoptosis of HTLV-1-infected cells by activating Akt and NF-κB pathways. Tax accelerates cell cycle progression by activating cyclins, cyclin dependent kinases (CDKs), and suppressing CDK inhibitors (CKIs). Aneuploidy is induced by Tax through a loss of spindle assembly checkpoint (SAC) function. Tax induces supernumeral centrosomes leading to multipolar mitosis. Finally, Tax causes clastgenic DNA damage by suppressing DNA damage checkpoints, DNA repair, and inhibiting hTERT transcription. BER, base excision repair; NER, nucleotide excision repair; MMR, DNA mismatch repair

activated by Tax binding to IKKγ and p100, in an IKKα–IKKγ–p100 complex that lacks IKKβ [26].

5.3.1.3 Upregulation of miRNAs that Inhibit Apoptosis

Recently miRNA expression in HTLV-1-transformed cell lines and primary ATL cells has been profiled using microarray techniques. It was found that six miRNAs are consistently upregulated in these ATL cells [27]. Among them, miR-93 and miR-103b target a tumor suppressor protein, tumor protein 53-induced nuclear protein 1 (TP53INP1). TP53INP1 activates an apoptosis pathway that guards against cellular transformation. It was postulated that HTLV-1 Tax oncoprotein can transcriptionally activate both miR-93 and miR-103b to reduce the expression of TP53INP1 and thus suppress cellular apoptosis.

5.3.2 Cell Cycle Progression

Tax also provides significant mitogenic activity, especially at the G1–S-phase transition [28, 29, 30], by provoking several cellular activities.

5.3.2.1 Upregulation of G1 D Cyclins

Increased cyclin D2 expression occurs through direct activation of its promoter by Tax. Cyclin D2 expression is also enhanced by interleukin-2 (IL-2) receptor signaling [31], which is observed in Tax-expressing cells. Indeed, it has been shown that high IL-2 secretion occurs in HTLV-1-infected T lymphocytes.

5.3.2.2 Activation of Cyclin-Dependent Kinases (CDKs)

Tax can activate CDKs such as CDK4 through direct protein binding [32, 33]. Activated CDK4 hyperphosphorylates the retinoblastoma (RB) protein. Tax can also interact with RB directly, leading to its proteosomal degradation [34]. The phosphorylation and degradation of RB free the otherwise sequestered E2F1 transcription factor. Unfettered E2F accelerates the cell's cell-cycle transition from G1 to S [30]. There is also evidence that Tax can activate the transcription of the *E2F1* gene [35].

5.3.2.3 Downregulation of CDK Inhibitors (CKIs)

Tax has been reported to transcriptionally repress CKIs such as INK4C [28], INK4D, and KIP1 [36]. The mechanism for this activity is through the direct binding of Tax to E-box proteins, which would otherwise activate the promoters for INK4C and INK4D [37, 38]; a secondary mechanism may be through Tax binding to and inactivating INK4A and INK4B [39, 40].

5.3.2.4 Others

Although a cellular factor p21/WAF1 was identified as a CDK inhibitor that can act to suppress cell cycle progression, subsequent findings show that it can also be an assembly factor which promotes G1–S transition via formation of an active p21/WAF1–cyclin D2–CDK4 complex. This complex can phosphorylate RB. Because Tax can increase the levels of WAF1 in cells, this is another way that Tax can contribute to increased cell-cycle progression [41]. DLG1 is the human homologue of the *Drosophila melanogaster* discs large

tumor-suppressor protein, containing PDZ domains [42]. DLG1 signals downstream of Wnt and frizzled [42], and binds the *C*-terminus of the adenomatous polyposis complex (APC); binding of DLG1 and APC regulates cellular proliferation and cell-cycle transition [43]. Tax has a PDZ-binding motif (PBMs) in its *C*-terminus. Tax has been reported to interact with DLG1 through this motif, and inactivates DLG1 by inducing its hyperphosphorylation and subcellular mis-localization [44, 45].

5.3.3 Cellular Aneuploidy

Structurally damaged DNA and chromosomal numerical abnormalities (aneupoidy) are common in cancers. Because most human cancer cells, including ATL cells, are aneuploid, aneuploidy has been proposed to be causal of transformation. It has been reported that Tax can induce aneuploidy by several mechanisms.

5.3.3.1 Induction of Multipolar Mitoses

Aneuploidy can arise from multipolar mitoses, which happen when more than two spindle poles (supernumerary centrosomes) emerge in a single cell. Tax creates over-amplification of centrosomes by targeting the cellular TAX1BP2 protein, which normally blocks centriole replication [46]; conversely, Tax engages RANBP1 during mitosis and fragments spindle poles, provoking multipolar segregation [47]. These complementary mechanisms can explain the long-standing observations of aneuploidy and frequent multipolar spindles in ATL cells.

5.3.3.2 Inhibition of Mitotic Spindle Assembly Checkpoint (SAC)

Cells have a SAC machinery that preserves the correct number of chromosomes by monitoring the fidelity of chromosomal segregation [48]. SAC proteins include MAD1, MAD2, MAD3/BUBR1, BUB1, BUB3, and MSP1. These proteins function at kinetochores to check for proper microtubule attachment and orderly mitotic chromosome partitioning [48]. The emergence of aneuploid ATL cells implies that these cells lack intact SAC function. Indeed, the loss of SAC function has been experimentally verified in several ATL cell lines [49]. Tax can bind MAD1 and inactivate its function, thus creating a loss of SAC activity [50]. Tax has also been shown to bind and activate the anaphase-promoting complex/cyclosome (APC/C), which functions downstream of the SAC [51]. By interaction with the APC/C, Tax is thought to promote premature mitotic exit and contribute to aneuploidy.

5.3.4 DNA Structural Damage

The chromosomes in ATL cells have clastogenic DNA damage [52]. Although other oncoproteins can induce direct DNA damage through increased reactive oxygen species, such activity has yet to be demonstrated for Tax. However, HTLV-1 infection appears to abrogate the cellular checkpoints and DNA repair functions that monitor and censor ambient structural DNA damage.

5.3.4.1 Inhibition of DNA Damage Checkpoints

It has been suggested that structurally damaged DNA is prevalent in cancer cells because ~ 50% of human cancers have checkpoint-disabling mutations in *TP53*. Interestingly, mutation of *TP53* is infrequent in ATL cells [53]. Nevertheless, the p53 checkpoint is functionally inactivated in ATL cells by the Tax protein [54]. How Tax inactivates p53 is still unclear since several competing mechanisms have been reported. These mechanisms include Tax abrogation of p53 function by competition for the binding of the p300/CBP transcriptional coactivator [55] ; Tax acting through an NF-κB/RelA(p65) pathway to perturb p53 function [56] ; and/or Tax inactivating p53 through another as yet uncharacterized pathway. Finally, Tax can also physiologically interact with CHK1 [57] and CHK2 [58] kinases to attenuate their activities. These two kinases contribute to the proper execution of G1 and G2/M checkpoints.

5.3.4.2 Perturbation of DNA Repair

Tax represses the expression of the DNA polymerase β enzyme used for base excision repair (BER) [59, 60]. In addition, Tax suppresses an alternate repair process, nucleotide excision repair (NER) following UV irradiation through its induction of PCNA (61,62). Further, it has been reported that the expression of human DNA mismatch repair (MMR) genes (such as human MutS homologue 2 (hMSH2)) are reduced in primary ATL cells [63]. These findings support Tax inhibition of DNA repair by several means.

5.3.4.3 Suppression of Telomerase

Chromosome end-to-end fusions and shortened telomeres are common in cancer cells; these findings are also prevalent in ATL cells [64]. It has been demonstrated that Tax suppresses the expression of human telomerase reverse transcriptase (hTERT) [65] and targets the DNA-end-binding and protective activity of the KU80 protein,

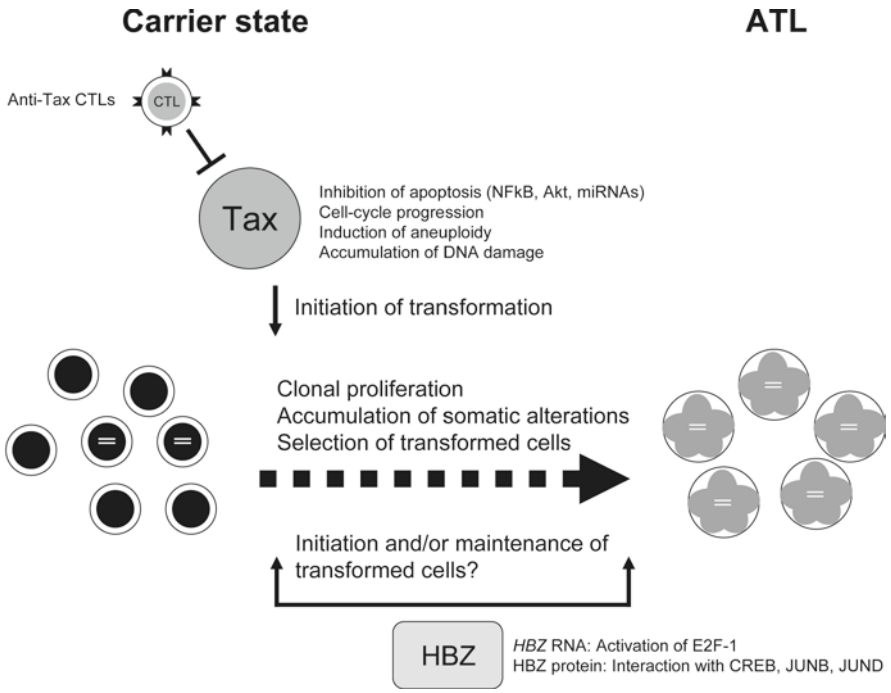

Fig. 5.3 The natural history of ATL development. HTLV-I-infected cells, marked by the = symbol, proliferate clonally and require Tax and HBZ. In the carrier state, proliferation of HTLV-I-infected cells is controlled by the host immune system. During the long latency period, Tax initiates cellular transformation in several ways; and in later stages fully transformed ATL cells do not appear to need Tax expression. The suppression of Tax expression in these cells allows cells to evade immune surveillance and to proliferate in vivo, resulting in ATL. HBZ has a cell proliferative function, and is expressed constitutively supporting that it has a role in leukemogenesis

which normally has the capacity in cells to protect newly formed double strand breaks as well as normal chromosome ends [66].

5.3.5 The Requirements for Tax in ATL Leukemogenesis

Tax is required for the virus to transform cells [67] ; however, since Tax is a major target of host immunity, ATL cells frequently (in about 60% of the cases) lose Tax expression by several mechanisms (Fig. 5.1). The first is through genetic changes in the *tax* gene (~10% of ATLs) [68, 69]. In some cases, ATL cells mutate the class I MHC recognition site of the Tax protein, resulting in an escape from immune recognition [68]. The second mechanism is an epigenetic change in the viral promoter/enhancer in the 5′-LTR; in this setting, DNA hypermethylation and histone modifications silence the transcription of viral genes (~15% of ATLs) [69, 70, 71]. The third mechanism is a deletion of 5′-LTR (~27% of ATL cases) [69, 72]. Although the significance of Tax in leukemogenesis has not been fully clarified, it is recognized that Tax is needed early after infection to initiate cellular transformation, but it may not be required after cells are fully transformed and have acquired Tax-independent proliferative ability (Fig. 5.3).

5.4 HBZ RNA and Protein In Leukemogenesis

As noted above, the proviral 5′-LTR is often deleted or epigenetically inactivated in ATL cells. Therefore, transcription of viral genes, including *tax*, encoded on the plus HTLV-1 strand is frequently suppressed. Conversely, the 3′-LTR is conserved and hypomethylated in all stages of ATL developments [71], suggesting its significance in the initiation and/or maintenance of leukemia. HBZ was identified to be encoded by the minus strand of the provirus as a protein that interacts with CREB-2 through its bZIP domain; HBZ was originally reported to suppress Tax-mediated viral transcription [14]. It also interacts with and modulates the transcriptional activities of other bZIP proteins, such as CREB, CREM-Ia, ATF-1 [73], c-Jun [74, 75], JunB [76], and JunD [77]. The *HBZ* gene is transcribed from the 3′-LTR and expressed in all ATL cells [13]. Because there are no mutations such as nonsense stop codons or deletions in its sequence *HBZ* has been suggested to be indispensable for ATL leukemogenesis. The findings that knocking down *HBZ* transcription by short hairpin RNA inhibits ATL cell proliferation support that HBZ enhances T cell growth and that over-expression of *HBZ* promotes proliferation of a human T-cell line [13]. Mutational analysis of *HBZ* genes shows

that *HBZ* (as an RNA form) can support T-cell proliferation by regulating the E2F-1 pathway. The *HBZ* RNA is predicted to form a stem-loop structure that may be essential for its growth-promoting activity, perhaps through interactions with uncharacterized cellular factors. Thus, the *HBZ* gene may have bimodal functions both as an RNA and as a protein. In these regards, the RNA form of *HBZ* is likely important in the proliferation of HTLV-1-infected cells while the protein suppresses viral transcription by Tax.

Several animal models have been established to evaluate the functions of HBZ *in vivo*. Transgenic mice expressing HBZ under control of the mouse CD4 promoter/enhancer, compared with wild type littermates, showed that their percentage of $CD4^+$ T-lymphocytes increased in splenocytes, and their proliferation induced by cross-linking with an immobilized anti-CD3 antibody was augmented in thymocytes [13]. It has been reported that when an HTLV-1 with a mutation in the leucine zipper domain of HBZ was inoculated into rabbits, the proviral load was reduced [78]. These data indicate that *HBZ* promotes cellular proliferation and support the idea that *HBZ* plays an important role in leukemogenesis of ATL (Fig. 5.3).

5.5 Other Viral Genes

5.5.1 $p12^I$

$p12^I$, which is encoded by ORF I of pX region, localizes to the endoplasmic reticulum and Golgi apparatus (79). $p12^I$ interacts with the interleukin-2 receptor (IL-2R) β and γc chains, modulating their surface expression. $p12^I$ activates signal transducers and activators of transcription 5 (STAT5) [80, 81], and it increases intracellular calcium levels through interaction with calnexin and calreticulin and leads to activation of nuclear factor of activated T cells (NFAT)-mediated transcription [79, 82]. $p12^I$ also binds the major histocompatibility complex I heavy chain (MHC I Hc), and this complex is degraded by the proteasome [83]. These findings suggest that $p12^I$ may support proliferation of HTLV-1-infected cells and contribute to escape from surveillance by the host immune system.

5.5.2 p27Rex

The pX ORF III encodes Rex in a doubly spliced *tax*/*rex* mRNA. Rex increases nuclear export of mRNA that encode viral structural genes such as *gag*, *pol*, and *env*, and Rex functions to enhance viral production [84]. Rex also suppresses the expression of fully spliced mRNA encoding regulatory proteins, and it negatively regulates viral transcription [85].

5.5.3 $p30^{III}$ and $p13^{II}$

ORF II of the pX region encodes both $p30^{II}$, a nuclear protein, and the $p13^{II}$ mitochondrial protein [79]. $p30^{II}$ interacts with CREB binding protein (CBP)/p300 and affects Tax-mediated viral transcription [86]. Conversely, $p30^{II}$ inhibits the transport of doubly spliced mRNA encoding Tax and Rex proteins to the cytoplasm, resulting in inhibition of their translation [87]; $p30^{II}$ can repress Tax/Rex expression in HTLV-1-infected cells both transcriptionally and post-transcriptionally. It has been suggested that strict control of viral expression by $p30^{II}$ contributes to viral persistence and escape from immune elimination. It has also been shown that $p30^{II}$ enhances the transforming potential of c-Myc and can transcriptionally activate the human cyclin D2 promoter through interaction with the c-Myc-60-kDa Tat-interacting protein (TIP60) complex [88]. These findings suggest a contribution of $p30^{II}$ to cellular proliferation. The $p13^{II}$ protein interacts with farnesyl pyrophosphate synthetase (FPPS), a protein that functions in the posttranslational modification of Ras. $p13^{II}$ alters Ras-mediated apoptosis in T lymphocytes [89, 90].

5.6 Other Host Factors

Familial clustering of ATL cases suggests that the host's genetic background influences ATL onset [91]. Polymorphism of the promoter region of tumor necrosis factor alpha (TNF-α) was reported to be associated with ATL susceptibility [92]. HLA haplotypes are also candidate genetic factors controlling the immune response against viral antigens. Specific HLA alleles are reportedly associated with an increased risk of ATL onset [93]. Such HLA alleles are genetically defective in their recognition of HTLV-1 Tax peptides, and allow HTLV-1-infected cells to escape from immunity.

5.7 Concluding Remarks

Advances in our understanding of the mechanisms for ATL leukemogenesis have been made since the first discovery of HTLV-1. Intensive studies on Tax biology have revealed molecular strategies used by HTLV-1 for cellular transformation. Tax appears to be required for the initiation of transformation but may be dispensable in later stages of leukemogenesis. Therefore, other factor(s) could contribute to the additional steps need for transformation. Emerging evidence on HBZ indicate its proliferative capacity. Further investigation of HBZ function may shed light on its role in leukemogenesis. Despite the progress in our molecular

understanding of ATL, the prognosis for this disease is still poor. Future research is needed to uncover new strategies for treatment and prophylaxis; hopefully these applications will be based on the accumulated knowledge of the virus' leukemogenic mechanisms.

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Part II
Microbiology: Human Immunodeficiency Virus (HIV)

Chapter 6
Roles for Chemokine Receptors in HIV Pathogenesis

Philip M. Murphy

6.1 Chemokines, Chemokine Receptors, and HIV-1/AIDS

HIV-1 is an enveloped virus that enters cells by receptor-dependent membrane fusion (Fig. 6.1). The viral fusion determinants are the envelope glycoproteins gp120 and gp41, which form a heterodimer and are the products of the Env gene. The target cell fusion determinant for HIV-1 includes the CD4 molecule, an accessory component of the antigen receptor expressed primarily on helper T cells [1]. Although CD4 is necessary for infection, it is not sufficient. This was appreciated by early workers in the field from the observation that HIV-1 isolates, which are all able to infect peripheral blood mononuclear cells (PBMCs), can be subclassified by their ability to infect cultured T cell lines versus primary macrophages, both of which express CD4. The clinical significance of HIV cytotropism rests on the strong, but still unexplained, correlation of macrophage (M)-tropic strains with both primary HIV infection and the period of clinical latency and also of T cell line (T)-tropic strains with immune system collapse and AIDS in a minority of HIV+ patients. Many primary clinical isolates are dual-tropic.

The complete solution to the cytotropism problem, reported within two months in the spring of 1996, involved the discovery of two HIV coreceptors, both chemokine receptors, one of which was specific for M-tropic HIV, and the other for T-tropic HIV (Fig. 6.2). The T-tropic HIV coreceptor was discovered first in the laboratory of Dr. Edward Berger of the Laboratory of Viral Diseases, NIAID, by means of a genetic screen designed to identify a cDNA that encoded a factor able to reconstitute infectivity of HIV-resistant CD4+ cells with HIV [1]. The factor, originally named fusin, turned out to be a known member of the G protein-coupled receptor superfamily, an orphan receptor at the time, that was later shown to be specific for the chemokine CXCL12 (also known as SDF-1), which justified its renaming to CXCR4, according to a standard nomenclature system for chemokine receptors. Importantly, the receptor could reconstitute infectivity with T-tropic but not M-tropic HIV. This seminal finding suggested that a related receptor might exist that could function as the putative M-tropic HIV coreceptor.

However, the discovery of the correct M-tropic receptor did not result from a second tedious genetic screen. Instead it was rapidly identified as the single best candidate thanks to the convergence of knowledge from three previously unrelated research areas [1]. The first was the discovery of CXCR4 by the Berger laboratory, which had a longstanding interest in the cytotropism problem, as described above. The second was the discovery by Paul Lusso and colleagues in the laboratory of Robert Gallo at the National Cancer Institute, NIH that three CC chemokines, MIP1α, MIP-1β, and RANTES, found in supernatants from a particular CD8+ T-cell line named PM1, were potent HIV suppressive factors for M-tropic but not T-tropic HIV. This research was aimed at understanding how some HIV+ individuals managed to resist immunologic deterioration and clinical progression to AIDS. That CD8+ T-cells could suppress virus replication in cultures of CD4+ T-cells was thought to be highly relevant to this clinical question, and the responsible soluble factors had long been a holy grail in HIV research both from the standpoint of pathogenesis and therapeutics. The third was the molecular cloning of CCR5 and discovery that its three principal chemokine ligands were also MIP1α, MIP-1β, and RANTES. Three laboratories independently cloned CCR5, including the LMI [2].

The simplest hypothesis that took into account all of these results was that the HIV-suppressive chemokines acted selectively on M-tropic HIV strains by blocking their access to their specific coreceptor CCR5. In a collaboration between LMI scientists and the Berger laboratory, this hypothesis was validated by a variety of approaches [3]. Most importantly, introducing CCR5 by gene transfection into HIV resistant CD4+ T-cells rendered the cells infectable with M-tropic but not T-tropic strains of HIV, the converse of the CXCR4 specificity. Moreover, CCR5 ligands could specifically block the activity and an antiserum directed against CCR5 could block infection of PBMCs. Four other laboratories independently obtained the same or

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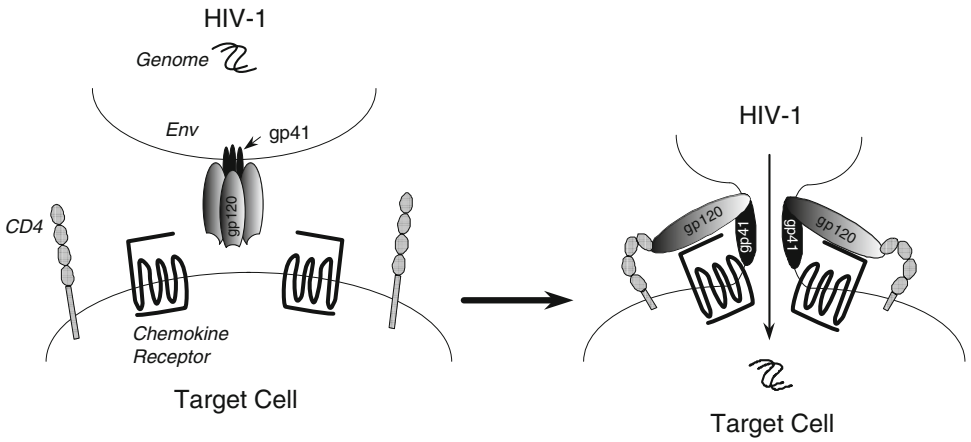

Fig. 6.1 Proposed biochemical mechanism of action of HIV coreceptors. HIV entry involves fusion of the viral envelope with the plasma membrane of the target cell. Fusion determinants of the virus include gp120 and gp41. Upon binding to both CD4 and a chemokine receptor on the target cell, gp120 undergoes a conformational change revealing a cryptic fusogenic peptide in gp41

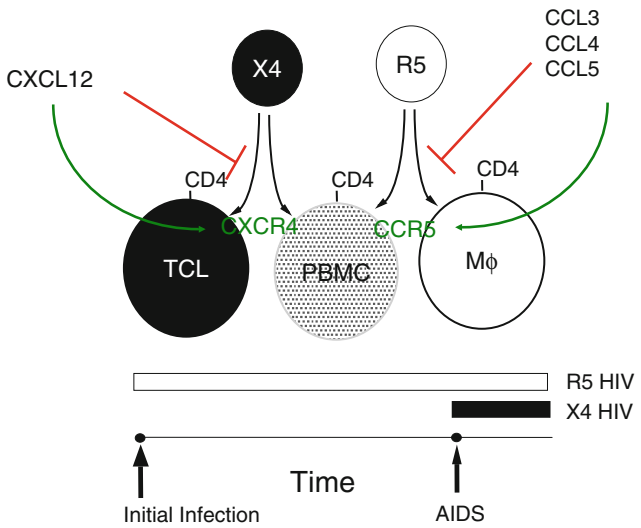

Fig. 6.2 Exploitation of chemokine receptors by HIV for cell entry. HIV strains all use CD4 and a chemokine receptor for cell entry. The two main chemokine receptors used are CCR5 and CXCR4. CCR5-tropic strains appear to be involved in person to person transmission and are typically found throughout the course of infection. CXCR4-tropic strains are typically found in a minority of patients after disease progresses to AIDS

similar results; all five papers were published within one week in *Nature, Science,* and *Cell* in June, 1996, establishing a new paradigm in HIV research.

We also found that CCR3, CCR8, CXCR6, and CX3CR1 could function *in vitro* as coreceptors for some HIV and SIV strains; other groups identified additional chemokine receptors and related receptors that possessed this activity. Thus, the next important question was which, if any, of these receptors was important *in vivo* in populations at risk of HIV. To date, this question has been answered unequivocally only for CCR5. LMI scientists addressed this question by asking whether a common loss-of-function mutation in CCR5 might exist, and, if so, whether it would protect homozygotes from HIV infection [4]. This mutation search was carried out in collaboration with Peter Zimmerman and Thomas Nutman of the Laboratory of Parasitic Diseases, NIAID. A remarkable mutant allele of CCR5 named *CCR5Δ32* was discovered in ~20% of healthy North American Caucasian random blood donors from the NIH blood bank. The allele has a 32 base pair deletion in the open reading frame of CCR5 and encodes a truncated, inactive receptor. Homozygous *CCR5Δ32* is highly protective against initial infection, since it is found ~20-fold less frequently among HIV-1+ people than the general population and ~6-fold less frequently among HIV-1+ individuals than among individuals who have been highly exposed to HIV-1 but remain uninfected (so called exposed-uninfected phenotype or EU). Nevertheless most EU individuals do not have the resistant CCR5 genotype, indicating that other resistance factors must exist. Moreover, some HIV+ individuals have been identified in spite of having the protective genotype, indicating that protection is not absolute. CXCR4-tropic strains have been characterized in some of these individuals, arguing for the biological importance of CXCR4 as an HIV coreceptor in populations. The protective effect is independent of route of transmission. Heterozygous *CCR5Δ32* does not protect against initial infection but is associated with a mean 2 year delay in progression to AIDS and is found ~50% more commonly among long-term non-progressors. Heterozygosity correlates with reduced CCR5 on T cells (50% of wild type controls). *CCR5Δ32* appears to have originated in Northern European Caucasians in whom it occurs with an average allelic frequency of 10%; it is not

found in native Asians and Africans. Three other groups independently identified *CCR5Δ32* and reported its association with resistance to HIV infection in homozygotes and delayed progression to AIDS in heterozygotes, establishing this as one of the most powerful restriction genes for any infectious disease of man. Additional genetic resistance factors for HIV/AIDS affecting CCR5 promoter function and expression of CCR5 ligands have also been identified. Importantly, *CCR5Δ32* homozygotes do not appear to have any overt medical problems, and CCR5 knockout mice also appear healthy in the unstressed state. Thus, the study of this allele provided proof of principle for safely targeting CCR5 in HIV/AIDS. In 2007, a piperazine derivative named maraviroc, developed by Pfizer Corp., was approved by the FDA as a small molecule antagonist of CCR5 for the oral treatment of patients with HIV/AIDS. This is the first licensed drug that acts by targeting the chemokine system, and the first anti-infective that acts by blocking a host determinant of infection.

Many enigmas remain regarding CCR5 such as why CCR5 deficiency selectively causes HIV-1 resistance when so many other coreceptors exist and how *CCR5Δ32* came to be fixed in the Caucasian genome over evolutionary time; however, to LMI scientists the question of the beneficial roles of CCR5 seemed preeminent, particularly when large numbers of HIV/AIDS patients, already immunosuppressed, stood to receive a CCR5 blocking agent. Studies of pathogenesis led to the discovery that CCR5 is critical in control of symptomatic West Nile virus infection, the first beneficial role discovered for the receptor in man (Fig. 6.3) [5,6,7,8].

In response to the recent emergence of West Nile virus in the United States and the almost complete lack of information about its molecular immunopathogenesis, LMI scientists initiated investigation of pathogenesis. An excellent mouse model of infection that involved intradermal injection was established by the lab in an NIAID BSL-3 facility [5]. West Nile virus is a flavivirus with a single-stranded RNA genome. It is a classic example of an emerging and reemerging pathogen, responsible for periodic outbreaks in Africa, Asia, and Europe where clinical manifestations were limited mainly to West Nile fever. The virus arrived in the United States from Israel in 1999 and was discovered first in a flamingo in the Bronx Zoo; by 2004, it had spread across the entire country. Approximately 20% of infected individuals develop clinical manifestations of disease, including West Nile fever, meningitis, and encephalitis, and, in 4% of these individuals, infection is fatal. In its neurovirulence and lethality, the U.S. experience with this agent differs from all previous experience. The pathologic correlates of fatal infection have not been well-delineated, although there are reports of infiltration by mononuclear cells. There is no specific treatment or vaccine available. In nature, WNV is a vector-borne zoonosis in which infected mosquitoes transmit the virus primarily to various bird species and accidentally and uncommonly to mammals such as horses, mice, and man, which are dead-end hosts. The mouse model of WNV infection therefore is biologically relevant and recapitulates the pathologic features of the human infection. Even with very high inocula, a maximum of only 40% of animals die. The virus passes from the infected site to the draining lymph node and spleen where it is cleared; however, viremia delivers the virus to the central nervous system where it crosses the blood brain barrier in a TLR3 dependent manner and infects neurons. Animals die of encephalitis. CD4, CD8 and γδ T-cells, NK cells, and monocyte/macrophages have all been shown to play a role in protection from lethal infection in this model. A variant in *Oas1b*, which regulates the Type I interferon antiviral response, is responsible for resistance to WNV and other flaviviruses in mice. At the time this project was begun, there was no information about the role of the chemokine system in WNV infection. We hypothesized that since leukocytes do accumulate in the brains of both infected mice and humans, that specific chemokines may be responsible for directing this process. No clear genetic risk factors had been established for WNV infection of man. Nor were there repositories of clinical materials that could be readily accessed. In order to generate specific hypotheses, we first carried out an inductive phase of experimentation, profiling mRNAs for chemokines, cytokines, chemokine receptors, and cytokine receptors present in uninfected adult mouse brain and in brain after infection with WNV[5]. This survey yielded a list of induced factors. We decided to focus on the chemokine part of the list and verified expression of each of the induced factors at the protein level. In particular, we noted inducible expression of the chemokine receptors CCR1, CCR2, CCR3, CXCR3, CCR5, and CX3CR1 and their ligands. Of these, CCR5 and its ligand CCL5/RANTES were most highly induced. We found that the infected brains accumulated CCR5+ CD4 and CD8 T cells, NK cells, and infiltrating macrophages and that infection was uniformly fatal

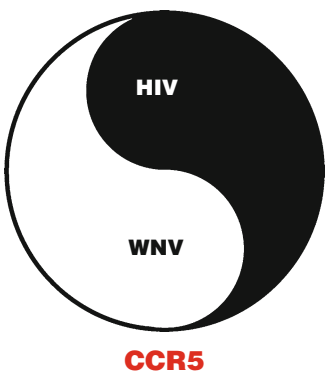

Fig. 6.3 CCR5 can be beneficial (WNV) or harmful (HIV), depending on the virus

in CCR5 knockout mice, a dramatic phenotype. In contrast, mortality was only slightly increased in CCR1-/- mice and CX3CR1-/- mice.

CCR5 appears to operate in this model at least in part at the level of leukocyte trafficking into the brain. Thus, clearance of WNV from the spleen and induction of cytokines and chemokines in the spleen and blood are normal in infected CCR5-/- mice, but the virus fails to clear normally in the brain; moreoever, there is a marked reduction in accumulation of CD4 and CD8 T-cells, NK cells, and infiltrating macrophages in the brains of these animals. Conversely, CCR5+ cells from each of these subsets do accumulate in the brains of infected CCR5 knockout mice when the animals are adoptively transferred with splenocytes from infected wild type mice. Most importantly, cell transfer is specifically associated with reversal of the mortality phenotype back to the expected value for control infected wild type mice.

The *CCR5Δ32* allele is an ideal genetic probe, since it is common (10% in North American Caucasians) and nonfunctional (provides plausibility that any statistical association with disease may reflect a direct cause and effect biological relationship). Given our results in mouse WNV infection, we took advantage of these properties of *CCR5Δ32* to probe the role of CCR5 in human WNV infection, hypothesizing that the allele would be overrepresented in cases of symptomatic infection. We located four independent centers in possession of serum samples from symptomatic WNV cases, one developed in Arizona in 2003, one in Colorado in 2004, one in California, and one in Illinois [6,7]. The Arizona and Colorado centers captured most of the cases of symptomatic WNV reported in those states in those years. One control cohort, in 1997, was developed from NIH random blood donors before WNV appeared in the United States. The second was developed in Arizona, in 2003, from individuals presenting with symptoms consistent with WNV infection but in whom WNV was excluded by serologic testing. In each cohort, we found an ~4.5-fold increase in the percentage of *CCR5Δ32* homozygotes relative to expectation from the two control cohorts. This difference was highly statistically significant and of a magnitude commensurate with the statistical association of this genotype with the HIV exposed-uninfected phenotype, one of the strongest and best accepted examples of genetic restriction of risk of infectious disease. The genotype was not clearly a risk factor for fatal WNV infection and is not a risk factor for initial infection, as determined by studies with samples from healthy blood donors screened as WNV+.

Control of WNV is the first result identifies a beneficial role for CCR5 in man, and *CCR5Δ32* the first strong genetic risk factor for symptomatic WNV infection in man. The results illustrate a yin-yang relationship of CCR5 with HIV and WNV and suggest that therapeutic blockade of CCR5 in HIV/AIDS, and perhaps in other diseases, may carry a cost of increased risk of symptomatic WNV in dually infected individuals. Clinical trials of CCR5 blockers should monitor this issue prospectively. Meanwhile, the findings should serve to reinforce to patients the importance of infection prophylaxis in the form of avoiding agents of transmission. Finally, the findings raise new questions regarding risk factors for the majority of symptomatic individuals who are not CCR5 deficient and precise mechanisms by which CCR5 regulates WNV pathogenesis. In this regard, it is not known which of the CCR5+ leukocyte subtypes that accumulate in the brains of infected mice are most important for controlling outcome and whether any are important in human infection of the brain with WNV. Nor is there any knowledge about how CCR5 controls initiation of the immune response to WNV.

6.2 Conclusions

The results of this research indicate clearly that chemokine receptors are critical factors in viral pathogenesis. However, the same receptor CCR5 can play either a beneficial (WNV) or harmful (HIV) role in pathogenesis depending on the virus. How HIV evolved to use chemokine receptors for cell entry is unknown, but it is interesting to note that there is at least one other pathogen that also uses a chemokine receptor for this step in its life cycle, *Plasmodium vivax*, a protozoan that causes a form of malaria. The general importance of chemokine receptors in host defense in humans is still not well known. The example of CCR5 in WNV infection suggests it may work in a conventional way, directing leukocytes to infected sites although other roles have not been ruled out. Whether it functions in a similar way in other infectious diseases or whether it is a prototype for the biological roles of other chemokine receptors is not yet known.

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Chapter 7
HIV-1 Accessory Proteins: Crucial Elements for Virus-Host Interactions

Klaus Strebel

7.1 Overview

The primary goal of a virus is the infection of host cells so it can replicate its genome and so it can produce progeny virions for the infection of new target cells. Some viruses cause long-lasting chronic infections while others replicate in fast, lytic cycles. However, replication of all viruses depends to a large degree on specific host factors from the recognition of specific cell surface receptors required for virus entry into a target cell to the packaging of cellular factors into virions. HIV penetrates target cells through fusion with the host plasma membrane. Penetration is followed by partial uncoating and reverse transcription of the viral RNA and subsequent integration of the double-stranded cDNA into the host genome. The integrated provirus then serves as template for the synthesis of viral proteins, which ultimately assemble into progeny virions that are released from the infected host cell. We are far from understanding all of the complex virus-cell interactions that take place during a single replication cycle; however, our current knowledge on the replication of HIV suggests that such interactions occur at virtually every step during the course of virus replication. Recent years have brought rapid progress in the identification and characterization of novel host factors supporting HIV replication. In particular, the recent identification of host restriction factors such as Trim-5α or APOBEC3G as well as the identification of Bst-2/Tetherin as a target of Vpu has significantly advanced our understanding of HIV cell tropism. The molecular mechanisms that dictate host restrictions and govern virus-host interactions, however, remain poorly understood.

Primate immunodeficiency viruses, including HIV-1, are characterized by the presence of a series of viral accessory genes that encompass *vif*, *vpr*, *vpx*, *vpu*, and *nef*. The *vif*, *vpr,* and *nef* genes are expressed in most HIV-1, HIV-2, and SIV isolates (Fig. 7.1). In contrast, the *vpu* gene is found exclusively in HIV-1 and some SIV isolates. The *vpx* gene, on the other hand, is not found in HIV-1 isolates but is common to HIV-2 and most SIV isolates. Current knowledge indicates that none of the primate lentiviral accessory proteins has enzymatic activity. Instead, there is increasing evidence that these proteins serve primarily, if not exclusively, as adapter molecules to mediate the physical interaction of other viral and/or host factors. Such a function explains the host-specific activity noted for most of the accessory proteins.

Our primary objective is to investigate the biological and biochemical functions of HIV accessory proteins, in particular Vif and Vpu, and to define their role in virus replication. Our research focuses on the detailed biological and biochemical characterization of the individual viral proteins as well as the investigation of their engagement with distinct host factors. Understanding the molecular mechanisms of viral proteins will benefit our general understanding of the HIV replication process and may reveal new targets for antiviral therapy. In addition, the investigation of virus-host interactions will give us new insights into general cellular processes involved in protein trafficking, degradation, post-translational modification etc. Our early work has focused primarily on the function of Vpu, and we were able to gain significant insights into the molecular mechanisms of Vpu-dependent CD4 degradation, regulation of virus release, and induction of apoptosis. The recent identification of bone marrow stromal cell antigen 1 (Bst-2; also referred to in the literature as tetherin) as a Vpu-sensitive host factor, whose expression interferes with the release of Vpu-deficient HIV-1 virions, has reignited interest in the mechanism of Vpu-mediated enhancement of virus release. In addition, we have put significant energy into the characterization of functional interactions between Vif and APOBEC3G. Here we summarize recent progress of our work.

7.2 HIV-1 Vpu

7.2.1 Introduction

Vpu is an 81 amino acid type 1 integral membrane protein that is composed of three discrete α-helices: The *N*-terminal helix constitutes the transmembrane anchor and is followed by a cytoplasmic tail containing two amphipathic α-helices [for review see [91]]. The *vpu* gene is not always functional and several primary isolates contain mutated initiation

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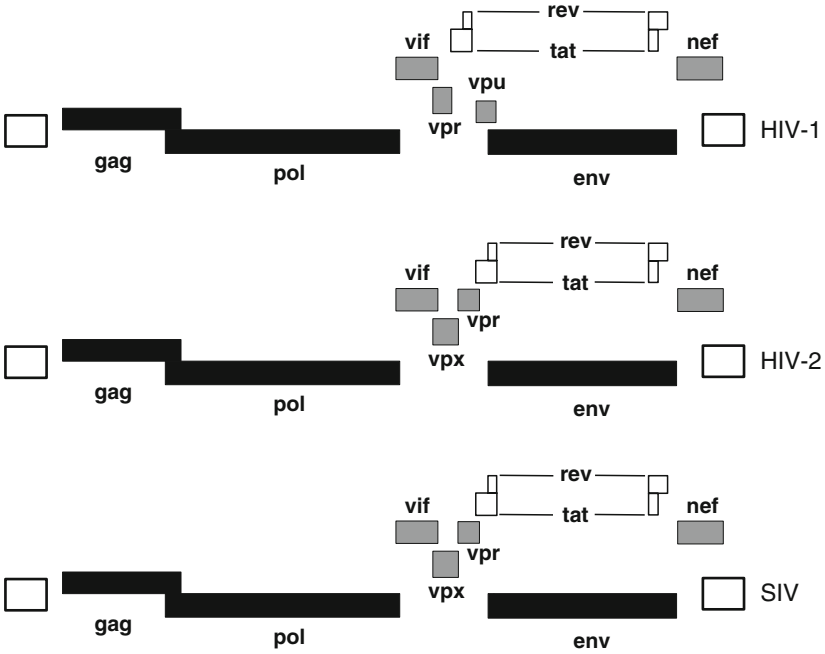

Fig. 7.1 Genome organization of primate lentiviruses. Aside from the prototypic retroviral *gag*, *pol*, and *env* genes, primate lentiviruses encode several additional genes, including the regulatory *tat* and *rev* genes as well as *vif*, *vpr*, and *nef* accessory genes. The *vpu* gene is characteristic of HIV-1, while HIV-2 and SIV isolates typically encode a *vpx* gene

codons or internal deletions, suggesting a mechanism by which Vpu expression is regulated by the virus [83]. Although the *vpu* gene is only found in HIV-1, the envelope protein of certain HIV-2 isolates have been shown to assume some of the functionality of the HIV-1 Vpu protein [10,12,78]. The Vpu protein has two main roles in the viral life cycle: it promotes the efficient release of viral particles from the cell surface and it induces the degradation of CD4, and possibly other transmembrane proteins, in the endoplasmic reticulum (ER). In a process that is mechanistically related to Vpu-induced CD4 degradation, Vpu also induces host-cell apoptosis. It remains to be determined, however, whether Vpu-induced apoptosis is an unfortunate side-effect of Vpu's ability to degrade CD4 or whether induction of apoptosis offers the virus a separate selective advantage upon infection of a host.

7.2.2 Vpu Induces Rapid Degradation of the HIV-1 Receptor CD4

Aside from Nef, the gp160 envelope glycoprotein precursor (Env) and Vpu both significantly contribute to the viral effort to downregulate CD4. Gp160 is a major player in CD4 down-modulation that can, in most instances, quantitatively block the bulk of newly synthesized CD4 in the endoplasmic reticulum (ER) [1,22,37]. However, this strategy has two principal shortcomings. First, in contrast to Nef, Env is unable to remove pre-existing CD4 molecules that have already reached the cell surface. Second, the formation of CD4-gp160 complexes in the ER blocks the transport and maturation of not only CD4 but also of the Env protein itself [8]. ER retention of Env by CD4 could lead to the depletion of cell-surface Env and thus the production of Env-deficient, non-infectious virions [1,15]. An important function of Vpu is to induce the degradation of CD4 molecules trapped in intracellular complexes with Env thus allowing gp160 to resume transport toward the cell surface [104].

In Vpu-expressing cells, CD4 is rapidly degraded in the endoplasmic reticulum (ER) and its half-life is reduced from 6 hours to approximately 15 minutes [105]. The importance of ER localization for CD4 susceptibility to Vpu-mediated degradation suggests that cellular factors essential for CD4 degradation are located in the ER and/or that the rate-limiting step of CD4 degradation is binding by Vpu [18]. In support of the latter option, co-immunoprecipitation experiments showed that CD4 and Vpu physically interact in the ER and that this interaction is essential for targeting CD4 to the degradation pathway [11]. The domains in Vpu required for CD4 binding are less well defined, suggesting that three-dimensional rather than linear structures are involved. While two conserved serine residues at positions 52 and 56 in the cytoplasmic domain of Vpu are critically important for CD4 degradation [76,85], they are not required for CD4 binding, since phosphorylation-defective mutants of Vpu retained the capacity to interact with CD4 [11]. We therefore hypothesized that Vpu binding to CD4 was necessary but not sufficient to induce degradation [11]. We subsequently found that the Vpu phosphoserine residues are important for interaction

with βTrCP, a host factor, which is a component of an E3 ubiquitin ligase complex [58]. Serine-phosphorylation plays the major regulatory role in the stability of βTrCP target proteins. For example, activation of the IκB kinase complex (IKK) by external stimuli such as TNF-α induces the serine-phosphorylation of IκBα followed by rapid TrCP-mediated proteasome degradation [33]. Fig. 7.2 summarizes our current model for Vpu-mediated CD4 degradation. According to this model, phosphorylated Vpu simultaneously binds CD4 and TrCP. The latter recruits the proteasome degradation machinery through SkpI, which is an F-box binding protein [58]. SkpI, in turn, interacts with Cul-1, a Nedd8-modified entity that provides a docking site for the Cdc34 E2 ubiquitin conjugating enzyme. Cul-1 also interacts with Rbx1, which can bind both TrCP and Cdc34 and stabilize the E2-E3 complex [89].

7.2.3 Vpu Induces Host Cell Apoptosis

Vpu has one intriguing property that distinguishes it from all other known substrates of βTrCP: its resistance to proteasome degradation. Indeed, while the SCF^{TrCP} usually degrades the serine-phosphorylated protein directly bound to the TrCP WD domains (i.e., Vpu), CD4 – bound to the Vpu cytoplasmic domain – is degraded instead. The resistance of Vpu to degradation has serious implications for the regulation and availability of the SCF^{TrCP} in cells that express Vpu. Indeed, due to the fact that Vpu is constitutively phosphorylated [85], it binds βTrCP with high affinity [58] and is not released from the complex by degradation [9]; Vpu expression in HIV-infected cells was likely to perturb the physiological function of the SCF^{TrCP} through competitive trapping of TrCP. Indeed, the dysregulation of IκB by Vpu was shown to lead to inhibition of both HIV- and TNF-α-induced activation of NF-κB [9]. The dysregulation of NF-κB in Vpu expressing cells has far-reaching consequences, since NF-κB is a central transcription factor that regulates the expression of key cellular genes involved in cell proliferation, cytokine production and the inhibition of apoptosis [4,75]. Inhibition of NF-κB activity by Vpu might therefore contribute to the induction of apoptosis in HIV-1 infected cells [3,17]. This was confirmed experimentally by showing that in a population of Jurkat cells expressing wild-type HIV-1, twice as many cells underwent apoptosis than in cells infected with a Vpu-defective virus [1]. Mechanistically, Vpu was shown to inhibit the NF-κB-dependent expression of anti-apoptotic genes such as Bcl-2 family proteins, leading to enhanced intracellular levels of the apoptosis-promoting caspase-3 [1]. Active caspase-3 then triggers a reaction that results in the cleavage of a number of target proteins including Bcl-2 family proteins and leads to cell death [1]. Fig. 7.3 summarizes our current understanding of Vpu-induced host cell apoptosis.

7.2.4 Vpu Enhances the Release of Virions by Targeting Bst-2/tetherin

Vpu enhances the release of virions from infected cells [46,94] and prevents endocytosis of nascent viral particles [30,31,66,93,97,99]. These activities of Vpu are mechanistically distinct from CD4 degradation and involve different structural domains in Vpu. Vpu does not affect assembly of viral particles per se but facilitates the detachment of virions from the cell surface [46,66]. Several mechanisms of Vpu

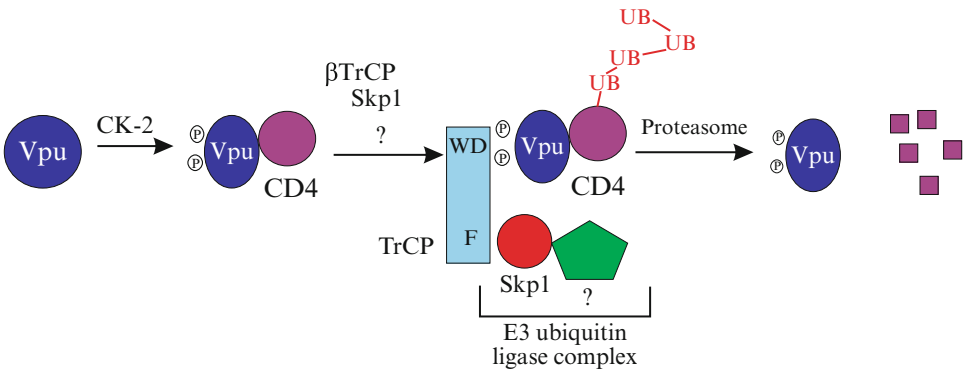

Fig. 7.2 Vpu-induced degradation of CD4. Vpu is constitutively phosphorylated by casein kinase II (CK-2) at two highly conserved serine residues (S52, S56) located in the cytoplasmic domain of Vpu. The Vpu phosphoserine motif constitutes a binding site for βTrCP, which binds Vpu via its WD domain. Interaction of Vpu and bTrCP results in the assembly of an active E3 ubiquitin ligase complex. Vpu also engages CD4 presumably via a membrane-proximal polar domain. After binding to Vpu, CD4 is ubiquitinated and ultimately degraded by proteasomes. Vpu itself is resistant to degradation and may be recycled

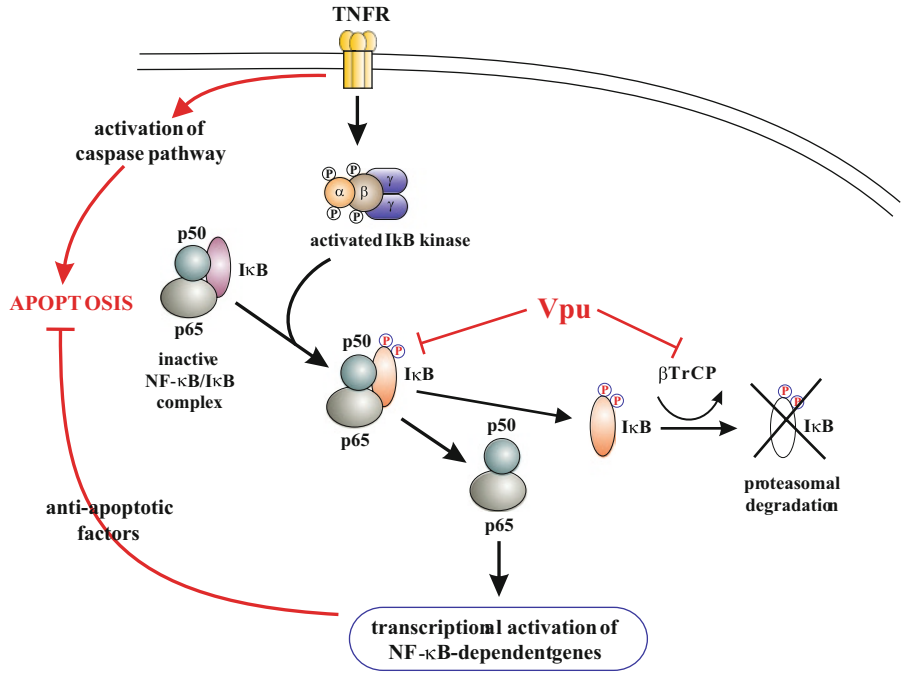

Fig. 7.3 Vpu induces host cell apoptosis. Stimulation of cells through the TNF receptor triggers two pathways: (i) activation of the pro-apoptotic caspase pathway; (ii) activation of the NF-κB pathway. The latter results in activation of IκB kinase, which in turn catalyzes the phosphorylation of IκB at two serine residues. Phosphorylated IκB is targeted by βTrCP for proteasomal degradation. The IκB-deficient NF-κB complex can now traffic to the nucleus and activate expression of anti-apoptotic genes. The simultaneous stimulation of pro- and anti-apoptotic pathways results in a balanced equilibrium that allows the cell to survive. Expression of Vpu results in competitive inhibition of IκB phosphorylation due to sequestration of βTrCP. Consequently, Vpu expression prevents activation of NF-κB and, as a result, inhibits expression of anti-apoptotic genes. This shifts the balance towards pro-apoptotic signals and results in cell death

mediated virus release have been proposed. First, Vpu has the ability to assemble into a cation-specific ion channel [23,54,57,77,84]. Randomization of Vpu's transmembrane (TM) domain did not affect membrane association or the ability to induce CD4 degradation but inhibited Vpu's ion channel activity and, at the same time, impaired its ability to regulate virus release [82,84]. This suggested a functional correlation between Vpu ion channel activity and virus release. However, it remained unclear exactly how a Vpu ion channel activity might trigger increased detachment of particles from the cell surface. It also became clear that Vpu dependence of virus release was tissue-specific, suggesting the involvement of host factors in Vpu function [80,101]. Several host factors have since been implicated in the Vpu-sensitive inhibition of virus release, including Task-1 and UBP [16,35]. More recently, two additional host factors, Bst-2/tetherin/CD317/HM1.24 [67,98] and CAML [100] have been correlated with Vpu-sensitive restriction of HIV-1 virus release. We recently began characterizing the functional interaction of Vpu and Bst-2/tetherin/CD317/HM1.24 (for simplicity referred to here as Bst-2).

Bst-2 was originally identified as a membrane protein in terminally differentiated human B-cells of patients with multiple myeloma [27,70]. Bst-2 is a 30 to 36 kDa type II transmembrane protein consisting of 180 amino acids [36]. The protein has an interesting topology because it has both an *N*-terminal transmembrane domain and a *C*-terminal glycosyl-phosphatidylinositol (GPI) anchor [48]. Bst-2 associates with lipid rafts at the cell surface and was identified on internal membranes, presumably the *trans*-Golgi network [48]. However, the precise function of Bst-2 remains unknown. Bst-2 mRNA was constitutively expressed in cell types such as HeLa, Jurkat, and at low levels in $CD4^+$ T-cells but not 293T or HT1080 cells [68] and thus correlated with cell types known to depend on Vpu for efficient virus release. Bst-2 expression was inducible by interferon treatment in 293T or HT1080 cells consistent with the previous observation that cell lines that did not normally require Vpu for efficient virus release became Vpu-dependent upon interferon treatment [67]. While Vpu had no overt effects on ectopically expressed Bst-2 levels and cellular distribution [68], Vpu clearly downregulated cell surface expression of Bst-2 in 293T cells [98] and caused a reduction of endogenous Bst-2 expression in HeLa cells [5]. Randomization of the Vpu TM domain abolished Vpu's ability to overcome the inhibitory effects of Bst-2 on virus release [98] consistent with our previous data on the importance of Vpu's TM domain for virus release [82].

We analyzed endogenous Bst-2 with respect to its effect on virus release from HeLa cells, T cells, and macrophages [63]. We noted significant cell type-dependent variation of total intracellular Bst-2 expression. However, with the exception of Jurkat cells, all Vpu-dependent cell types analyzed so far expressed Bst-2 on their surface. Interestingly, while Vpu caused a reduction in Bst-2 expression in transfected HeLa cells and long-term infected macrophages, HIV-1 replication in PBMC as well as CEMx174 and H9 cells did not result in cell surface downmodulation of Bst-2 or a reduction of intracellular Bst-2 levels; however, virus release was Vpu-responsive (Fig. 7.4). Surprisingly, Bst-2 was undetectable in virions that had been removed from the surface of cells by physical agitation, an observation that is difficult to explain by the tethering model of Bst-2 [63]. We currently believe that enhancement of virus release by Vpu does not require

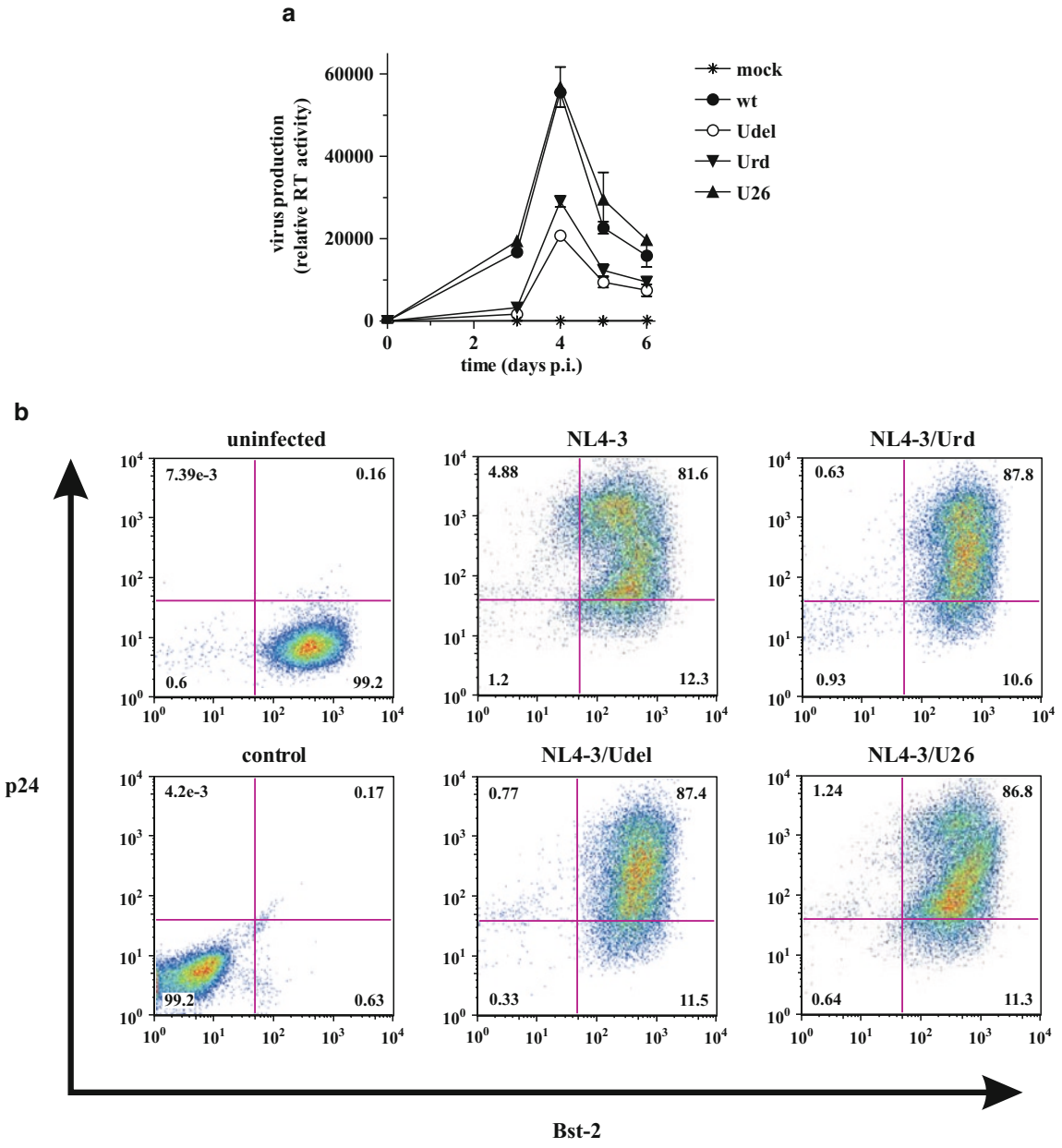

Fig. 7.4 Vpu enhances virus release in the absence of surface downregulation of Bst-2. **(a)** CEMx174 cells were infected with wild type NL4-3 (wt), a vpu-deficient variant (Udel), a variant expressing Vpu with a scrambled transmembrane domain (Urd), and a variant expressing Vpu lacking two conserved phosphoserine residues (U26). Virus replication was monitored by determining the reverse transcriptase activity in the culture supernatants. Note that in all infections virus production peaks on the same day (day 4). Also note that the βTrCP binding mutant (U26) replicates like wild type virus while Urd exhibits a Vpu(-) phenotype. **(b)** Cell surface expression of Bst-2 was determined by FACS on day 4 using a Bst-2 specific polyclonal antibody. Cells were gated for p24-positivity (intracellular p24 staining). Note that wt Vpu induces a minor reduction of surface Bst-2 while Vpu26 does not reduce Bst-2 surface levels

cell-surface downmodulation or intracellular depletion of Bst-2 nor does it involve exclusion of Bst-2 from virus particles. This could be an indication that Vpu functions at an intracellular site, and it is possible that cell surface downregulation of Bst-2 and reduction of Bst-2 levels are downstream consequences of Vpu activity. Current work in the lab focuses on mechanistic aspects of Vpu-Bst-2 interactions and in particular the question of where in the cell Bst-2 exerts its inhibitory effect on virus release.

7.3 Vif and APOBEC3G

7.3.1 Introduction

The HIV-1 accessory protein Vif plays an important role in regulating virus infectivity. The lack of a functional Vif protein results in the production of virions with reduced or abolished infectivity [92]. This effect of Vif on virus infectivity is producer cell-dependent and can vary by several orders of magnitude [92]. Virus replication in non-permissive cell types such as primary T cells and macrophages as well as a small number of T cell lines, including H9, is strictly dependent on Vif while Vif-defective viruses can efficiently replicate in permissive hosts such as Jurkat cells. Results from heterokaryon analyses, which involved the fusion of restrictive with permissive cell types, suggested the presence of an inhibitory factor in restrictive cell types such as cellular factor, CEM15, that was identified in 2002 by the Malim laboratory and was found to be expressed in non-permissive cell types but not in permissive cell types [86].

CEM15 is a member of the cytidine deaminase family of proteins and is now commonly referred to as APOBEC3G. APOBEC3G is a 384 amino acid protein that primarily localizes to the cytoplasm but is also packaged into HIV and other viruses. The number of APOBEC3G molecules packaged is dependent on the intracellular expression level. Based on our own calculations, Vif-negative HIV-1 particles produced in H9 cells, which express significant levels of APOBEC3G and are non-permissive, contain between 40 and 50 molecules of APOBEC3G per virion. Virions from transiently transfected HeLa or 293T cells can contain 5-10 times this amount. Packaging of APOBEC3G into HIV virions is thought to require an interaction with the viral NC protein. However, the interaction of APOBEC3G *in vitro* with viral Gag proteins was found to be RNase-sensitive and our own data suggest that the function of NC in packaging APOBEC3G may be at least in part due to its role in the packaging of the viral RNA genome. Packaging of even small amounts of APOBEC3G in the absence of Vif can cause significant damage to the HIV genome during reverse transcription, due to the introduction of mutations by deamination. Interestingly, APOBEC3G deaminase activity selectively targets single-stranded DNA. As a result, the vast majority of APOBEC3G-induced C-to-U mutations occurs on the minus-strand cDNA and results in G-to-A mutations on the coding strand following second-strand synthesis. The exact mechanism of APOBEC3Gs antiviral activity remains to be investigated. While it is obvious that the accumulation of mutations in the viral genome is detrimental to viral replication in the long run, it is surprising that APOBEC3G consistently and effectively abolishes viral infectivity in single cycle assays that are relatively insensitive to mutations in the coding region of the viral genome. We predict that the main inhibitory effect of APOBEC3G may be interference with the production of double stranded, integration-competent viral cDNA.

Vif is a 23-kDa basic protein that is expressed late during infection. Immunocytochemical analyses revealed a largely cytoplasmic localization of Vif [41]. In particular, confocal microscopy revealed that a significant amount of Vif associates with the intermediate filament network in virus-producing cells causing severe alterations of the intermediate filament structure [41]; however, the domain(s) in Vif responsible for this association as well as its functional significance remain to be determined. Recent reports also suggested that Vif associates, with viral genomic RNA *in vivo* and *in vitro* and deletions in the *N*-terminal and central regions, were found to affect the ability of Vif to bind to poly(G)-conjugated agarose beads *in vitro*. Aside from its affinity to RNA and intermediate filaments, Vif was reported to associate with cellular membranes through a mechanism involving a basic *C*-terminal domain in Vif. This same domain was also reported to be responsible for the interaction of Vif with the Gag precursor $Pr55^{gag}$ and was found to influence multimerization of Vif *in vitro* and *in vivo*.

One of the unresolved and still debated issues surrounding Vif is the significance of Vif encapsidation into HIV-1 particles. While the fact that Vif is packaged into HIV particles is undisputed, its functional relevance is less clear. The two main arguments against a functional role for virus-associated Vif are the following: supposed non-specific packaging mechanism and the generally low copy number of Vif in virus particles. Published estimates on the number of Vif molecules packaged per virion have varied significantly, ranging from less than 1 to as much as 100 molecules. As far as specificity of packaging is concerned, we found that Vif packaging is, in fact, specific and dependent on its interaction with viral genomic RNA and possibly NC [2,42]. Helper viruses devoid of genomic RNA did not package significant amounts of Vif unless packageable genomic RNA was provided *in trans*. Not surprisingly, we found that virus-associated Vif is stably associated with the nucleoprotein complex [41,42]. Intriguingly, we found that not only is Vif packaged into virions but it is also proteolytically cleaved by the viral protease [43].

7.3.2 Vif Reduces Intracellular Expression and Blocks Packaging of APOBEC3G

Our studies on Vif provide increasing evidence that packaging of Vif into virus particles is functionally relevant. For example, Vif packaging is specific and mediated through an interaction with viral genomic RNA [42]. In addition, virus-associated Vif interacts with Gag and/or Gag-Pol precursor molecules and is stably associated with the viral nucleoprotein complex. Next, virus-associated Vif is proteolytically cleaved by the viral protease at a conserved sequence located near the *C*-terminus of the protein [43]. Finally, in the case of SIVagm Vif, virus infectivity can be restored without excluding APOBEC3G from SIVagm virions [96].

The effect of Vif on virus infectivity is producer cell-dependent and can vary by several orders of magnitude [for review see [13,26]]. Virus replication in non-permissive cell types such as primary T cells and macrophages as well as a small number of T-cell lines, including H9, is strictly dependent on Vif. In contrast, Vif-defective viruses can efficiently replicate in permissive hosts such as Jurkat cells. Work by the Malim laboratory identified APOBEC3G, which was expressed in cell types restrictive for the replication of Vif-defective viruses but was not expressed in permissive cell types. Expression of APOBEC3G in permissive cell types imposed a restrictive phenotype on these cells providing intriguing evidence that APOBEC3G is indeed a cellular inhibitor whose activity must be overcome by Vif for HIV replication to proceed [86]. Interestingly, APOBEC3G - like Vif - is packaged into virions and induces hypermutation of viral cDNA in the absence of Vif [7,56, 81,106]. Surprisingly, APOBEC3G induced mutations were found to be widely spread across the entire HIV-1 genome with a preference for CC dinucleotides. Such mutations were not observed in viruses produced in the presence of Vif. To characterize the effect of APOBEC3G on HIV infectivity in the presence or absence of Vif and to gain insights into the mechanism of APOBEC3G neutralization by Vif, we cloned APOBEC3G from a human kidney cDNA library. Expression of APOBEC3G in HeLa cells confirmed that the protein was biologically active and severely inhibited virus infectivity in the absence of Vif. Analysis of virus preparations revealed that packaging of APOBEC3G was inhibited by wild type Vif but not by biologically inactive Vif variants. The inhibition of APOBEC3G packaging by wild type Vif was dose-dependent. Interestingly, increasing levels of APOBEC3G did not adversely affect Vif packaging. Analysis of the intracellular APOBEC3G expression levels suggested that wild type Vif but not biologically inactive Vif variants decreased the steady state levels of APOBEC3G [40].

7.3.3 Degradation of APOBEC3G

Vif inhibits packaging of APOBEC3G into virus particles. The inhibition of APOBEC3G encapsidation by Vif is generally accompanied by a reduction of the intracellular expression levels, which have been attributed to degradation by the cellular ubiquitin-dependent proteasome machinery [21,52, 60,62,87,90,103,108]. With respect to APOBEC3G degradation, Vif appears to exe rt an adaptor function: Vif binds to APOBEC3G as well as to components of a cullin-ubiquitin ligase complex that includes Cullin5 (Cul5), Elongin B, Elongin C and the RING protein Rbx and induces ubiquitination and subsequent degradation of APOBEC3G [62,108,109]. (For a more detailed review of this topic see [26].)

7.3.4 Degradation of APOBEC3G and Rescue of Viral Infectivity are Separable Sctivities of HIV-1 Vif

Most of our studies involve the expression of Vif from a proviral vector, expressing not only Vif but all other viral accessory and regulatory proteins as well. Expressing Vif from a proviral vector has raised concerns that some of the other viral factors (e.g., Tat, Rev, Vpu, Nef, or Env) might contribute directly or indirectly to our observations on Vif. Analyzing Vif function in the context of a proviral vector is undoubtedly more complex than expressing Vif from an autonomous vector. On the other hand, Vif is never expressed on its own *in vivo* and as such functional studies on the interaction of Vif and APOBEC3G in the context of a whole viral complement are likely to be more relevant.

To study the effect of Vif on APOBEC3G, we characterized the stability of APOBEC3G in transiently transfected HeLa and 293T cells and its correlation with the production of infectious virions. As part of this project, we analyzed three different types of HIV-1 Vif, including (i) Vif, expressed from a proviral vector (A1-Vif); (ii) Vif expressed from a codon-optimized vector in the absence of other viral proteins (hVif); and (iii) Vif fused at its *N*-terminus to yellow fluorescent protein (YFP-Vif). The YFP-Vif chimera was previously shown to efficiently induce proteasome degradation of APOBEC3G [102]. Our own results are consistent with these observations. In fact, in all of our experiments, YFP-Vif and hVif were more effective in inducing APOBEC3G degradation than A1-Vif protein. Interestingly, despite their different effects on APOBEC3G stability, A1-Vif and hVif both efficiently counteracted APOBEC3G's antiviral activity [39]. Surprisingly, however, YFP-Vif was severely impaired in its ability to direct the production of infectious HIV-1 particles from APOBEC3G-expressing cells. The lack of correlation

between APOBEC3G degradation and infectivity of virus particles suggests that these two functions of Vif can be separated. The efficiency with which Vif induced APOBEC3G degradation appeared to be dependent on the expression system and was most pronounced when Vif was expressed from autonomous (i.e., Tat- and Rev-independent) expression vectors, (e.g., hVif and YFP-Vif). The inefficient degradation of APOBEC3G by A1-Vif in HeLa cells was not due to isolate-specific differences or the co-expression of additional pNL-A1-encoded viral proteins. However, there were cell type-specific differences as APOBEC3G degradation by A1-Vif was somewhat more efficient in 293T cells than in HeLa cells. Taken together, our data demonstrate that the ability of HIV-1 Vif to counteract the anti-viral effects of APOBEC3G is not directly linked to the efficiency of Vif-induced APOBEC3G degradation by cellular proteasomes. Our results also demonstrate that the functional properties of Vif vary depending on the expression system used. Importantly, our results suggest that intracellular degradation of APOBEC3G may not be the sole function of Vif required for the production of infectious virions from APOBEC3G-expressing cells [39].

These results are consistent with data from a degradation resistant APOBEC3G variant whose virus encapsidation and antiviral activity was nonetheless efficiently controlled by Vif (Fig. 7.5) [73]. We had previously shown that APOBEC3G has the propensity to form multimers [74], a property that it shares with other members of the APOBEC family of proteins [38,49,65,74,88,103]. The multimerization of APOBEC3G was sensitive to RNase treatment, suggesting an involvement of viral or cellular RNA in this process. Interestingly, multimerization was also exquisitely sensitive to mutation of a single cysteine residue (C97) that is part of an *N*-terminal zinc-finger motif. However, the C97A mutation neither

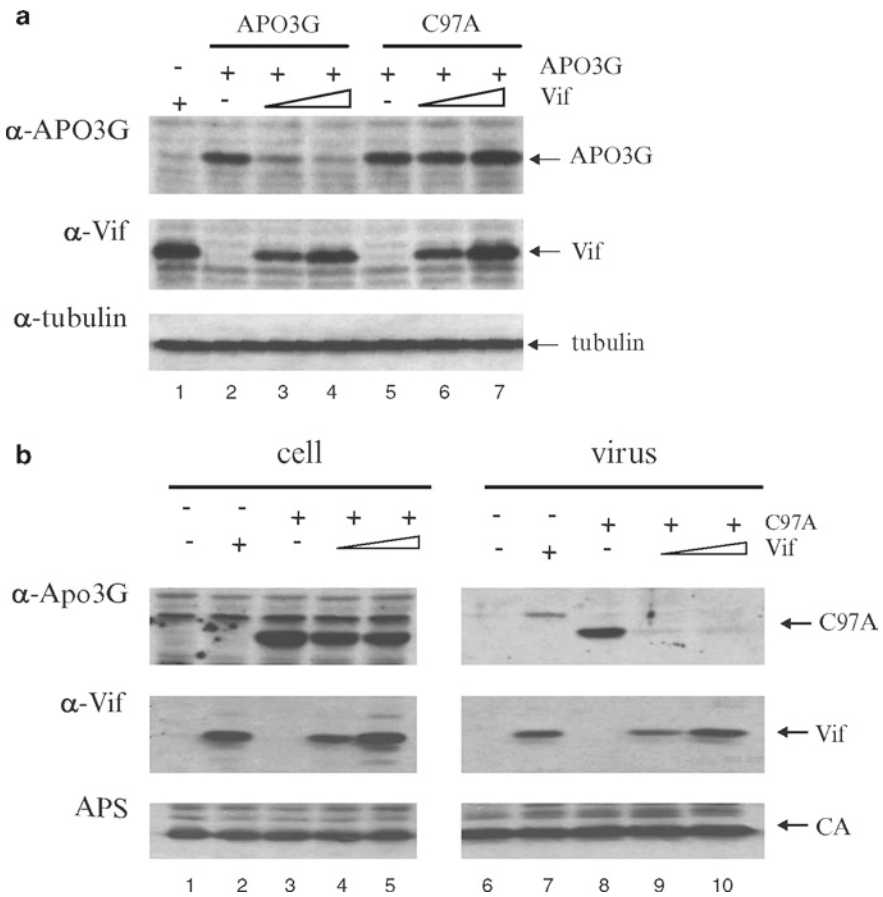

Fig. 7.5 Vif inhibits packaging of APOBEC3G in the absence of APOBEC3G degradation. **(a)** Mutation of C97 (C97A) in APOBEC3G results in resistance to Vif-induced degradation. Wild type APOBEC3G (APO3G) and the C97A mutant were co-expressed in HeLa cells with increasing amounts of Vif. This results in the dose-dependent degradation of wt APO3G (lanes 3-4) while APO3G C97A is resistant to Vif (lanes 6-7). APO3G is shown in the top panel; Vif is shown in the middle panel; tubulin expression serves as an internal loading control and is shown in the lower panel. **(b)** Vif inhibits packaging of APO3G C97A into HIV-1 virions. APO3G C97A was co-expressed in HeLa cells together with NL4-3(Vif-). Increasing amounts of Vif were provided in trans from a separate vector. APO3G C97A is packaged into HIV-1 virions in the absence of Vif (lane 8) but not when Vif is present (lanes 9-10). Viral capsid protein (CA) is shown in the lower panels; APO3G and Vif are shown in the upper and middle panels, respectively

abolished catalytic nor antiviral activity of APOBEC3G [74]. Mutation of C288 and C291, on the other hand, did not impair APOBEC3G oligomerization but completely abrogated APOBEC3G deaminase activity. Nevertheless, this deaminase-deficient APOBEC3G mutant was packaged into *vif*-defective HIV-1 virions and retained partial antiviral activity when transiently expressed in HeLa cells [74].

APOBEC3G assembles into intracellular high-molecular-mass (HMM) ribonucleoprotein complexes in activated $CD4^+$ T-cells and transfected cell lines [19,20,24,47]. APOBEC3G associated with HMM complexes was reported to be enzymatically inactive and is presumed to lack antiviral activity [19]. Since both multimerization of APOBEC3G and assembly into HMM complexes are RNA-dependent, we investigated the ability of the multimerization-incompetent APOBEC3G C97A mutant to form HMM complexes in transfected HeLa cells. We found that APOBEC3G C97A retained its ability to form HMM complexes. Assembly into HMM complexes was sensitive to RNase treatment although the effect of RNase-treatment was different for wt and mutant APOBEC3G protein [74]. We also tested the sensitivity of APOBEC3G C97A to inhibition by Vif. Interestingly, we found that APOBEC3G C97A was resistant to degradation by Vif. In addition to the C97A mutation, mutation of the nearby C100 residue in APOBEC3G C100S also rendered the protein resistant to degradation by Vif while mutation of cysteine residues in the *C*-terminal zinc finger motif did not affect sensitivity to Vif. These results suggest that sensitivity of APOBEC3G to degradation by Vif is dependent on determinants in the *N*-terminal zinc-finger domain [74]. Importantly, we found that despite resistance to degradation, encapsidation and antiviral activity of APOBEC3G C97A were still efficiently controlled by Vif. These results suggest that Vif has evolved distinct mechanisms to exclude APOBEC3G from HIV-1 virions. One involves intracellular degradation of APOBEC3G; the other regulates packaging of APOBEC3G through a degradation-independent pathway [74].

7.3.5 Viral RNA is Required for the Association of APOBEC3G with Viral Nucleoprotein Complexes

It is well established that APOBEC3G is packaged into virus particles; in fact, viral packaging is a prerequisite for the APOBEC3G-induced hypermutation of the viral minus-strand cDNA during reverse transcription [40]. Interestingly, human APOBEC3G is not only packaged into human immunodeficiency viruses but it is also incorporated into simian immunodeficiency viruses and murine leukemia virus. The efficient packaging of APOBEC3G into such diverse viruses suggests that the mechanism of APOBEC3G packaging is either relatively non-specific or that it requires signals shared by these viruses. Indeed, the recent observation that deletions in various regions of APOBEC3G can prevent its packaging into virus particles point towards a specific APOBEC3G packaging mechanism.

APOBEC3G was found to bind RNA *in vitro*, a property that it shares with other members of the APOBEC family of proteins. Indeed, while viral RNA was not absolutely essential for the packaging of APOBEC3G, its presence clearly enhanced APOBEC3G encapsidation [95]. We investigated the packaging of APOBEC3G into HIV-1 virions, and we found that viral RNA promotes the stable association of APOBEC3G with viral nucleoprotein complexes. Mutations of the viral nucleocapsid protein or its target binding site on the viral RNA interfered with both the virus incorporation and the nucleoprotein complex association of APOBEC3G. A target sequence located within the 5′-untranslated region (5′-UTR) of the viral RNA was identified as necessary and sufficient for efficient APOBEC3G packaging [45]. Fine mapping of this region revealed a stem-loop motif (SL1), normally involved in viral genomic RNA dimerization and Gag binding, to be necessary for APOBEC3G association with viral nucleoprotein complexes. Mutation of the SL1 motif alone or elimination of the entire RNA packaging signal resulted in reduced virus-associated APOBEC3G and destabilized APOBEC3G: nucleoprotein complexes. These results corroborate and extend our previous observations, which suggest that viral RNA is required for stable association of APOBEC3G with the viral nucleoprotein complex. During virus production, proper assembly of APOBEC3G into viral nucleoprotein complexes may be necessary to exert its mutagenic activity and could explain why APOBEC3G, when expressed in target cells rather than producer cells, generally does not block HIV infection [45].

In an extension of this study, we investigated the possible involvement of cellular RNAs in the encapsidation of APOBEC3G into HIV-1 virions. We focused on RNAs previously identified in intracellular APOBEC3G complexes (e.g., human Y RNAs [20] or HIV-1 RNA [47]) or previously found in retroviral particles [7SL [71,72,79]; snRNAs (U1-U6) [25]]. We also analyzed mRNAs previously reported to be excluded from intracellular APOBEC3G complexes (α-tubulin and β-actin [20,47]), and we randomly chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to study APOBEC3G binding and virus encapsidation of its mRNA. Our results confirmed the presence of hY1 and hY3 RNAs in intracellular APOBEC3G complexes. In addition, we identified 7SL RNA, U6 snRNA, and GAPDH mRNA as novel components of intracellular APOBEC3G complexes [44]. Only small amounts of α-tubulin mRNA were recovered from APOBEC3G immune complexes as reported before [20]; On the other hand, β-actin mRNA was clearly associated with APOBEC3G complexes in our analysis contrasting earlier

reports. Most of these RNAs were also packaged into HIV-1 virions. Interestingly, packaging of hY RNAs appeared to be inhibited by the presence of genomic RNA while packaging of other cellular RNAs including 7SL RNA was largely independent of viral genomic RNA. Taken together, our data strongly support a role of viral genomic RNA in the specific encapsidation of APOBEC3G. Our results also demonstrate that cellular RNAs are not sufficient for the encapsidation of APOBEC3G into HIV-1 particles and for the functional association with viral nucleoprotein complexes [44].

7.3.6 Enzymatically Active APOBEC3G is Required for Efficient Inhibition of HIV-1

In the absence of Vif, APOBEC3G is efficiently packaged into HIV virions and inhibits virus replication. A number of studies reported that the presence of APOBEC3G in the virus can result in hypermutation of the viral minus-strand cDNA during reverse transcription [32,50,55,59,107,110], inhibition of reverse transcription [28], tRNA annealing or tRNA processing [29,61], DNA strand transfer [51,61], or integration [53,61].

Some of these effects do not require catalytically active APOBEC3G [51,53], and several reports suggested that deaminase-defective APOBEC3G and APOBEC3F have antiviral activity when transiently co-expressed with HIV-1 in 293T cells [6,34,69,88]. Our own data concerning the antiviral properties of the deaminase-defective APOBEC3G C288S/C291A mutant supported these conclusions [74]. However, in our previous study we found that comparable inhibition of viral infectivity required higher levels of deaminase-defective APOBEC3G protein than that of wild type (wt) [74]. Using transient transfection protocols as well as stable cell lines expressing wt and deaminase-defective APOBEC3G (Fig. 7.6), we performed side-by-side analyses of wt and deaminase-defective APOBEC3G. Our results demonstrate that wt APOBEC3G has significantly higher antiviral activity than does deaminase-defective APOBEC3G [64]. In particular, deaminase-defective APOBEC3G in stably transfected HeLa cells had negligible antiviral activity as compared to the wt protein, despite both proteins being encapsidated with very similar efficiency and were equally sensitive to Vif. Deaminase-defective APOBEC3G, like wt APOBEC3G, co-purified with the viral nucleoprotein complexes. Our finding that the deaminase-defective APOBEC3G C288S/C291A variant had antiviral activity only when expressed at high levels raises questions about the physiological relevance of deaminase-independent activities of APOBEC3G [64].

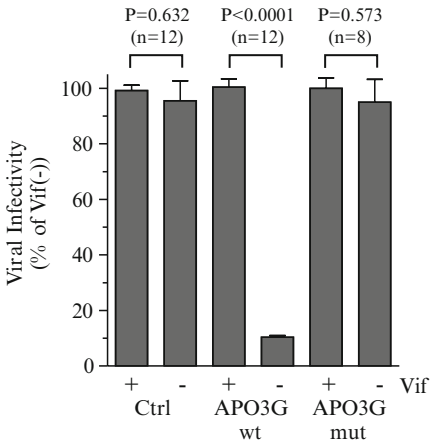

Fig. 7.6 Deaminase activity is required for efficient antiviral activity of APOBEC3G in stable HeLa cells. Stable HeLa cell lines expressing wt or deaminase-defective APOBEC3G were transfected with pNL4-3 (Vif+) or pNL4-3vif(-) (Vif-) DNA. Transfected cells and virus-containing supernatants were harvested 24 h later. Cell-free virus was normalized for equivalent reverse transcriptase activity and used to determine the viral infectivity in a single round infectivity assay. Error bars reflect standard deviations from eight independent infections of each of the three producer cell types. P values were calculated using a two-tailed unpaired t-test (GraphPad Prism 4)

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Part III
Microbiology: Bacteriology

Chapter 8
Exploring the Cause of Human Q Fever: Recent Advances in *Coxiella burnetii* Research

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8.1 Introduction

The obligate intracellular bacterium *Coxiella burnetii* is the causative agent of the zoonosis Q fever, a disease that generally manifests as an acute, debilitating flu-like illness [1]. Rare but potentially severe chronic disease can occur that commonly manifests as endocarditis. The animal reservoirs of *Coxiella* are extensive with human infection occurring primarily through contact with infected livestock and their products. In most animals, *Coxiella* does not cause overt disease, although extensive proliferation of the organism in the female reproductive system can result in late-term abortion. Parturition can consequently deposit large numbers of *Coxiella* into the environment. The debilitating nature of Q fever, along with pathogen environmental stability, aerosol transmission, and a very low infectious dose has resulted in classification of biosafety level-3 *Coxiella* as a Centers for Disease Control and Prevention category B select agent with potential for illegitimate use [2].

In natural infections, *Coxiella* has a tropism for mononuclear phagocytes such as alveolar macrophages [3]. Central to the pathogenesis of *Coxiella* is its unique ability among intracellular bacteria to replicate in a parasitophorous vacuole (PV) that appears indistinguishable from a phagolysosome [4, 5]. Due to experimental restrictions imposed by *Coxiella*'s obligate intracellular lifestyle, there is a paucity of molecular information regarding genetic determinants that allow the organism to withstand this harsh environment and to cause disease. Indeed, lipopolysaccharide (LPS) is currently the only defined *Coxiella* virulence factor [6]. Full-length "phase I" LPS is required for pathogen virulence and is produced by isolates from natural sources and infections. Serial *in vitro* passage of phase I *Coxiella* in embryonated eggs or tissue culture selects for bacteria synthesizing shortened LPS molecules, a process that culminates in the severely truncated LPS of "phase II" organisms [7]. Clonal isolates of phase II *Coxiella* are considered avirulent for an immunocompetent animal [6].

Important insight into *Coxiella* genetic potential was recently provided by the complete genome sequence of the Nine Mile reference strain [8]. Highlights of the ~ 2 megabase sequence include the identification of putative virulence genes with potential roles in host cell invasion, intracellular trafficking, and lysosomal detoxification. Abundant transporters also point to *Coxiella* exploiting the degradative milieu of its lysosome-like niche for nutrients. Interestingly, the genome differs substantially from those of obligate intracellular *Rickettsia* and *Chlamydia* in having abundant mobile genetic elements, less genome reduction, and coding for extensive metabolic functions. These characteristics are consistent with a bacterium that is evolutionarily at an early stage of obligate parasitism and pathoadaptation [8, 9].

Here, we summarize recent advances in *Coxiella* research that have provided insight into the pathogen's cellular and developmental biology, including unconventional pathogen interactions with the innate and adaptive immune systems. Moreover, a genomics approach to discover *Coxiella* diagnostic antigens and a host cell-free (axenic) culture method are discussed. Finally, development of a genetic transformation system for *Coxiella* is reviewed.

8.2 The *Coxiella* Parasitophorous Vacuole (PV)

8.2.1 *Parasitophorous Vacuole Characteristics*

Coxiella replicates in a membrane-bound compartment (termed the parasitophorous vacuole [PV]) that, to a first approximation, resembles an exceptionally large phagolysosome (Fig. 8.1). Following *Coxiella* internalization, the nascent pathogen-containing phagosome matures through the canonical endolysosomal pathway to fuse with lysosomes, albeit with slower kinetics than phagosomes containing inert particles [5, 10, 11]. The benefit of phagosome stalling to the pathogen is unknown. The "lysosome-like" character of the mature *Coxiella* PV (> 1 day post-infection [PI]) was noted over 20 years ago owing to an acidic pH (~ pH 4.5) and the presence of lysosomal enzymes such as acid phosphatase

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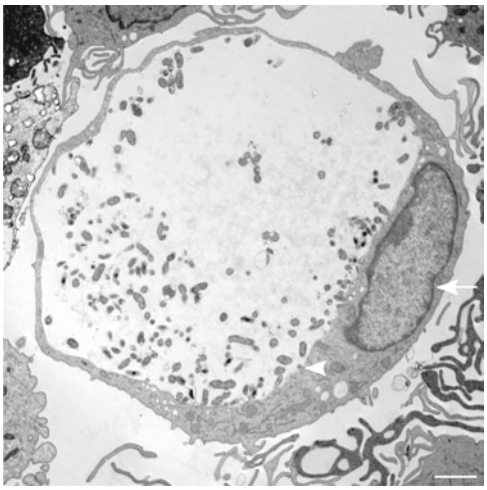

Fig. 8.1 Large and spacious *Coxiella* PV in a human dendritic cell. Transmission electron micrograph of a human peripheral blood monocyte-derived dendritic cell infected with *Coxiella* for two days. Rapid expansion of the vacuole occurs concomitant with the onset of pathogen replication at 1 to 2 days post-infection. Arrow and arrowhead denote the host cell nucleus and *Coxiella* PV, respectively. Bar, 2 μM

[12, 13]. In fact, *Coxiella* is strictly reliant on a moderately acidic pH to sustain metabolism and has exploited the only intracellular niche supporting this condition [4]. During maturation, the nascent phagosome also quickly engages the autophagic pathway with the autophagosome marker microtubule-associated protein light-chain 3 (LC3) decorating the vacuole as early as five minutes post-infection [11, 14]. The mature PV retains autophagosome markers and also contains vacuolar H^+ ATPase, Rab7 and various lysosome-associated membrane proteins (LAMPs) and hydrolases [3].

8.2.2 Pathogen Subversion of Vesicular Tafficking

The ability of *Coxiella* to prosper in a normally bacteriostatic/cidal vacuole is central to its pathogenesis. While the *Coxiella* PV is clearly endolysosomal in origin, it also has characteristics that distinguish it from a phagolysosome containing inert particles such as latex beads. For example, at ~ 2 days PI, and coincident with entry of *Coxiella* into its exponential growth phase, the maturing PV dramatically expands to often occupy the majority of the host cell cytoplasm [15]. Furthermore, the large and spacious PV promiscuously fuses with endolysosomal vesicles and fluid phase endosomes. PV structure and behavior suggested *Coxiella* actively subverts vesicular trafficking to generate this unusual compartment. Indeed, we demonstrated that *Coxiella* protein synthesis, but not replication, is required for PV maturation and fusogenicity [16, 17]. In cells treated with chloramphenicol to inhibit pathogen protein synthesis, organisms are harbored as single bacteria in tight-fitting, acidic, LAMP-positive vacuoles scattered throughout the cytoplasm. These vacuoles homotypically fuse to form a large and spacious PV upon removal of the drug. Similar treatment of infected cells containing a pre-existing large and spacious PV also inhibits fusion between the PV and latex bead-containing phagosomes and causes the PV to collapse.

Coxiella protein synthesis is also required for early autophagosome interactions, an event that is thought to mediate stalling of PV maturation [10, 11]. Autophagy degrades cellular components via trafficking of targeted material to autophagolysosomes [18]. *Coxiella* clearly benefits from autophagy, as inducers of the pathway promote pathogen replication and PV development [19]. Because the *Coxiella* PV is not passively permeable to small molecules [20] and requires an exogenous source of membrane for expansion, continuous PV fusion with autophagosomes and other vesicles is presumably essential for acquisition of nutrients and membrane. Collectively, these results demonstrate that *Coxiella* is an active participant in the biogenesis of its biologically unique PV.

8.2.3 Role of Cholesterol in PV Formation

While some host protein constituents of the PV have been described, the composition of the PV membrane remains largely undefined. *In vivo,* the vacuole is resistant to mechanical disruption, a characteristic observed during microinjection experiments [20]. Moreover, the PV membrane has a low buoyant density when fractionated by density gradient centrifugation [21] and contains lipid raft-associated proteins such as flotillin 1 and 2 [22]. These features suggested the PV membrane was cholesterol-rich. Therefore, we investigated whether *Coxiella* infection influences cellular cholesterol metabolism and if disruption of cholesterol uptake and biosynthetic pathways affects PV development.

In infected cells, the *Coxiella* PV membrane stains for cholesterol at roughly the same intensity as the cholesterol-rich plasma membrane, and the total host cell cholesterol content is 73% greater than that of uninfected cells. Host cell genes encoding proteins involved in both cholesterol uptake (e. g., low density lipoprotein receptor) and synthesis (e. g., C-24 sterol reductase) are upregulated during the exponential phase of *Coxiella* growth, when the PV is rapidly expanding, then down regulated during stationary phase, when PV size has maximized. Together, these data indicate the PV is a bioactive organelle that is integrated into the cellular cholesterol distribution machinery. Demonstrating the critical importance of cholesterol to *Coxiella* infection

is the dramatic alteration in PV morphology and inhibition of pathogen replication observed when infected cells are treated with pharmacological inhibitors of cholesterol uptake and/or biosynthesis [21]. Among other possibilities, cholesterol may strengthen the PV membrane, decrease vacuole ion permeability, and/or serve along with sphingolipids to form lipid raft microdomains that have important signaling roles.

8.3 *Coxiella* Pro-survival Signaling

8.3.1 Inhibition of Apoptosis

Development of a large and spacious PV that contains hundreds of *Coxiella* organisms presumably imposes considerable metabolic stress on the host cell. Yet, little cytopathic effect is observed during *Coxiella*'s lengthy infectious cycle (> 6 days to stationary phase). *Coxiella*-infected cells also divide normally and distribute the PV to only one daughter cell [23]. Collectively, these observations suggested *Coxiella* employs an active mechanism to sustain host cell viability. We investigated *coxiella* maintenance of host cells by examining the activation status of host cell pro- and anti-survival pathways during infection.

Using infection of human THP-1 monocyte-derived macrophage-like cells and primary monkey alveolar macrophages as model systems, we demonstrated that *Coxiella* infection protects macrophages from apoptotic death following treatment with staurosporine, a potent inducer of intrinsic apoptosis [24]. This protection correlates with reduced cleavage of caspase-9, caspase-3, and poly (ADP-ribose) polymerase (PARP), all proteolytic events that occur during apoptosis. Reduced PARP cleavage is also observed in infected cells treated with tumor necrosis factor-α to induce extrinsic apoptosis. *Coxiella* infection affects expression of multiple apoptosis-related genes and results in increased synthesis of the anti-apoptotic proteins A1/Bfl-1 and c-IAP2. Apoptosis inhibition requires *Coxiella* protein synthesis, indicating this is a pathogen directed process [24]. Similar events, along with reduced mitochondrial release of cytochrome *c,* were shown by Luhrmann and Roy to occur in epithelial cells [25].

8.3.2 Activation of Erk1/2 and AKT kinases

In a separate study, we conducted a multiplex analysis of 15 host signaling proteins involved in stress response, cytokine production, and cell survival [26]. Interestingly, the pro-survival kinases AKT and Erk1/2 show sustained activation during infection (at least 72 h PI). Activation is abolished if cells are treated with rifampin to inhibit bacterial RNA synthesis, indicating a *Coxiella* a directed process. Pharmacological inhibition of Akt or Erk1/2 significantly decreases *Coxiella'*s anti-apoptotic activity. These results show that *Coxiella* actively modulates several signaling pathways to inhibit host cell death, thus providing a stable intracellular niche for the course of its lengthy infectious cycle.

8.4 Dot/Icm Type IV Secretion System (T4SS) and Effector Proteins

As described above, *Coxiella* protein synthesis is necessary for PV biogenesis/maintenance and protection against apoptotic host cell demise. Pathogen effectors of these and other host cell functions are often directly translocated into the cytosol via specialized secretion systems [27]. The *Coxiella* genome encodes a Dot/Icm type IV secretion system (T4SS) that is nearly identical to that of its phylogenetically-close relative *Legionella pneumophila* [28]. Secretion of Dot/Icm substrates by *Legionella* is absolutely required for establishment of the pathogen's replication PV, with a similar requirement invoked for *Coxiella* [3].

As an initial probe of *Coxiella* type IV secretion, we co-infected a variety of cell types with *Legionella* and *Coxiella* and evaluated pathogen trafficking and replication. This dual infection approach allowed examination of whether the effects of Dot/Icm T4SS substrates are restricted to pathogen vacuoles. Without exception, *Legionella* and *Coxiella* replicate in co-infected cells with unaltered kinetics in closely juxtaposed but distinct PVs [29]. These findings suggest *Legionella* and *Coxiella* Dot/Icm T4SS substrates have highly specific effector functions that do not globally affect host processes which inhibit formation of a different PV. This unique biology likely reflects the fact that, although the genomes of *Legionella* and *Coxiella* encode similar Dot/Icm secretion systems, to date nearly all defined and putative *Legionella* type IV effector proteins lack homologs in *Coxiella* [27].

Identification and functional characterization of *Coxiella* T4SS substrates is critical to gaining a better understanding Q fever pathogenesis. However, direct genetic manipulation of *Coxiella* to achieve this goal is currently not possible. Fortunately, the close relationship of *Legionella* and *Coxiella,* and their production of similar Dot/Icm systems, has allowed use of *Legionella* as a surrogate host to screen candidate *Coxiella* T4SS substrates. A commonly used type IV secretion assay involves translocation of candidate effector proteins fused to the calmodulin-activated adenylate cyclase (CyaA) of *Bordetella pertussis*. *Legionella* expressing a

Coxiella protein-CyaA chimera is used to infect mammalian cells. If the fusion protein is translocated into the host cytosol, then CyaA is activated by binding calmodulin thereby resulting in production of high levels of intracellular cAMP that can be detected using a sensitive enzyme immunoassay. Using this approach, four *Coxiella* eukaryotic-like ankyrin repeat domain-containing proteins (Anks) have recently been identified as Dot/Icm substrates [30].

The identification of Anks as *Coxiella* T4SS substrates is consistent with the growing number of secreted bacterial effector proteins containing eukaryotic-like motifs/domains, which often functionally mimic or alter the activity of host cell proteins [31]. Indeed, both *Coxiella* and *Legionella* encode numerous proteins with eukaryotic-like domains such as Anks, tetratricopeptide repeats, coiled coil domains, leucine-rich repeats, GTPase domains, and ubiquitination-related motifs [9, 32, 33]. Known *Legionella* effectors of this nature have diverse cellular activities, including guanine nucleotide exchange and E3 ubiquitin ligase activities [34].

Using the CyaA assay, we have currently identified 27 *Coxiella* proteins, most with eukaryotic-like features, that are secreted in a T4SS-dependent fashion (unpublished data). Two particularly interesting groups of effectors are plasmid-encoded proteins and a heterogeneous group of Anks. All *Coxiella* isolates maintain a large (~33-54 kb) autonomously-replicating plasmid or plasmid sequences integrated into their chromosome [9]. Approximately 14 kb of plasmid sequences are conserved among isolates, encoding only five shared hypothetical proteins [35]. The absolute maintenance of these sequences/genes suggests they are indispensable for *Coxiella* survival. CyaA screening of the five conserved hypothetical proteins revealed that three are T4SS substrates. Two proteins unique to the QpH1 plasmid of the Nine Mile reference isolate, one with ubiquitination-related F-box homology and the other with a kinesin-like domain, are also secreted in a T4SS-dependent fashion. These results suggest a hitherto unknown role for conserved and unique plasmid sequences in host cell modulation.

The Ank gene family, the second group of effectors, displays striking heterogeneity between *Coxiella* isolates of differing virulence potential. For example, the naturally attenuated Dugway rodent isolate encodes eleven secreted Anks while the highly virulent Nine Mile isolate encodes only five. Thus, it is intriguing to speculate that Ank gene content plays a role in *Coxiella* pathotype-specific virulence, and, perhaps in the case of Dugway, downregulates virulence by modulating unique host cell processes (Voth *et al.,* manuscript submitted).

Coxiella T4SS effector molecules represent a treasure trove of potential virulence factors and elucidation of their cellular targets and activities will further our understanding of the cellular biology and virulence of this pathogen. Indirect approaches are currently necessary to define *Coxiella* effector function. A commonly used method that provides functional clues is to ectopically express an effector in mammalian cells as a fluorescent fusion protein where the effector is predicted to modulate an activity associated with a targeted sub cellular site. Ectopically expressed *Coxiella* Dot/Icm substrates localize to a variety of sub cellular locations including the PV membrane and host organelles. For example, AnkO and AnkJ fused to red fluorescent mCherry traffic to the PV membrane and mitochondria, respectively, in infected HeLa cells (Fig. 8.2). PV localization of AnkO-mCherry suggests the protein may mediate vesicular fusion events required for vacuole biogenesis and/or maintenance. Localization of AnkJ-mCherry to host mitochondria suggests this protein may subvert apoptotic signaling. Other effectors localize to autophagosomes, endoplasmic reticulum and microtubules (Voth and Heinzen, unpublished observations). Identification of the effector-host protein binding partner(s) is critical to defining effector protein function and can be accomplished using yeast two-hybrid, co-immunoprecipitation, or pull-down assays.

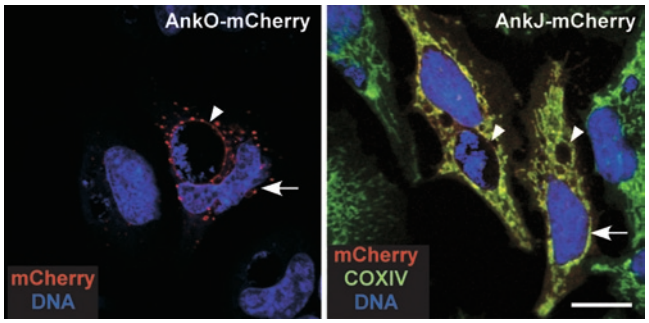

Fig. 8.2 Trafficking of ectopically expressed *Coxiella* Dot/Icm substrates in mammalian cells. *Coxiella*-infected HeLa cells (2 days PI) were transfected with plasmids encoding AnkO or AnkJ C-terminally fused to the *N*-terminus of mCherry (red). A primary antibody was used to stain the mitochondrial protein COXIV (green) and DRAQ5 was used to stain host and *Coxiella* DNA (blue). Cells were visualized by confocal fluorescence microscopy. AnkO-mCherry localizes to the PV membrane and punctate vesicular structures. AnkJ-mCherry traffics to the mitochondria as evidenced by the yellow fluorescence resulting from overlapping mCherry and COXIV fluorescent signals. Arrows and arrowheads denote the host cell nucleus and *Coxiella* PV, respectively. Bar, 10 μM

8.5 Biphasic Development

8.5.1 *Biology of Coxiella Developmental Forms*

Coxiella's resistance to the degradative conditions of its PV correlates with remarkable environmental stability. A biphasic developmental cycle producing environmentally stable small

cell variant (SCV) and unstable large cell variant (LCV) morphological forms was proposed by McCaul and Williams in 1981 and is considered important for *Coxiella* virulence [36]. Ultrastructural characteristics of the SCV and LCV have been defined; however, little is known about the biological roles of cell variants such as relative infectivities for animal hosts and roles in *in vivo* persistence. Furthermore, the molecular events leading to morphological differentiation are largely unknown. As most cases of human Q fever are acquired through inhalation of contaminated aerosols originating from products shed by chronically infected livestock, the environmentally resistant SCV likely initiates most natural infections. Thus, rationally designed vaccines to protect against Q fever should specifically target this cell form.

To gain a greater understanding of *Coxiella* biphasic development, we defined the growth kinetics and some transcriptional properties of SCV and LCV. One-step growth curves, using both genome equivalents and infectious foci forming units as readouts of replication, were generated to correlate morphological differentiations observed by transmission electron microscopy with growth cycle phase. Aiding this study, was the discovery that *Coxiella* harvested from aged infected cell cultures (~ 28 days PI) consist almost entirely of highly infectious SCV. This cultivation procedure allowed synchronous infection of host cells with purified SCV [15].

Following infection, SCV to LCV morphogenesis occurs over a lag phase lasting approximately 2 days. Exponential replication of the LCV then ensues and is associated with dramatic expansion of the PV. During roughly 4 days of exponential growth, the LCV has a doubling time of approximately 11 h. Stationary phase begins at approximately 6 days PI and coincides with the reappearance of SCV that increase in number for the duration of the infectious process. In general, quantitative reverse transcriptase PCR shows maximal expression of metabolic, porin, and T4SS genes during mid-log phase. Collectively, these results indicate that the overall growth cycle of *Coxiella* is characteristic of a closed bacterial system and that the replicative form of the organism is the LCV [15].

8.5.2 Composition and Antigenicity of SCV and LCV Developmental Forms

The antigenic uniqueness of *Coxiella* cell forms was noted prior to description of the pathogen's developmental cycle [37]. A handful of proteins have been subsequently identified that are differentially-expressed by SCV and LCV [38]. In particular, two highly basic DNA-binding proteins termed ScvA and Hq1 are selectively synthesized by the SCV. These proteins are thought to promote SCV chromatin condensation and metabolic quiescence, as well as protecting the DNA from environmental insult [39, 40].

To provide a more complete assessment of proteins that may confer the different biological and ultrastructural properties of SCV and LCV, we conducted a proteome analysis of cell forms purified from a mixed population by cesium chloride density gradient centrifugation. Two-dimensional (2-D) gel electrophoresis and silver staining revealed multiple proteins that are differentially expressed by SCV and LCV. Proteins identified by mass spectrometry show functional roles consistent with a metabolically active LCV (e.g., FtsZ, a cell division protein) and a structurally resistant SCV (e.g., TolB, a protein involved in outer membrane stabilization). Interestingly, multiple stress-related proteins, such as thioredoxin peroxidase, were also upregulated by the LCV, suggesting the lysosome-like PV presents a stress situation for this cell form. Common and unique antigens are produced by cell forms as indicated by immunoblots probed with convalescent serum from human Q fever patients. These may be important in the immunopathophysiology of clinical Q fever and/or the induction of protective immunity [41].

8.6 *Coxiella* Phagocyte Interactions and Immune Function

8.6.1 Human Dendritic Cell Interactions

Acute Q fever is generally self-limiting and resolves within two weeks. However, in most cases resolution of disease does not result in complete clearance of *Coxiella* [42]. Consequently, in predisposed individuals, latent organisms can reactivate months or years after initial exposure to cause serious chronic disease such as endocarditis. Components of innate and adaptive immunity that resolve acute Q fever infection are largely undefined. Furthermore, mechanisms by which *Coxiella* evades clearance by the host immune response during persistent infection to recrudesce are poorly resolved.

To achieve a better understanding of the host response to primary infection and mechanisms of protective immunity, we investigated *Coxiella's* interaction with dendritic cells (DC), professional antigen presenting cells that bridge the innate and adaptive immune responses. Functional roles of DCs include priming the adaptive immune response by secreting cytokines that promote development of naïve $CD4^+$ T-cells into Th1 type effectors, the T-cell type associated with control of a number of intracellular parasites [43]. Infection by the Nine Mile phase I (NMI) strain, which is virulent and produces full-length LPS, does not induce DC maturation. In contrast, infection by the avirulent Nine Mile

phase II (NMII) strain, with severely truncated LPS, results in Toll-like receptor 4 (TLR4)-independent DC maturation and upregulation of the inflammatory cytokines IL-12 and TNF. Differential DC activation has no effect on pathogen growth as NMI and NMII replicate with similar kinetics *in vitro*. Chemical extraction of LPS from NMI dramatically increases its ability to stimulate DCs. Collectively, these data indicate *Coxiella* LPS is non-stimulatory and that the full length molecule of virulent organisms masks other TLR ligands, such as lipoproteins, from innate immune recognition [44]. In a separate study, we determined that the masking effect is a common property of the three defined phase I LPS chemotypes of *Coxiella* [45]. Infection of DCs by virulent *Coxiella,* without stimulating significant maturation or inflammatory cytokine production, may be a mechanism of immune evasion that contributes to persistent infection of an otherwise immunocompetent host.

8.6.2 Antibody- Mediated Immunity to Coxiella Infection is Fc Receptor- and Complement-Independent

Cell-mediated immunity is clearly important for protection against infection by intracellular pathogens [43]. This immunological paradigm extends to *Coxiella* where infection stimulates a strong cellular host response as indicated by bacterial antigen-driven proliferation and IFN-γ secretion by T-lymphocytes from convalescent Q fever patients [46]. IFN-γ stimulation of infected mononuclear phagocytes and fibroblasts effectively controls *Coxiella* growth with reactive oxygen and nitrogen species considered important effector molecules [47, 48, 49].

A growing body of evidence indicates that humoral immunity also plays an important role in protective immunity to intracellular pathogens [50]. Indeed, shortly after the discovery of *Coxiella*, Burnet and Freeman described the protection of mice and guinea pigs from infection by passive immunization with *Coxiella* immune serum [51, 52]. Antibody-mediated immunity (AMI) can occur through a variety of different mechanisms including direct bactericidal activity, complement activation, antibody-dependent cellular cytotoxicity, and opsonization that can result in altered intracellular trafficking [50].

The mechanism of AMI to *Coxiella* is unknown. As discussed above, virulent *Coxiella* replicate within human DCs without inducing their maturation or activation. Interestingly, antibody-opsonized *Coxiella* are more efficiently internalized by human monocyte-derived and murine bone marrow-derived DCs and cause these cells to mature and produce inflammatory cytokines without negative effects on pathogen replication. Similar effects are observed in cultured macrophages [53, 54]. Thus, enhanced phagocytosis and/or altered intracellular trafficking likely do not contribute to *Coxiella* AMI. Activation of DC by opsonized *Coxiella* is dependent on the Fc receptor (FcR) as evidenced by a reduced response of DCs from FcR knockout (k/o) compared to C57Bl/6 (B6) mice. However, the FcR is dispensable for AMI *in vivo* as passively immunized FcR k/o mice show equal protection from *Coxiella* infection as B6 control mice based on splenomegaly and spleen organism counts. Similar protection is observed in passively immunized C2/fB k/o mice that are deficient in all complement pathways. Thus, AMI to *Coxiella* is independent of both FcR and complement function and remains to be defined (Shannon et al., manuscript in preparation).

8.7 *Coxiella* Pathogenomics

8.7.1 Comparative Genomics

Comparative genomics is a powerful tool to identify pathogenetic determinants that confer unique disease potential between strains of a given bacterial species. This analysis is particularly informative with obligate intracellular bacteria where, because of limited opportunities for genetic exchange, strains can show dramatically different virulence phenotypes while lacking broad genetic variance. *Coxiella* isolates are genetically distinct and show different degrees of pathogenicity for laboratory animals [6, 55, 56]. Moreover, an interesting, but controversial, hypothesis suggests *Coxiella* isolates have pathogenetic potential to cause human acute or chronic disease [57]. Therefore, to provide a more complete understanding of *Coxiella* genetic diversity, evolution, and pathogenic potential, we conducted microarray-based whole-genome comparisons between the Nine Mile reference isolate and multiple isolates from different sources.

The genomes of 24 *Coxiella* isolates were hybridized to a high-density custom Affymetrix GeneChip containing all open reading frames (ORFs) of the Nine Mile reference isolate [35]. Significant findings of this study include the following: (i) identification of conserved plasmid gene content; (ii) placement of isolates into 8 phylogenetically-distinct genomic groups; (iii) identification of possible pathotype-specific ORFs; (iv) the discovery that large chromosomal deletions are not necessarily involved in LPS phase transitions; and (v) confirmation that QpDG from the naturally attenuated Dugway rodent isolate is a distinct plasmid.

Although highly informative, microarray-based whole genome comparisons suffer from being unidirectional (i.e., all test isolates are compared to the reference isolate). Thus, in a separate study, we sequenced the genomes of the K and G

human chronic endocarditis isolates and the Dugway isolate [9]. Cross-genome comparisons that included the previously sequenced Nine Mile isolate revealed both novel gene content and disparate collections of pseudogenes and potential secreted effector proteins that may contribute to isolate virulence and other phenotypes. The major force behind *Coxiella* genome plasticity appears to be extensive recombination between abundant insertion sequence (IS) elements, resulting in chromosomal rearrangement of syntenic blocks (i. e., same gene content and order) and DNA insertions/deletions. The numerous IS elements, genomic rearrangements, and pseudogenes of *Coxiella* isolates is consistent with genome structures of other bacterial pathogens that have recently emerged from non-pathogens with expanded niches [58]. The observation that the severely attenuated Dugway isolate has the largest genome (by ~ 200 kb) and the fewest pseudogenes and IS elements, suggests this isolate lineage is at an earlier stage of pathoadaptation than the NM, K, and G lineages [9]. Polymorphic ORFs associated with isolates of differing virulence potential can now be examined for function in *Coxiella* pathogenicity and disease outcome. This information will improve our ability to model *Coxiella* pathogenesis and speed development of rationally designed subunit vaccines with efficacy against a range of *Coxiella* isolates.

8.7.2 Candidate Q Fever Serodiagnostic and Subunit Vaccine Antigen Discovery using Protein Microarray Technology

The complex nature of the whole cell antigen used in current Q fever serological tests results in lack of uniformity and specificity in test results. Furthermore, current vaccines based on formalin-killed organisms can cause severe local reactions in individuals previously sensitized to *Coxiella.* Thus, skin testing is required to assess pre-existing immunity prior to vaccination. Control of Q fever would be aided by a specific/sensitive serodiagnostic test and an efficacious/safe vaccine based on recombinant antigen.

To this end, we identified immunodominant antigens recognized by antibody in the context of human *Coxiella* infection or vaccination by using high-throughput protein microarray technology [59]. Protein microarrays allow rapid identification of consensus immunodominant antigens on a whole proteome basis. Transcriptionally active PCR products of *Coxiella* ORFs were used a template in cell-free *in vitro* transcription and translation (IVTT) reactions. Full-length proteins corresponding to 75% of the Nine Mile strain proteome were synthesized and crude IVTT lysates spotted onto nitrocellulose microarrays. Approximately 50 strongly immunoreactive *Coxiella* proteins were identified by probing arrays with sera from acute Q fever patients and individuals vaccinated with a killed whole-cell vaccine. Recombinant protein corresponding to selected array-reactive antigens was generated and immunoreactivity confirmed by ELISA. These proteins may prove efficacious as Q fever serodiagnostic antigens.

The ability to identify *Coxiella* T-cell antigens by using IVTT-produced protein would aid development of a Q fever vaccine based on recombinant protein. A recent innovation in this regard is a method developed by Lopez *et al*., [60], which employs IVTT-expressed protein that is bead-affinity purified. Bead-bound antigens are added to antigen presenting cells which process and present antigen to T-lymphocytes from immunized animals. Using this bead procedure novel T-cell antigens of the obligate intracellular bacterium *Anaplasma marginale* were identified based on their ability to consistently stimulate T-cell recall responses. This method will allow high-throughput screening of *Coxiella* IVTT-expressed proteins to identify T-lymphocyte antigens with potential as subunit vaccine candidates [60].

8.8 Genetic Manipulation of *Coxiella*

The four-way genome comparisons described above provided a comprehensive view of *Coxiella* gene content. Isolate-specific genes were identified that likely contribute to *Coxiella* virulence. However, the absence of gene inactivation methods precludes the fulfilment of molecular Koch's postulates [61] for these putative virulence factors. A single report of *Coxiella* genetic transformation by electroporation was published over 10 years ago [62]. Although a significant achievement, the system suffered in using resistance to ampicillin as a method of positive selection. Spontaneous mutation to ampicillin resistance resulted in significant outgrowth of non-transformed organisms. To circumvent this problem, we investigated chloramphenicol resistance as a selectable marker [63]. Tetracycline or quinolone antibiotics are used in the clinical treatment of acute Q fever; therefore, use of the chloramphenicol acetyltransferase gene (*cat*) is appropriate for *Coxiella* transformation studies. *Coxiella* growth is completely inhibited in Vero cells treated with a low concentration of chloramphenicol (e. g., 5 μg/mL) that does not cause obvious host cell toxicity. The lack of organism outgrowth suggests spontaneous mutation to chloramphenicol resistance by *Coxiella* is rare or does not occur.

The *cat* gene was incorporated into the *mariner* family transposon *Himar1* to attempt random mutagenesis of the *Coxiella* genome. This transposon integrates nonspecifically with high frequency at T/A base pairs in the absence of specific host factors [64, 65]. *Coxiella* was co-electroporated with a plasmid encoding the *Himar1* C9 transposase variant

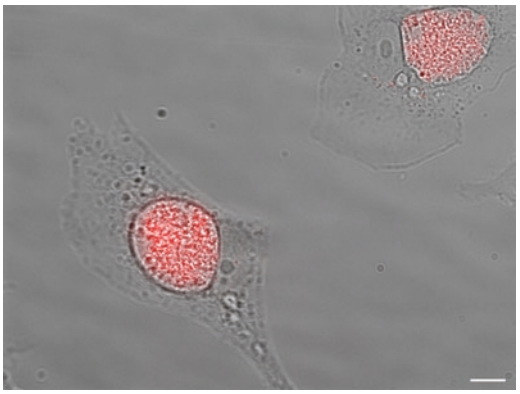

Fig. 8.3 Genetic transformation of *Coxiella* by transposon mutagenesis. Vero cells were infected with a *Coxiella Himar1* transposon mutant for 5 days, then they were imaged live by phase contrast and epifluorescence microscopy. The fluorescent image is overlaid onto the phase contrast image. Red fluorescent *Coxiella,* resulting from expression of mCherry carried by the transposon, are apparent in the PV of infected cells. Bar, 5 μM

and a plasmid containing a *Himar1* transposon (Tn) encoding chloramphenicol acetyl-transferase (CAT) and mCherry red fluorescent protein as a single transcriptional unit, as well as a ColE1 origin of replication for rescue cloning. Expression of both the transposase and CAT-mCherry was driven by the constitutive *Coxiella* Hsp20 (CBU1169) promoter. This transposon mutagenesis resulted in successful transformation of *Coxiella* as evidenced by mCherry-expressing organisms replicating in Vero cells in the presence of chloramphenicol (Fig. 8.3). Rescue cloning of the Tn-contained ColE1 origin of replication revealed 35 Tn insertion sites dispersed throughout the *Coxiella* chromosome and large cryptic plasmid [63].

Phenotypic evaluation of *Coxiella* transposon mutants requires clonal isolation. Historical methods of cloning *Coxiella* such as plaque formation and end-point limiting dilution are laborious and technically challenging. We developed a new procedure that takes advantage of the clonal nature of *Coxiella* residing in the large, phase-translucent PV of infected cells. The method relies on excision of individual PV using micromanipulation. Harvested organisms can be used to infect fresh cells for clonal expansion with or without prior genotyping by whole genome amplification methods [66].

Micromanipulation was used to derive a clone from the *Himar1* transformant mixture that harbors a Tn insertion within the cell division gene *ftsZ*. Characterization of the FtsZ::Tn mutant revealed a doubling time almost twice as long as wild type *Coxiella* and severe septation defects. Our FtsZ mutant is the first example of a cloned and characterized genetically-transformed *Coxiella.* The red fluorescent organisms generated in this study will be useful for host-pathogen interaction studies. Moreover, *Himar1*-based transformation can now be used to introduce transgenes into the *Coxiella* chromosome, allowing dominant/negative and complementation approaches to study gene function. Finally, the technical hurdles overcome in this study will aid development of additional genetic tools, most importantly site-specific allelic exchange.

8.9 Host Cell-Free Growth of *Coxiella*

The obligate intracellular nature of *Coxiella* has made elucidation of pathogen-specific processes technically challenging. Attempts to culture *Coxiella* outside of a host cell were tried upon its discovery in the late 1930s, but over six decades later laboratory growth of the organism was still strictly reliant on a viable eukaryotic host cell. Axenic (host cell-free) buffers have been developed that activate *Coxiella* metabolism *in vitro*. However, metabolism is short-lived with bacterial protein synthesis halting after a few hours [4].

A medium that supports prolonged *Coxiella* metabolic activity outside of host cells would dramatically aid biochemical studies and potentially lead to a method of axenic cultivation. *Coxiella* is genetically sophisticated relative to other obligate intracellular bacteria with roughly twice the genome size of obligate intracellular *Rickettsia* and *Chlamydia* [8]. The lack of extensive genome reduction correlates with complete pathways of central carbon metabolism and bioenergetics with the organism's abundant transporters likely compensating for existing auxotrophies [8]. The predicted metabolic complexity of *Coxiella* led us to hypothesize that a systematic evaluation of pathogen responses to different nutritional and biophysical conditions could yield a medium that supports sustained *Coxiella* axenic metabolic activity and potentially growth. Utilizing this strategy, we developed an axenic medium termed Complex *Coxiella* Medium (CCM) that supports *Coxiella* metabolic fitness for at least 24 h [67]. Critical components of CCM are citrate buffer (pH 4.5), three complex nutrient sources (neopeptone, fetal bovine serum and RPMI cell culture medium), and a salt solution containing 140 mM chloride. While *Coxiella* incubated in CCM had enhanced metabolic activity in the form of protein synthesis and a stable ATP pool, they did not replicate.

To gain insight into potential nutritional deficiencies of CCM, the transcriptome of *Coxiella* incubated in CCM was compared to that of bacteria replicating in Vero cells. Organisms metabolizing in CCM showed dramatically reduced expression of ribosomal genes, suggesting the medium was deficient in amino acids. A significant improvement in metabolic activity was observed when organisms

were incubated in CCM supplemented with additional amino acids and peptide sources (e.g., casamino acids) to generate Acidified Citrate Cysteine Medium (ACCM) [68]. *In silico* metabolic pathway reconstruction and phenotype microarray analysis further suggested that *Coxiella* is a microaerophile. Robust cell division (approximately 3 $\log_{10}$ over 6 days) was achieved when *Coxiella* were incubated in ACCM under microaerobic (2.5% O_2) conditions. The exponential phase doubling time of *Coxiella* in ACCM is 1-2 h less than that of Vero cell grown bacteria and, importantly, axenically cultured organisms are as infectious as host cell culture-propagated organisms [68]. *Coxiella* axenic replication is the first demonstration of host cell-free growth of a prototypical obligate intracellular bacterial pathogen. Axenic cultivation of *Coxiella* will dramatically aid studies of the organism's developmental biology, identification of genes required for intracellular growth and virulence, and vaccine antigen discovery. Furthermore, the systematic approach employed to develop ACCM may be broadly applicable to development of axenic media supporting growth of other medically important obligate intracellular bacterial pathogens.

8.10 Conclusions

Over six decades ago, *Coxiella* was defined as the etiologic agent of human Q fever. Despite experimental obstacles inherent in working with a highly infectious, obligate intracellular bacterium lacking genetic tools, novel aspects of the *Coxiella*-host relationship have been defined, as have strategies employed by the pathogen to avoid immune recognition and to ensure viability outside of its intracellular niche. The entry of *Coxiella* research into the post-genomic era, with several complete genomes currently available, has increased our understanding of genome structure, genetic diversity, and pathogenetic potential. This genomic information is critical for developing new countermeasures against Q fever such as rationally designed subunit vaccines, new serodiagnostic reagents, and tools for molecular epidemiology and forensics. We predict that the recent technological achievements in axenic growth and genetic transformation of *Coxiella* will result in a paradigm shift in how the pathogen is viewed and studied (i.e., a tractable *facultative* intracellular parasite amenable to genetic manipulation). These advances should dramatically accelerate progress in understanding the unique biology and pathogenicity of *Coxiella*.

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Chapter 9
Plague in the 21st Century: Global Public Health Challenges and Goals

B. Joseph Hinnebusch

9.1 Introduction

Yersinia pestis, the Gram-negative bacterial agent of plague, is a zoonotic pathogen that primarily infects wild rodents and is transmitted by fleas. *Y. pestis* is one of the most invasive and virulent bacterial pathogens and has caused devastating pandemics, including the Black Death of 14th century Europe. The last plague pandemic began in Asia in the last half of the 19th century and lingered well into the 20th century, causing tens of millions of deaths as it spread across the world.

Three main forms of plague occur in humans. Bubonic plague, the most common, usually follows transmission by flea bite. Bacteria deposited in the dermal bite site travel through the lymphatic system to the regional draining lymph node. There, bacterial multiplication leads to the swollen, painful lymph node known as a bubo. At this stage, the disease can usually be treated successfully with antibiotics. The infection quickly spreads systemically via the blood stream to produce septicemic plague. If the flea deposits bacteria directly into the dermal vasculature, then primary septicemic plague can sometimes occur in the absence of bubonic plague [1]. In about 5% of humans, hematogenous spread to the lungs results in pneumonic plague. Because it can be spread directly from person-to-person via aerosolized respiratory droplets, this form of the disease presents a public health emergency. The case fatality rate of septicemic and pneumonic plague is high, even with antibiotic treatment.

Plague remains an international public health concern. Periodic plague outbreaks illustrate the ability of *Y. pestis* to suddenly cross over from established enzootic reservoirs and reemerge in human populations [2]. Multidrug resistant strains of *Y. pestis* have been isolated from human plague patients, and *Y. pestis* has recognized potential as an agent of bioterrorism. Plague has been weaponized in the past, and the World Health Organization (WHO) estimated that dissemination of 50 kg of *Y. pestis* in an aerosol cloud over a large metropolis could result in 150,000 cases of pneumonic plague [3]. If an antibiotic resistant strain were used, then the fatality rate would be high.

Despite these well-recognized threats, significant gaps in our understanding of many areas of plague biology impede progress in developing effective public health countermeasures. For example, there is currently no vaccine available for plague, but immune correlates of protection against plague have not been well characterized. There is also a recognized need for new diagnostic tests; most plague cases are based on presumptive clinical diagnosis [2].

9.2 Ecology and Epidemiology of Plague in the World Today

The highly virulent *Y. pestis* strain responsible for the last pandemic originated in Asia, but it spread across Pacific and Indian Ocean shipping routes to geographic areas where it did not previously exist, including North and South America. An adaptable pathogen, *Y. pestis* quickly established itself in many different species of rodents, among which it circulates using many different flea species as vectors. Thus, plague is more widespread today than it has ever been. It is certain that *Y. pestis* is permanently entrenched in wild rodent reservoir hosts in many parts of the globe, but the geographic extent of the enzootic foci is often poorly defined and subject to rapid expansion and contraction depending on factors such as climate and rodent and flea population dynamics [4].

After the last pandemic waned in the middle of the 20th century, the incidence of human plague declined dramatically. On average, a few thousand cases are reported to the WHO each year, although the actual disease incidence is almost certainly much higher. Beginning in the 1990s, however, outbreaks have occurred in parts of Africa, India, and South America where plague had previously been dormant for many years (Fig. 9.1).

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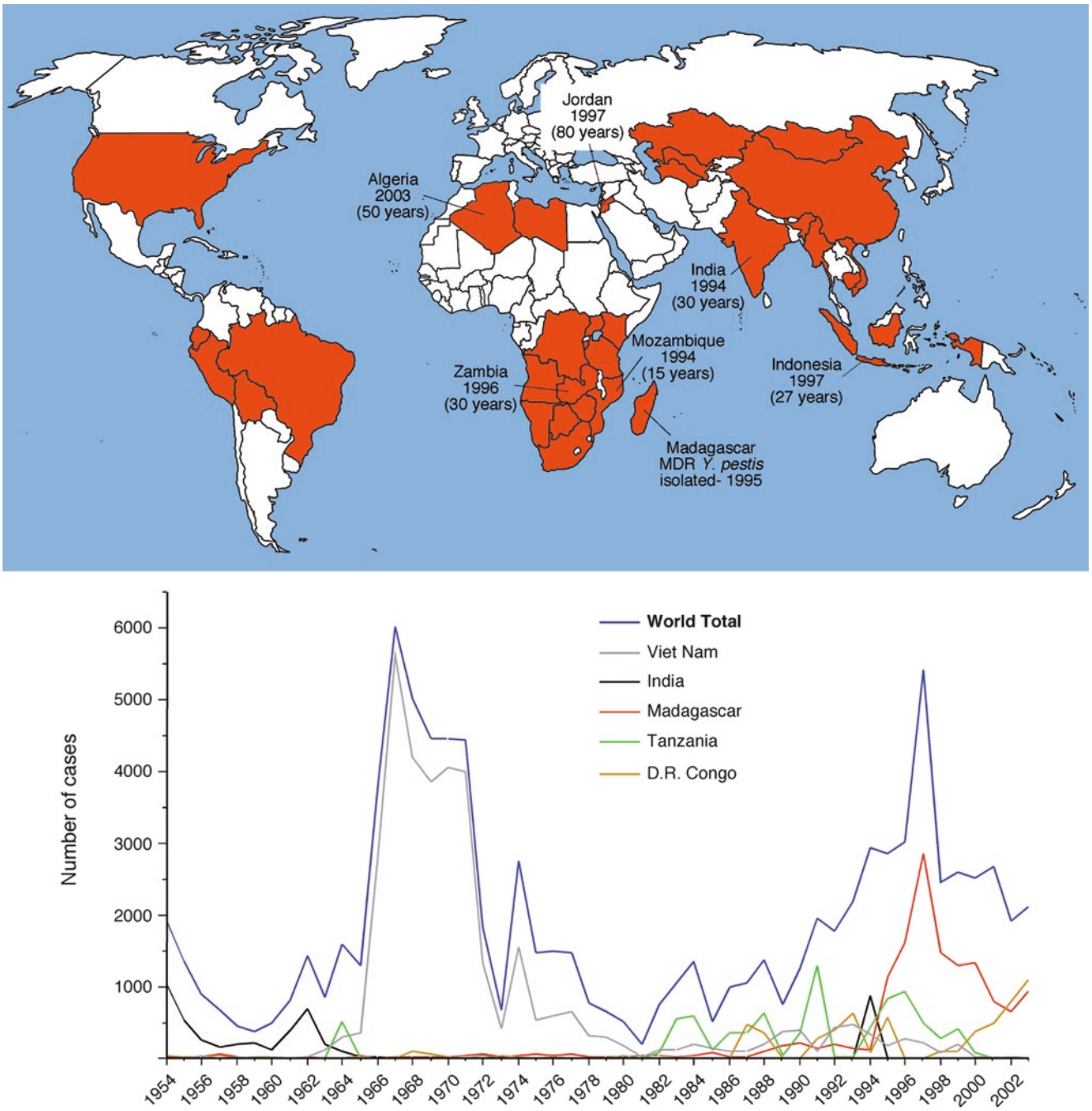

Fig. 9.1 Epidemiology of plague. Countries reporting plague cases during the last fifty years are depicted in red. In the last ten years, the majority of plague outbreaks have occurred in Africa. Examples of countries in which plague has re-emerged recently after many years of absence are indicated as well as the first isolation of antibiotic-resistant (MDR) *Y. pestis* strains during the recent plague epidemic in Madagascar. Sources: WHO and CDC

9.3 Plague: A Case Study in the Sudden Emergence of New Epidemic Infectious Diseases

Virulent *Y. pestis* strains from around the world constitute a highly uniform clone that diverged from the closely related *Yersinia pseudotuberculosis* only within the last 1,500 to 20,000 years [5, 6]. Despite their recent common ancestry and genetic relatedness, the two sister-species differ radically in parasitic lifestyle. *Y. pseudotuberculosis* is a relatively benign food- and water-borne enteric pathogen. Evolutionarily speaking, therefore, plague is a new disease. The hypervirulence and arthropod-borne transmission of *Y. pestis* evolved quite recently and involved relatively few

genetic changes, making plague an interesting historical case study in the sudden emergence of an epidemic infectious disease. It is sobering to reflect on the fact that only a modest number of minor genetic changes were needed to convert the relatively mild recent ancestor of *Y. pestis* into one of the most feared human pathogens.

9.4 Retracing the Genetic Steps that Led to Flea-borne Transmission and Hypervirulence of *Y. pestis*

Our laboratory participated in an international team effort that reported the complete annotated genome sequence of *Y. pseudotuberculosis* and the comparative genomics of *Y. pseudotuberculosis* and *Y. pestis* [6]. Remarkably, only 32 chromosomal genes occur in *Y. pestis* that do not have identical or highly similar orthologs in *Y. pseudotuberculosis*, as determined from comparative analyses of a panel of isolates of the two species from around the world.

The most obvious genetic difference between the two species is the presence of two *Y. pestis*-unique plasmids that were acquired since the divergence from the ancestral *Y. pseudotuberculosis* strain. Using the flea infection and transmission model systems we developed to investigate the interactions between *Y. pestis* and the rat flea *Xenopsylla cheopis*, the principal vector of human bubonic plague, we reported that both plasmids are essential for flea-borne transmission and virulence of plague. One plasmid encodes a phospholipase D (PLD) that had been originally characterized as murine toxin and presumed to have a role in virulence. We determined that this gene is not required for virulence and identified its true biological function: it is required for *Y. pestis* survival in the flea gut [7].

The other *Y. pestis*-unique plasmid encodes a plasminogen activator (Pla) that is required for dissemination from an intradermal or subcutaneous injection site. Using a *pla*-negative mutant strain, we demonstrated that two distinct pathologies can ensue from a fleabite: bubonic plague, which depends on Pla, and primary septicemic plague, which does not [1]. Our results suggested an evolutionary scenario in which acquisition of the PLD-encoding plasmid was a crucial initial step in the transition to arthropod-borne transmission, and that subsequent acquisition of the Pla-encoding plasmid greatly increased flea-borne transmissibility and epidemic potential.

A major focus of our laboratory has been to understand the genetic and molecular mechanisms that mediate flea-borne transmission. To produce a transmissible infection, *Y. pestis* grows in the flea digestive tract in the form of a bacterial biofilm, a dense multicellular community surrounded by a polysaccharide extracellular matrix (ECM), and usually attached to a surface [8]. In *X. cheopis* fleas, the *Y. pestis* biofilm often adheres to the hydrophobic, cuticle-coated spines that line the interior surface of the proventriculus, the valve that connects the esophagus to the midgut (Fig. 9.2). Consolidation and continued growth of the biofilm can eventually fill the proventriculus, interfering with its valvular action and blocking the normal flow of blood into the midgut when the flea attempts to feed. Complete or partial blockage of the proventriculus is prerequisite to efficient biological transmission, because it results in regurgitation of bacteria into the flea bite site [9, 10].

The chromosomal *hms* genes of *Y. pestis* are responsible for the synthesis of the biofilm ECM, a polymer of β-1,6-*N*-acetyl-D-glucosamine, and therefore are required for the ability of *Y. pestis* to grow as an adherent biofilm *in vitro* and to infect and block the flea proventriculus [8, 11, 12]. The *hms* genes are not required for virulence in the mammal; their biological function is specific to producing a transmissible infection in the flea. Intriguingly, *Y. pseudotuberculosis* never forms a biofilm in infected fleas, despite the fact that it contains identical orthologs to the *Y. pestis hms* genes and

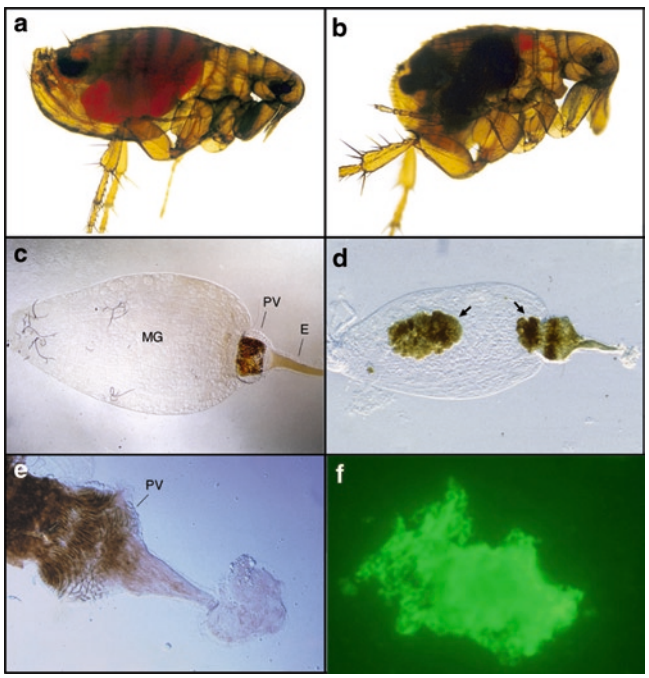

Fig. 9.2 Biofilm life stage of *Y. pestis* in the flea vector. Uninfected (**a**) and (**b**) infected, blocked *X. cheopis* fleas immediately after feeding. The presence of fresh blood in the esophagus, but not the midgut, is indicative of proventricular blockage. (**c**, **d**) Dissected digestive tracts from an uninfected (**c**) and a blocked (**d**) flea. Arrows indicate dense aggregates of *Y. pestis* embedded in an extracellular matrix in the midgut and proventriculus. (**e**) Micrograph of the anterior end of the digestive tract dissected from a blocked flea showing a viscous *Y. pestis* biofilm extruding from the esophagus. (**f**) Antibody to poly-β-1,6-*N*-acetyl glucosamine binds the ECM of *Y. pestis* biofilm in an immunofluorescence assay. (MG = midgut; PV = proventriculus; E = esophagus)

is able to form *hms*-dependent biofilms *in vitro* [13]. This suggests that regulatory pathways leading to ECM synthesis differ in the two species, and we have shown that loss of gene function in *Y. pestis* (pseudogene formation) has been important in this regard. About 7% of the ~4,000 orthologous gene pairs of the two species are pseudogenes in *Y. pestis* but are intact in *Y. pseudotuberculosis*. One of them, *nghA*, encodes a glycosyl hydrolase that specifically hydrolyzes β-1,6-*N*-acetyl-D-glucosamine and interferes with biofilm formation in the flea [12]. Another, *rcsA*, a negative regulator of the biofilm phenotype, is a functional gene in *Y. pseudotuberculosis* but a pseudogene in *Y. pestis*. Restoring the functional *Y. pseudotuberculosis rcsA* allele in *Y. pestis* essentially eliminated the ability to produce a proventricular-blocking biofilm in fleas [14].

In common with many other bacteria, ECM synthesis in *Y. pestis* is controlled by intracellular levels of cyclic di-GMP, which are determined by competing activities of the *hmsT* diguanylate cyclase and *hmsP* phosphodiesterase gene products [15, 16]. We have recently implicated a second phosphodiesterase gene (*rtn*), whose product is truncated and nonfunctional in *Y. pestis*, in biofilm ECM regulation. As was the case for the rcsA pseudogene, replacement of the *Y. pestis rtn* pseudogene with the functional *Y. pseudotuberculosis* allele resulted in a greatly reduced ability to form biofilm in fleas. Because of their transmission-enhancing effects, mutational loss of the ancestral *rcsA* and *rtn* functions would likely have been positively selected during the emergence of *Y. pestis*. In conclusion, we have shown that selective gene loss as well as gene addition via acquisition of the two *Y. pestis*-specific plasmids, played an important role in the evolution of arthropod-borne transmission.

9.5 The Sudden Emergence of both Flea-borne Transmission and Hypervirulence in the Genus Yersinia: A Case of Mutually Reinforcing Coevolution?

Y. pestis is a striking example of a pathogen that has taken the sinister evolutionary path to increased virulence. Modern epidemiological models hypothesize that pathogens evolve a level of virulence that maximizes their transmissibility [17, 18]. We used the flea infection model to quantitatively evaluate two basic components of vector competence – the oral infectious dose (ID_{50}) of *Y. pestis* for *X. cheopis* fleas and the transmission efficiency of blocked fleas [19]. The data indicated that the vector competence of fleas is low compared to other parasite-vector systems. The low infectivity (high ID_{50}) for fleas mandates that, in order to complete its life cycle, *Y. pestis* must achieve a high density, usually fatal septicemia in peripheral blood. In addition, the low number of bacteria transmitted by fleas mandates that *Y. pestis* must be invasive enough to produce this overwhelming sepsis starting from a small infectious dose that is deposited intradermally by flea bite; the LD_{50} of *Y. pestis* for mammals is in fact less than 10 bacteria. Thus, the evolutionary change to the flea-borne transmission cycle likely imposed new selective pressures that favored the coevolution of increased virulence. Further epidemiological modeling based on the data indicated that a high flea density per host is required to sustain epizootic plague, supporting the practice of flea control alone as an effective strategy to control plague outbreaks.

9.6 Functional Genomics Approaches to the Discovery of *Y. pestis* Transmission, Infectivity, and Virulence Mechanisms

A number of important virulence factors of *Y. pestis* have been discovered and an important basic pathogenesis strategy recognized – the ability to retard and attenuate the mammalian innate immune response [20, 21]. Thus, *Y. pestis* combines the dangerous attributes of stealth (the ability to prevent immune detection and stimulation) and aggression (rapid invasiveness) to produce rapidly fatal systemic sepsis. The molecular mechanisms by which it does so are incompletely known, however, and often based on *in vitro* evidence and inferences from studies of the enteropathogenic *Yersinia* species or attenuated *Y. pestis* strains. Thus, the molecular mechanisms underlying specific impairments of host defense remain to be determined or validated *in vivo*. To this end, we have developed, characterized, and used mouse and rat models of bubonic plague [22, 23]. The rat model had not been employed for many years, but disease progression and gross pathology in the rat more closely resembles human bubonic plague. In addition, the microbial pathogenesis of *Y. pestis* is seldom studied in the context of the natural flea-borne route of transmission. Nearly all studies have employed needle-injection of cultured bacteria. While this artificial transmission model is valuable, it ignores specific aspects of the natural transmission route, such as the unique biofilm phenotype assumed by *Y. pestis* in the flea and the effect of flea saliva. In fact, the true role and significance of some *Y. pestis* virulence factors were only revealed in studies employing transmission by flea bite [1, 24].

One way to discover new transmission and virulence factors is to analyze gene expression response patterns of the host-parasite interaction. Such microarray-based functional genomics strategies reveal genes that are specifically upregulated during infection, implicating them as being involved in the disease process. Furthermore, where and when a gene is

expressed provides important clues about what the gene does [25]. In collaboration with the Genomics Unit of NIAID's Research Technologies Branch, we have characterized the *in vivo* transcriptome, or gene expression profile, of *Y. pestis* in infected fleas and in infected lymph nodes (buboes) of rats, as well as the transcriptome of rat lymph node cells during bubonic plague. A comparison of normalized gene expression levels from the two data sets provides insight into the biology of the flea-mammal life cycle and supports the hypothesis that *Y. pestis* differentially expresses distinct subsets of genes to establish a productive infection in its two disparate hosts. About 24% of *Y. pestis* genes showed significantly higher relative expression levels in the bubo than in the flea; 15% were higher in the flea than in the bubo; and 61% were not differentially expressed in the two hosts.

9.7 Virulence Determinants Discovered by Analysis of *Y. pestis* Gene Expression in the Bubo

The microarray profile of *Y. pestis* gene expression in the rat bubo compared to the profiles of exponential and stationary phase *Y. pestis* cultured *in vitro* at 37° and 21°C revealed an adapative response of *Y. pestis* to iron limitation and nitric oxide (NO)-derived reactive nitrogen species (RNS) in the extracellular environment of the bubo [26]. Expression of 10 of the 14 proven or potential iron or heme transport systems, including three siderophore-based systems, five ABC iron transporters, a heme transport system, and an iron permease, was upregulated >5-fold in the bubo. Several genes that have been implicated in the bacterial protective response to RNS were also upregulated *in vivo* compared to *in vitro*. In particular, expression of *hmp*, which encodes a flavoglobin that is induced by NO and confers resistance to RNS, increased 10 to 20-fold in the bubo. NO-derived antimicrobial activity is generated by macrophages, neutrophils, and other host cells by the inducible NO synthase (iNOS) in response to bacterial infection [27]. In subsequent pathogenesis studies, we showed that large numbers of iNOS-expressing neutrophils are recruited to the infected bubo and are in close association with extracellular masses of *Y. pestis,* serum NO levels are elevated in rats with bubonic plague, and that mutation of the *Y. pestis hmp* gene resulted in decreased virulence.

Current models of plague pathogenesis stress the ability of *Y. pestis* to inhibit phagocytosis and the inflammatory response, thereby avoiding exposure to innate immune effector molecules. Our results show that *Y. pestis* encounters and responds to NO-derived reactive nitrogen stress in the bubo, where it is extracellular. Although the importance of NO-derived stress in innate defense against intracellular pathogens is well-established, evidence for a role against extracellular bacteria such as *Y. pestis* is lacking. In addition to its antibacterial role in innate immunity, NO has known concentration-dependent immunosuppressive and other regulatory effects on the developing adaptive immune response; and high NO levels contribute to septic shock, the ultimate cause of plague mortality [27]. Thus, the elevated systemic NO levels we detected during plague disclose a new potential factor in disease pathogenesis and immunomodulation.

9.8 The Infective Phenotype of *Y. pestis* Characterized by Analysis of Gene Expression in the Flea Vector

After transmission, many known virulence factors of *Y. pestis* are induced that confer resistance to innate immunity. However, these factors are not produced in the low-temperature environment of the flea, suggesting that *Y. pestis* is vulnerable to the initial encounter with innate immune cells at the flea bite site. Therefore, in a complementary project we compared the *Y. pestis in vivo* transcriptome in infective fleas to *in vitro* transcriptomes in temperature-matched biofilm and planktonic cultures, and to the previously characterized *in vivo* gene expression profile in the rat bubo. The results characterized a specific phenotype *Y. pestis* in the flea vector and implicated previously unrecognized genes involved in biofilm formation. Notably, several genes with known or predicted roles in resistance to innate immunity and pathogenicity in the mammal were upregulated in the flea; and *Y. pestis* from infected fleas had increased resistance to phagocytosis. The results indicate that cycling through the flea vector preadapts *Y. pestis* to face the mammalian innate immune response that it encounters immediately after transmission, and that the vector-specific phenotype of *Y. pestis* as it exits the flea and enters the mammal enhances infectivity [32].

9.9 Wanted: 21st Century Public Health Countermeasures Against Plague

9.9.1 Epidemiology and Surveillance

Until recently, all human cases of plague as well as cholera and yellow fever were required by international law to be reported to WHO. This somewhat outdated emphasis on only three diseases was eliminated in the latest version of the International Health Regulations of the WHO, which came into effect in June 2007. Now, notification to WHO is mandated only for an unexpected event presenting a risk of international spread, for example a SARS or avian influenza virus outbreak.

Although any unusual occurrence of plague would still be reportable under the new regulations, one fallout will likely be less complete data on the worldwide prevalence and incidence of human plague. However, because of poor diagnostic facilities in many countries where plague occurs, plague almost certainly has been chronically underreported.

Plague cannot be eradicated; it is widespread and firmly entrenched in many wild rodent reservoirs. As a zoonosis, the risk of plague in human populations is closely linked to the dynamics of plague in its natural reservoirs [4]. Surveillance to monitor the geographical extent of natural foci and to detect plague epizootics in wild rodents is well recognized as being an important public health measure. Surveillance typically entails serological testing of urban and wild rodents and testing their fleas for the presence of *Y. pestis*, but is rarely done. Consequently, the threat that plague poses at any given time to human populations in most parts of the world remains poorly understood.

9.9.2 Diagnostics, Treatment, and Prophylaxis

Globally, laboratory diagnosis is problematic because plague tends to occur in remote areas and in countries with limited public health resources and infrastructure. Diagnosis of human plague is most often based on the following: (i) compatible clinical and epidemiological features and microscopic observation of Gram-negative coccobacilli in a direct smear from a clinical sample (suspected case); (ii) detection of *Y. pestis* F1 capsular antigen in a clinical sample or anti-F1 antibody in a single serum sample (presumptive case); and (iii) *Y. pestis* culture and identification or a fourfold increase in anti-F1 antibody titer in paired sera (confirmed case). Most human plague that is reported to WHO are suspected or presumptive cases. Simple and rapid diagnostic tests for F1 antigen detection have been developed and are being assessed [2]. Such tests would provide much-needed new tools if their reliability, sensitivity, and specificity can be verified in field conditions. The use of PCR, real-time PCR, and other molecular biology tools for diagnosing plague from clinical specimens is also being assessed for use in reference laboratories. At their most refined, these methods can genotype different *Y. pestis* isolates with exquisite sensitivity, which is of great value in epidemiological and forensic (bioterrorism-related) investigations [28].

Our *in vivo* genomics studies have identified outer surface protein genes of *Y. pestis* that are highly expressed in the flea, indicating that their protein products are prominent antigens initially exposed to the mammalian immune system when the bacteria exit the flea and enter the mammal. We are investigating them as candidate markers for new efficacious vaccines, diagnostics, and therapeutic drug targets. Diagnostic markers specific for flea-borne versus primary pneumonic (aerosolized) plague transmission would be valuable in epidemiologic invesigation. For example, if a case of pneumonic plague occurred in Los Angeles, it could either be primary pneumonic plague, a highly unusual event that might indicate a deliberate, nefarious act of bioterrorism; or secondary pneumonic plague that developed rapidly from flea-borne plague, more easily explainable because enzootic plague foci have been reported in suburban parks of that city.

If it is diagnosed early enough, then plague can be successfully treated with several widely available antibiotics [3]. The public health response to a plague outbreak in most instances consists of isolation and antibiotic treatment of cases, prophylactic treatment of contacts, and local application of insecticide to kill fleas. Because of the explosive nature of plague outbreaks in certain conditions, rapid response is of the essence, making reliable and readily available diagnostic tests all the more critical. Although pneumonic plague has a reputation as being highly contagious via respiratory droplets, the simple precaution of wearing a mask, even an improvised one consisting of layers of cotton gauze, effectively prevents aerosol transmission [29, 30].

9.9.3 The Need for a Third-Generation Plague Vaccine

Two types of plague vaccines have been used in the past [30]. Beginning in 1914, live attenuated *Y. pestis* vaccine strains were administered to millions of people in some Asian countries and Madagascar and are still being used in parts of the former Soviet Union. These vaccines protected against both bubonic and pneumonic plague, but were associated with severe side effects and would never be approved today. A different whole-cell vaccine, consisting of formalin-killed, virulent *Y. pestis* in saline (the USP plague vaccine) was used from about 1950 to 1995. This formulation reportedly provided short-term protection against bubonic plague only, and is no longer available.

Two new subunit vaccines, both based on the surface-exposed F1 and V antigens of *Y. pestis*, were developed at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) and the United Kingdom's defense department. Mouse and non-human primate trials have been completed for the USAMRIID vaccine, and both are now in human trials. Protection against bubonic plague was good in all animal models but mixed results were obtained for efficacy against pneumonic plague–good protection against aerosol challenge occurred in cynomolgus macaques, but not in African green monkeys [30]. The mechanisms of vaccine protection and predictive correlates of protection are not well understood. For example, humoral immunity is important, but antibody titers were not predictive of protection against

aerosol challenge in non-human primates. Another potential shortcoming of these vaccines is the fact that the F1 capsular protein is not required for *Y. pestis* virulence; therefore, vaccinees might be unprotected against naturally occurring or deliberately engineered F1-negative strains. In addition, antigenic variants of the *Y. pestis* V antigen occur, and V antigen has been shown to have immunosuppressive effects, an undesired property of a vaccine component *(30)*. Thus, there is a need for further research on new vaccine candidates, but only limited information is available about the mammalian protective immune response to plague and the corresponding *Y. pestis* targets. Optimal delivery routes, vehicles, and adjuvants also remain to be established [30, 31].

Our microarray analyses of the *Y. pestis in vivo* transcriptome identified eight outer membrane protein genes that were highly expressed during bubonic plague. In collaboration with Dr. Susan Buchanan, a membrane protein biochemist at NIH, NIDDK, we are evaluating these proteins as potential vaccine candidates. In addition, X-ray crystallographic structural analyses of the highly expressed essential virulence factors required for iron acquisition and transport may point to new chemotherapeutic strategies with novel modes of action.

9.10 Summary

Plague is exceptional for its extreme virulence and potential for rapid epidemic spread in the right conditions. These capabilities of *Y. pestis* evolved relatively recently, making plague a sobering example of how a new epidemic can suddenly appear on the basis of just a few genetic changes. Far from being an historical curiosity, plague in the 21st century remains a public health threat that is incompletely understood. Plague still periodically reemerges, often after decades of quiescence, for enigmatic reasons. The occurrence of antibiotic resistant *Y. pestis* isolates and the documented potential of *Y. pestis* as an agent of bioterrorism increase the urgency for new medical countermeasures. Paramount among them is an effective plague vaccine, a goal that should be achievable. Successful development of these new tools, however, depends on further research to clearly define the molecular mechanisms of *Y. pestis* transmission, infection, and immunity.

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Chapter 10
Molecular Biology of Staphylococcal Pathogenesis

Michael Otto

10.1 Introduction

The Pathogen Molecular Genetics Section of the Laboratory of Human Bacterial Pathogenesis investigates the molecular basis of pathogenesis in staphylococci, focusing on the two most important staphylococcal pathogens, *Staphylococcus aureus* and *S. epidermidis*. Research projects include mechanisms of staphylococci to evade human innate host defense such as toxin production and biofilm formation and virulence gene regulation. In addition, recent studies have given important insight into the molecular underpinnings of the exceptional pathogenic potential of community-associated methicillin-resistant *S. aureus*.

10.2 Molecular Mechanisms of Immune Evasion

10.2.1 Staphylococcus epidermidis

S. epidermidis is the most prominent species within the family of coagulase-negative staphylococci, which comprise most members of the genus *Staphylococcus* with the exception of *S. aureus*. As common and frequent colonizers of human epithelia, coagulase-negative staphylococci usually have a benign relationship with the human host. However, after penetration under the skin, for example during the insertion of indwelling medical devices, *S. epidermidis* and other coagulase-negative staphylococci can cause long-lasting, chronic disease that commonly proceeds with the formation of bacterial agglomerations called biofilms.

In biofilms, bacteria are protected from antibacterial agents such as antibiotics and attacks by human innate host defense. This protection is in part due to decreased penetration of phagocytes and antibacterial agents through the extracellular biofilm matrix but also the specific physiology of bacteria in biofilms. We have shown that gene expression in *S. epidermidis* biofilms is characterized by a reduction of basic cell processes, providing an explanation for decreased efficacy of many antibiotics against bacteria in biofilms [1].

Furthermore, there is increased expression of specific immune evasion determinants, notably poly-γ-glutamic acid (PGA), in *S. epidermidis* biofilms. PGA is a polymeric substance composed of glutamic acid residues that are attached to each other via their γ-carboxyl groups. PGA is known mostly as the capsule substance of *Bacillus anthracis*. Despite low production in *S. epidermidis*, we found that PGA has an important role in providing protection from mechanisms of innate host defense and significantly affects persistence of *S. epidermidis* during infection of indwelling medical devices [2].

PIA (polysaccharide intercellular adhesin, or PNAG, poly-*N*-acetylglucosamine) is considered an important part of the biofilm matrix in *S. epidermidis* and other staphylococci (Fig. 10.1). In addition, it has a crucial function in immune evasion by protecting bacteria from innate host defense mechanisms, including antimicrobial peptides (AMPs) and neutrophil phagocytosis [3]. To interact with the bacterial surface, PIA has a positive charge, which is introduced by a dedicated enzyme called IcaB via partial deacetylation of *N*-acetylglucosamine residues [4].

Several lines of evidence support our overarching hypothesis that *S. epidermidis* is an "accidental" pathogen, as determinants that provide protection from host defenses and promote persistence during *S. epidermidis* infection have an original role in the non-infectious lifestyle of this opportunistic pathogen, rising to additional value during infection. For example, PGA contributes to salt tolerance, a function clearly associated with life on human skin [2].

10.2.2 Antimicrobial Peptides

AMPs are an evolutionarily ancient form of host defense. Especially in neutrophil phagosomes and on epithelia; they are also an important part of the innate immune system of humans. Most AMPs are cationic as they need to bind to the

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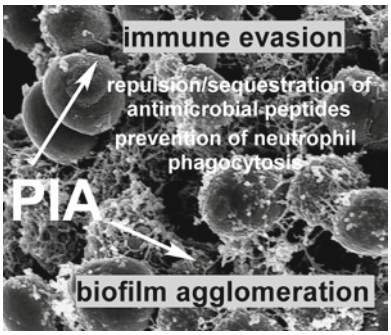

Fig. 10.1 Biological functions of PIA. The exopolysaccharide polysaccharide intercellular adhesin (PIA) surrounds many staphylococcal strains, functioning as a biofilm agglomeration substance and providing protection from mechanisms of innate host defense. It has a positive charge that is crucial for binding to the negatively charged bacterial surface

negatively charged bacterial surface. However, there are exceptions such as the human AMP dermcidin, which has a negative net charge.

Our research focuses on ways by which bacteria sense the presence of AMPs and trigger adaptive defenses. In Gram-negative bacteria, the PhoP/PhoQ system senses cationic AMPs and reacts with defensive gene regulatory changes, whereas a corresponding system in Gram-positive bacteria had not been previously identified. We found that a 3-component system that is unrelated to PhoP/PhoQ and which we called Aps (antimicrobial peptide sensing) system, up-regulates key mechanisms of AMP defense in *S. epidermidis* in response to cationic AMPs [5].This first example of an AMP-sensing system in Gram-positive bacteria consists of a membrane sensor (ApsS or GraS), a histidine kinase (ApsR or GraR), and an essential third component of unknown function (ApsX). Binding of cationic AMPs occurs to the sole extracellular loop of ApsS, which is only 9 amino acid residues long and contains 3 negatively charged amino acids that are believed to be crucial for interacting with the cationic AMPs. The same system also exists in *S. aureus* [6]. Interestingly, binding of cationic AMPs and activation of the *S. aureus* Aps system appear to discriminate between different cationic AMPs. Importantly, the Aps system is not activated by dermcidin, owing to its anionic character. However, both cationic AMPs and dermcidin trigger up-regulation of exoprotease production, which is mediated by global regulators such as Agr and SarA [7]. Together, these studies showed that staphylococci have dedicated mechanisms to sense AMPs and to react with protective measures.

10.2.3 Basis of CA-MRSA Virulence

Antibiotic resistance is a considerable problem for the treatment of *S. aureus* infections. Methicillin-resistant *S. aureus* (MRSA) have spread to such an extent that methicillin and other β-lactamase resistant penicillins cannot be used for many hospital-associated infections anymore. Whereas MRSA have traditionally been mostly limited to the hospital setting, since the late 1990s, particularly in the United States, we have seen the enormous spread of community-associated (CA)-MRSA. There, CA-MRSA infections are almost exclusively caused by strain USA300, which has undergone recent clonal expansion and replaced other CA-MRSA strains [8] such as MW2 (USA400). However, the molecular basis of USA300's extraordinary success in spreading fast and sustainably in the population, and causing severe cases of skin and soft tissue and sometimes very severe and fatal disease, is not understood.

USA300 and many other CA-MRSA strains that have emerged globally carry a phage, ΦSLT, that contains the *lukS* and *lukF* genes encoding the bicomponent toxin Panton-Valentine leukocidin (*PVL*). On that basis, it has been speculated that PVL is the major cause for the exceptional virulence and pathogenic success of USA300 and other CA-MRSA strains. However, using infection models in mice, rats, and rabbits studies performed in several laboratories, including ours, have not revealed a significant contribution of PVL to experimental disease caused by USA300 or USA400 CA-MRSA strains [9, 10, 1]. Furthermore, we showed recently that a prominent publication that indicated a contribution of PVL to murine pneumonia in an *S. aureus* laboratory strain [12], and which was mistaken by many as an indication for a role of PVL in CA-MRSA disease, was seriously flawed, because a major strain in that study contained a second site mutation with a major gene regulatory impact [13]. Of note, we repaired the mutation and were not able to detect a significant impact of PVL on gene regulation or virulence.

In a search for alternative explanations for the exceptional virulence potential of USA300 and other CA-MRSA strains, we identified and characterized a class of leukocidal peptides, the phenol-soluble modulins (PSMs) [14]. This class of peptides is characterized by pronounced α-helicity and amphipathy, typical features of pore-forming peptides. In fact, one of the PSMs, the δ-toxin, has long been recognized to form pores in eukaryotic cytoplasmic membranes. We detected the strongest leukolytic capacities in previously unknown peptides, the PSMα peptides PSMα1, PSMα2, PSMα3, and PSMα4, which are encoded in the *psmα* operon in the genome of all analyzed *S. aureus* strains. In contrast to the PSMα peptides (~ 20–25 amino acids), the β-type PSMs are longer (~ 45 amino acids) and have almost no cytolytic capacity. In addition to demonstrating significant *in vitro* and *in vivo* leukolytic capacities of the PSMα peptides, we showed that deletion of the *psmα* operon in USA300 and USA400 causes significantly decreased dermonecrosis in a murine skin and soft tissue infection model and significantly decreased murine bacteremia (Fig. 10.2). Moreover, PSMs

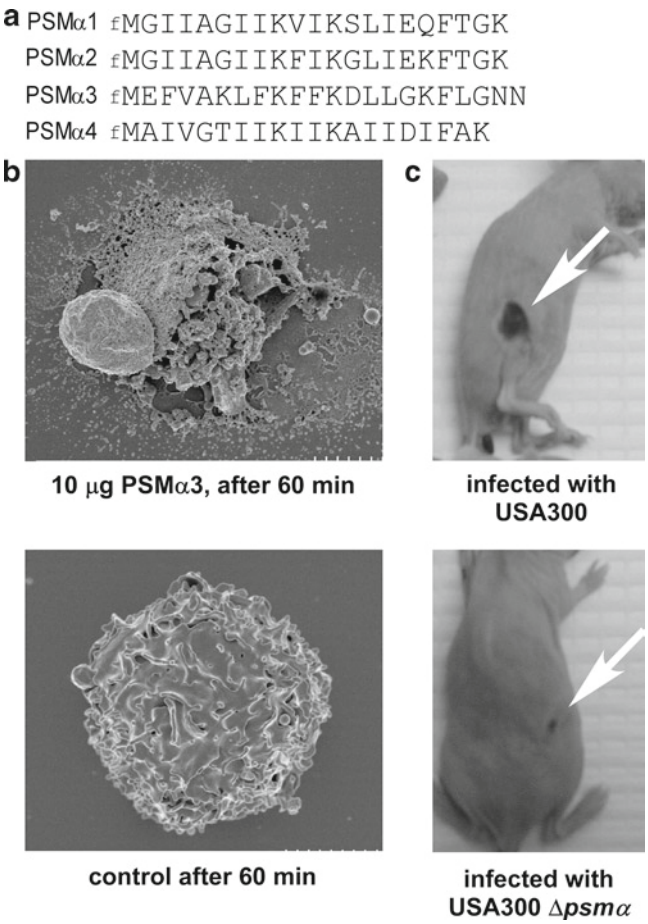

Fig. 10.2 Phenol-soluble modulins (PSMs). **a**. Amino acid sequences of the PSMα peptides. All PSMs are secreted without processing and thus carry *N*-formyl-methionine, which is incorporated at the *N*-terminus of every bacterial protein. **b**. The PSMα peptides have pronounced cytolytic activities, as shown here for the interaction between the most strongly cytolytic PSMα3 and human neutrophils. **c**. The PSMα peptides have a very significant impact on the development of skin and soft tissue infection. Shown is a murine abscess model comparing USA300 wild-type and *psmα* operon deletion strain, lacking peptides PSMα1 through PSMα4

have considerable chemotactic and pro-inflammatory activities [12], which, as we found in collaboration with Dr. Peschel's group from the University of Tübingen, Germany, are mediated by a dedicated receptor on the neutrophil surface whereas the lytic capacity is likely receptor-independent (unpublished).

Furthermore, in collaboration with Dr. Schneewind's group from the University of Chicago, we showed that the α-toxin has a crucial impact on experimental CA-MRSA pneumonia [15]. This toxin is a well known *S. aureus* cytolysin, which however does not lyse human neutrophils. Possibly, its role in pneumonia is linked to pro-inflammatory rather than lytic capacity or the lysis of other cell types.

Notably, both PSMs and α-toxin are core genome-encoded and thus present in virtually all *S. aureus*. Thus, to explain the exceptional pathogenic success of CA-MRSA, we hypothesized that CA-MRSA have higher expression than other MRSA of these key determinants of CA-MRSA disease. Therefore, in a recent study, we analyzed virulence and expression of these key virulence determinants in all prominent strains of the CC8 clonal complex, which includes USA300 and other CA- and HA-MRSA strains that have caused epidemic outbreaks [16]. We found that virulence, as assessed in mouse skin and soft tissue and bacteremia models, capacity to lyse human neutrophils, and expression of α-toxin and PSMs are strongly increased in USA300 compared to other MRSA of the same clonal complex. Moreover, all these phenotypes were at a comparable level already in strain USA500, the direct progenitor of USA300 (Fig. 10.3). USA300 differs from USA500 mostly in the acquisition of mobile genetic elements including the ΦSLT phage with the PVL-encoding genes. Therefore, our results indicate that the acquisition of PVL and other mobile genetic elements did not have a substantial impact on the evolution of virulence in USA300, which is rather determined by differential expression of core genome-encoded virulence determinants such as PSMs and α-toxin. These findings call for a paradigmatic shift in our notion about the development of virulence in CA-MRSA. Furthermore, they point to differences in transmissibility rather than virulence in an attempt to explain why USA300 has caused more widespread epidemic outbreaks than USA500.

10.3 Virulence Regulation

10.3.1 Biofilms

Gene expression in biofilms is substantially different from that in planktonic bacteria and subject to manifold regulatory influences. We have focused our research efforts in this field on the impact that global regulators, namely Agr, SarZ, and LuxS have on the phenotype and gene expression of *S. epidermidis* biofilms.

The quorum-sensing regulator Agr, a long-time interest in my laboratory (see below), is expressed in exposed layers of a biofilm [17], in which it likely triggers the detachment of biofilm clusters, a mechanism that is an important prerequisite for the systemic spread of a biofilm-associated infection. As a consequence, *agr* mutants produce thicker biofilms than *agr*-positive strains. Interestingly, *agr* mutants occur naturally and can be isolated more frequently from infections of indwelling medical devices [17], indicating that mutation in *agr* facilitates the establishment of a biofilm-associated infection. Accordingly, an *agr* deletion mutant of *S. epidermidis* caused more persistent indwelling-device associated infection than the corresponding wild-type in a rabbit infection model [17]. In contrast, the wild-type strain showed more pronounced infiltration of tissue, showing that permanent

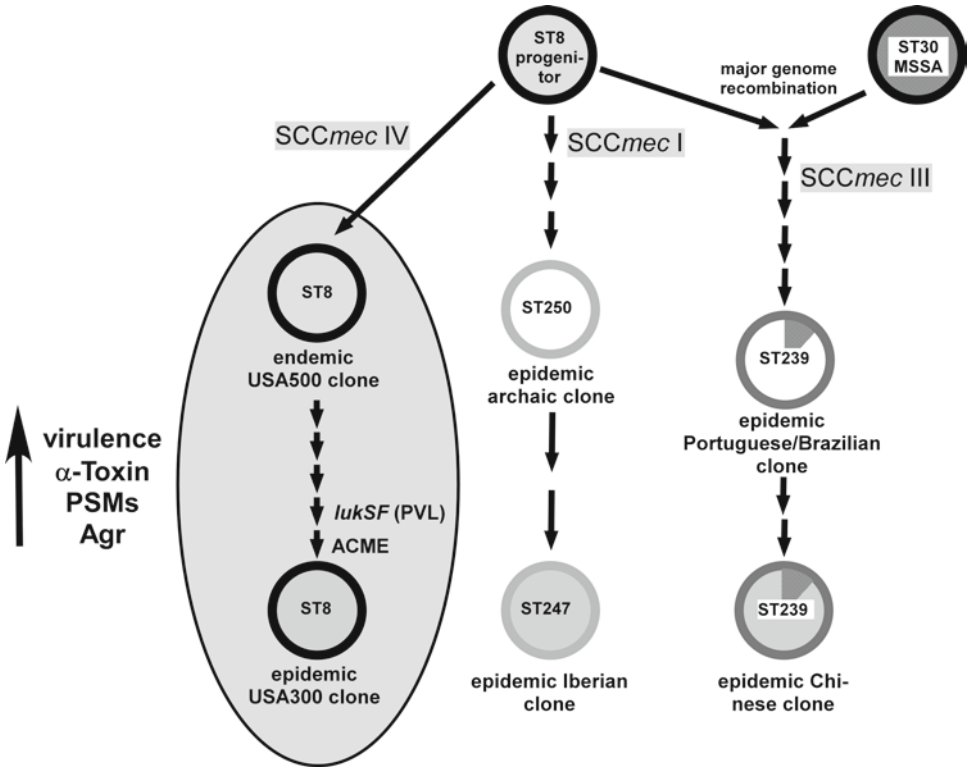

Fig. 10.3 Evolutionary relationship of subclones in the clonal complex 8 (CC8). The evolutionary tree architecture of major epidemic subclones in CC8, comprising the USA300 strain, which causes most CA-MRSA infections in the United States, was inferred by analysis of seven housekeeping and seven surface protein genes. The highlighted subtree has increased expression of major toxin genes such as PSMs and α-toxin, expression of the virulence regulator Agr, and virulence in murine bacteremia and skin infection models. It is further distinguished from the other sub-trees by the presence of methicillin-resistance encoding SCC*mec* element of type IV, whereas the other clones have different SCC*mec* types. Notably, virulence and toxin expression were comparable in USA300 and its progenitor USA500, indicating that mobile genetic element-encoded determinants such as PVL and ACME, which distinguish USA300 from USA500, have no significant impact on these phenotypes in USA300

mutation of *agr* leads to an impaired ability to spread, most likely owing to abolished detachment capacity. The mechanism by which Agr influences biofilm development is under current investigation in my group and most likely linked to the detergent-like activity of Agr-regulated PSM peptides.

The LuxS autoinducer-2 system is another quorum-sensing system that has a profound gene regulatory effect in *S. epidermidis* [18]. Similar to Agr, deletion of *luxS*, which we investigated in collaboration with Dr. Gao's group in Shanghai, causes more pronounced in vitro biofilm-associated infection [19]. However, in contrast to Agr [20], LuxS appears to impact biofilm formation via regulation of biofilm exopolysaccharide (PIA) production. Finally, Dr. Gao's and my group have identified another global regulator, SarZ, that has a key impact on *S. epidermidis* biofilm formation *in vitro* and *in vivo* [21]. Together, these studies showed that global regulators have key roles in determining biofilm development in *S. epidermidis*.

10.3.2 The Accessory Gene Regulator (Agr)

The Agr quorum-sensing system is a pivotal and the most intensively studied global regulator in *Staphylococcus*. In addition to our work on biofilm regulation by Agr [17, 20, 22], we showed that Agr has a key impact on evasion of other innate host defense mechanisms in *S. epidermidis* [23]. Furthermore, in contrast to the previous notion that all Agr target genes are under control of the regulatory RNA, RNAIII, we have recently shown that there are two different subsets of Agr-controlled target genes, one of which is under RNAIII control and comprises mostly virulence determinants and one which is RNAIII-independent, comprising mostly metabolic and *psm* genes [24]. Importantly, the *psm* genes are under direct control of the AgrA response regulator DNA-binding protein, indicating an evolutionarily old link of *psm* genes to quorum-sensing thereby emphasizing the key role of PSMs in staphylococcal physiology. Importantly, these

studies have given insight into how quorum-sensing and virulence control were connected during the evolution of pathogenicity in *S. aureus*.

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Chapter 11
Investigations of Relapsing Fever at Home and Abroad

Tom G. Schwan

11.1 Introduction

Relapsing fever is an ancient disease of humans first described by Hippocrates long before the development of the germ theory and achromatic microscopes that lay the foundation for the discovery of the causative agents. Two epidemiological forms of the disease exist; these include epidemic relapsing fever that is associated with only humans and the human body louse *Pediculus humanus* and endemic relapsing fever that is associated with enzootic cycles involving many species of wild animals and soft-bodied ticks in the family Argasidae [21].

The historical events leading to the discovery of the bacterial agents causing these diseases are fascinating, but they are beyond the scope of this presentation. Epidemic or louse-borne relapsing fever was most likely the disease noted by Hippocrates for which large numbers of human infections may erupt with high mortality rates [21]. During two epidemics in Berlin, Germany, in 1868 and 1873, Otto Obermeier discovered the spirochetal cause of this relapsing fever [3, 32]. However, many years lapsed before S. Percival Makie demonstrated what role the human body louse played in spreading such epidemics [28]. Several independent investigations, during the first decade of the 1900s in Central and East Africa, demonstrated that the disease long referred to as tick fever was also caused by spirochetes similar to the louse-borne bacteria [8, 17, 26, 27, 37], and that these spirochetes were transmitted by the soft tick *Ornithodoros moubata* [17]. These seminal investigations set the stage for elucidating the occurrence and distribution of relapsing fever throughout many regions of the world.

The pathogenic spirochetes that cause relapsing fever are taxonomically grouped within the genus *Borrelia*, which is defined in part by their obligate requirement for a blood-feeding, arthropod vector for transmission from one vertebrate host to another [6]. The body louse is the vector of *Borrelia recurrentis*; all other known species of borreliae are transmitted by ticks.

The presence of tick-borne relapsing fever in North America was first reported in 1915, when five patients acquired the infection in Colorado [30]. Ticks were not considered as the source of the infections during this investigation. Instead, C.N. Meader suggested that a band of gypsies that camped near the patients' cabin shed spirochete-infected vermin. Twenty years passed before the tick vector that was responsible for this first outbreak was discovered and named; this was due to extensive studies in the mountains of California where tick-borne relapsing fever was endemic [51, 52].

Today, three species of spirochetes and their respective tick vectors are responsible for relapsing fever in North America. Extensive studies performed at the Rocky Mountain Laboratory during the 1930s and 1940s demonstrated the ticks *Ornithodoros hermsi*, *Ornithodoros turicata,* and *Ornithodoros parkeri* were each responsible for transmitting spirochetes that were not transmissible by the other ticks [14]. The spirochetes were named *Borrelia hermsii*, *Borrelia turicatae,* and *Borrelia parkeri*, respectively, based on their strict specificity for only one of the three species of ticks. Thus the early taxonomic designations of these relapsing fever spirochetes were based on the species of tick that transmitted them in nature, which predated the ability to culture these organisms or compare them with numerous molecular techniques. If a patient contracted relapsing fever, then the identification of the spirochete detected in the blood smear by microscopy was based on the known or suspected tick vector.

Of the three species of *Ornithodoros* ticks involved with relapsing fever in western North America each has a unique set of ecological parameters that separate them spatially [9, 15]. *Ornithodoros hermsi* occurs in higher elevation coniferous forests with small diurnal tree squirrels and chipmunks, which serve as primary hosts [18]; *Ornithodoros turicata* lives in low elevation grass and shrub lands and inhabits caves and burrows of various animals; *Ornithodoros parkeri* is found primarily in low to mid elevation semiarid grasslands and lives in the burrows of various species of ground squirrels [9, 19]. Therefore, the ecological setting of the exposure site has been used to assume the identity of the spirochetes in the patient's blood: a mountain cabin

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suggested *B. hermsii,* a cave in Texas meant *B. turicatae,* and a dry river bank in California's Central Valley suggested *B. parkeri*.

Despite the early history of research and public health efforts to elucidate the distribution and maintenance of these zoonotic pathogens in North America, human infections of relapsing fever continue to present a challenge for doctors to promptly diagnose and treat the infection. A retrospective analysis of human cases of relapsing fever in Washington and California from 1995 to 2006 showed that 80% and 46% of the cases, respectively, required hospitalization [7]. This situation is troublesome and indicative of the need today to better define the geographic distribution of the endemic foci for these spirochetes, develop better serological tests to discriminate relapsing fever infections from Lyme borreliosis, and to better inform the medical community concerning the distribution and public health significance of tick-borne relapsing fever. These goals constitute one area of investigation for our laboratory, during the past two decades.

11.2 Distribution and Diversity of Tick-borne Relapsing Fever Spirochetes in North America

Most human outbreaks of tick-borne relapsing fever in western North America have been associated with patients who have slept in cabins located in higher elevation coniferous forests [20]. As stated above, the ecological setting for these outbreaks led to the conclusion that *B. hermsii* was the etiological agent and the tick vector involved was *O. hermsi*. In 1971, Richard Kelly developed a liquid culture medium that for the first time supported the continuous *in vitro* growth of *B. hermsii*, *B. turicatae,* and *B. parkeri* [25, 34]. This extremely important breakthrough, followed in more recent years by the development of many molecular approaches to characterize the genetic relatedness of all procaryotes, has provided the tools for us to better define the putative species of relapsing fever spirochetes, to elucidate their geographic distribution, and to determine the role they play in the repertoire of zoonoses impacting human health.

The first isolate of *B. hermsii* established in vitro originated from an *O. hermsi* ticks collected as part of a site investigation at a camp in eastern Washington where an outbreak of relapsing fever involving boy scouts occurred in 1968 [46]. This isolate, designated HS1, became the type strain of *B. hermsii* [ATCC # 35209, but no longer in stock] and was the only isolate available when we began our work. Acquiring new isolates of *B. hermsii* has been slow and laborious, but after 20 years we now have a basic understanding, albeit not complete, of the distribution and genetic diversity of this spirochete.

We have established 37 isolates of *B. hermsii* from California, Utah, Colorado, Idaho, Washington, Montana, and southern British Columbia, Canada [1, 22, 35, 39, 44] (Fig. 11.1). Most of these isolates came from the blood of patients who were acutely ill with relapsing fever, but a few of the isolates originated from *O. hermsi* ticks. The five-patient outbreak at Flathead Lake, Lake County, Montana, in 2002 resulted in the first isolation of *B. hermsii* and the first finding of the tick vector within the state [41, 49]. Samples of genomic DNA extracted from the 37 spirochete cultures have been analyzed by agarose gel electrophoresis for plasmid profiles, multilocus sequence typing with the highly conserved genes *16S rRNA*, *flaB*, *gyrB* and *glpQ*, sequence of vector-expressed variable tick protein gene *vtp* [35, 40], and for their repertoire of genes that encode the variable major outer surface proteins [24, 35, 44]. Our efforts as stated above have delineated two distinct genomic groups (GGI and GGII) in *B. hermsii* [35, 44]. Spirochetes in both genomic groups cause human disease and are transmitted by the same species of tick (*O. hermsi*); they are also sympatric with widely overlapping geographic distributions. During one outbreak, two children that slept in the same bed became infected with spirochetes belonging to the different genomic groups [44].

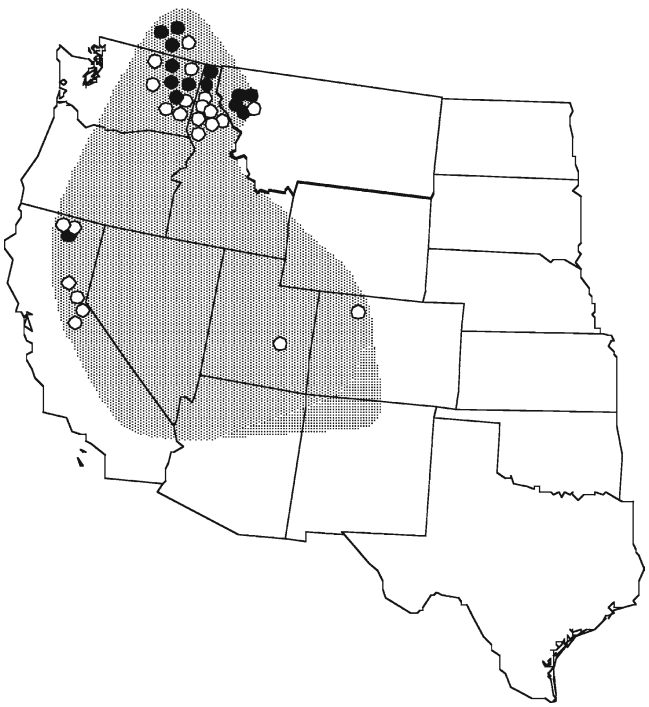

Fig. 11.1 Distribution of *Borrelia hermsii* in western North America. Shaded area encompasses the estimated endemic region, while dots represent 37 localities of origin for spirochete isolates. Open dots = GGI, solid dots = GGII

Inoculating mice with infected patient's blood is usually the first step in our attempt to isolate these spirochetes. Our experimental inoculations of *B. hermsii* in mice suggest that spirochetes in GGII may be more virulent than spirochetes in GGI. This developing hypothesis is based on the ability of some GGII spirochetes to produce extremely high spirochetemias (>10^8 per mL of blood) and morbidity which requires that we euthanize the infected mice. We initiated a comparative genomic analysis of spirochetes in both genomic groups to hopefully identify possible mechanisms for the difference in virulence and in growth in the host's blood. While the genomes of the spirochetes in the two genomic groups are essentially identical, we found that all members of GGII have an intact adenine deaminase gene while all members of GGI do not [33] (additional data unpublished). Therefore, *B. hermsii* spirochetes in GGII have a complete pathway for purine salvage while spirochetes in GGI do not. These observations have led us to another hypothesis: the difference in the potential for more efficient salvage of purine bases in the host's blood by spirochetes in GGII allow these bacteria to achieve higher cell densities in the blood than do the spirochetes in GGI. By constructing *B. hermsii* mutants in which genes involved with purine salvage have been inactivated, we are currently testing this hypothesis using our newly developed genetic tools [2]. If our hypothesis is correct, then we should see such mutants unable to achieve as high cell densities in the host's blood as those in wild-type spirochetes.

Relapsing fever has long been recognized in the midwestern and southwestern United States, and it has been associated with another argasid tick *Ornithodoros turicata* [9, 11, 12, 13, 16]. Yet, few human cases have been reported in recent years, and to date, no isolates of the probable causative agent, *Borrelia turicatae*, have been made from infected humans. However, we have isolated and characterized spirochetes from acutely ill domestic dogs and *O. turicata* ticks from Texas and Florida [43, 53]. Multilocus sequence typing of these isolates demonstrated that the spirochetes from ticks and dogs represented the same species and that an endemic focus of *B. turicatae* and the potential for relapsing fever exist in the southeastern United States [43].

Most recently our laboratory has expanded efforts to study an emerging potential zoonosis involving bats, bat ticks, and a group of newly discovered relapsing fever spirochetes [23, 42]. The argasid bat tick *Carios kelleyi* parasitizes many species of bats distributed across most of the United States. Through our collaborations with the Centers for Disease Control and Prevention and other local health care researchers, we have identified three novel species of spirochetes in bat ticks that are distinct from, but most closely related to, the relapsing fever spirochete *B. turicatae* [23, 42]. We have not yet isolated these bat tick-associated spirochetes, but we have visualized them by microscopy in tick tissues, including the midgut, salivary glands, and coxal fluid. To date, the spirochete identities are based on PCR analysis and multilocus sequence typing of the amplicons that are produced from infected tick tissues. We will continue our efforts to collect bat ticks and attempt to isolate these spirochetes for further characterization and for pathogenicity studies with experimental animals.

11.3 Serology for Relapsing Fever

For many years the serological detection of antibodies produced during infection with relapsing fever spirochetes was problematic for several reasons. First and foremost, the inability to culture the spirochetes *in vitro* prevented diagnostic laboratories from having a reliable source of bacterial cells for use as antigens. Second, the large repertoire of variable major immunodominant surface proteins (serotype antigens) led many diagnosticians to assume that borrelia cells of one serotype would be of little use in an assay if patients were infected with other serotypes of the spirochete [4, 21]. Third, with the emergence of Lyme borreliosis caused by *Borrelia burgdorferi* and related tick-borne spirochetes [5], investigators soon recognized there was considerable cross reactivity in serological assays for Lyme borreliosis and relapsing fever [29]. High titered convalescent serum samples from patients having had either disease were seropositive when tested with either *B. hermsii* or *B. burgdorferi* cells. Thus, we set out to develop a serological assay that was sensitive and specific for relapsing fever. Such an assay would facilitate the diagnosis and early treatment of human relapsing fever infections, it would help to elucidate the geographic distribution of human disease, and it would help to develop serological surveillance tools for detecting specific antibodies to relapsing fever spirochetes in wild animal populations.

We have identified a highly conserved, immunodominant protein in relapsing fever spirochetes that is absent in Lyme borreliosis spirochetes [45]. The protein, glycerophosphodiester phosophodiesterase (GlpQ), is an enzyme that hydrolyzes deacylated phospholipids, and like the purine salvage enzymes, may also contribute to the relapsing fever spirochetes achieving high cell densities in the host's blood [38]. Over 90% of relapsing fever patients infected with *B. hermsii* had antibodies that recognized this 39-kDa protein, while serum samples from Lyme borreliosis and syphilis patients were non-reactive [45].

11.4 Relapsing Fever in Africa

Tick- and louse-borne relapsing fevers probably affect human populations in Africa more than anywhere else in the world

[10, 50]. Therefore, we developed an ELISA with purified recombinant GlpQ from *Borrelia recurrentis*, which is the causative agent of louse-borne relapsing fever in Africa [36]. We tested paired acute and convalescent serum samples from spirochete-positive patients hospitalized in Ethiopia, and we found that, during a 2–3 week period, 91% of the patients had a 2–3 fold rise in titer with anti-GlpQ antibodies [36]. These results gave us confidence that this assay could be used as a surveillance tool in areas of Africa where both tick- and louse-borne relapsing fever may be an unrecognized public health problem.

Our next study was performed with Swedish collaborators in Togo, West Africa [31]. We hypothesized that here, and in other areas of sub-Saharan Africa where malaria is hyper-endemic, humans acutely ill with a febrile recurrent illness may be incorrectly assumed to have malaria rather than relapsing fever and are treated inappropriately without cure. Our retrospective serological study demonstrated that 13% of the human serum samples tested were positive in our ELISA with recombinant GlpQ as antigen. Therefore, we concluded that relapsing fever was endemic in some regions of Togo and the Swedish investigators are continuing efforts there.

We have shifted our focus to Mali, where our institute has established an International Center for Excellence in Research [ICER] at the Malaria Training and Research Center in Bamako. Currently we are performing field studies (Fig. 11.2) to identify areas where *Borrelia crocidurae* and *Ornithodoros sonrai*, the respective etiological agent and tick vector of relapsing fever in other areas of West Africa [47, 48, 50] may exist in Mali. Once determined, we will establish a prospective study with clinics in the endemic areas to determine the incidence and prevalence of human relapsing fever.

11.5 Conclusions

Despite the antiquity of relapsing fevers, these infections continue to place challenges on health care providers regarding early recognition and prompt antibiotic treatment. For many patients in North America, a diagnosis may never be made; eventually, however, an acquired immune response will cure them but only after many weeks to months of recurrent acute episodes, after many person-hours lost, and after many large medical bills have accumulated. We believe that relapsing fever is under-diagnosed and under-reported in all endemic areas. The epidemic of louse–borne relapsing fever in southern Sudan in 1999–2000 with 20,000 cases and ~2,000 deaths was first thought to be a viral hemorrhagic fever. Recent work in Senegal also demonstrated that in most villages studied tick-borne relapsing fever was the second most frequent bacterial infection after tuberculosis that caused patients to seek medical help at clinics [50]. We will continue our efforts to define the public health significance of tick-borne relapsing fever in the United States and West Africa. We sincerely hope that by increasing the awareness

Fig. 11.2 Small fishing hamlet on the Bani River, Mali, where NIAID investigators are searching for relapsing fever spirochetes in the wild rodents and shrews

of this potentially lethal zoonosis, infected patients will receive prompt diagnosis and treatment.

In summary, one primary area of investigation of the Medical Entomology Section of the Laboratory of Zoonotic Pathogens involves a multifaceted approach to elucidate the distribution and public health significance of tick-borne relapsing fever in North America and West Africa. In North America, most human cases of this disease are caused by *Borrelia hermsii*, which is transmitted by the argasid tick *Ornithodoros hermsi*. We have defined two genomic groups of *B. hermsii* that are geographically sympatric, that are transmitted by the same species of tick, and that cause severe human disease. We have also developed serological tests to discriminate relapsing fever infections from Lyme borreliosis. These serological tests are now being applied both to wild animals and to humans in Mali in the effort to help define the prevalence of infection and to determine if, in some areas, this recurrent illness is possibly being misdiagnosed as malaria.

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Chapter 12
Molecular Sleuthing with the Lyme Disease Agent

Patricia A. Rosa

Abbreviations

(ORF)	open reading frame
(Osp)	outer surface protein
(PGF)	paralogous gene family
(DMC)	dialysis membrane chamber
(B. burgdorferi)	*Borrelia burgdorferi*
(E. coli)	*Escherichia coli*
(lp)	linear plasmid
(cp)	circular plasmid
(kbp)	kilobase pairs

12.1 Introduction

Borrelia burgdorferi, the spirochete that causes Lyme disease, is an obligate parasite of both its tick vector and mammalian or avian host. This bipartite lifestyle demands the ability to sense and to adapt in order to stay alive and move between these two different environments and persist in nature. A major focus of our research has been the development and application of a genetic system for *B. burgdorferi*, in order to investigate, at the molecular level, the roles of specific spirochetal components at different stages of the infectious cycle. Part of this endeavor has been an investigation of the structure and function of the unusual segmented genome of *B. burgdorferi*, both in terms of utility as genetic tools and as a biological role in infection. It would be misleading to argue that this research is designed to have a direct impact on the diagnosis, treatment, or prevention of Lyme disease. However, *B. burgdorferi* is a wide spread human pathogen and our research has contributed to a better understanding of how this tick-borne pathogen infects a mammalian host. The genetic system that we have developed facilitates not only the study of the Lyme disease spirochete by a number of labs, but has also provided guidance for the development of genetic tools in other pathogenic bacteria. The basic mechanisms underlying the genomic structure of *B. burgdorferi* have broader applicability in molecular biology. Finally, determining the roles of specific *B. burgdorferi* components in the tick vector and mammalian host will contribute to our understanding of the infectious strategy of this and other vector-borne pathogens.

12.2 Tools for Genetic Manipulation of *B. burgdorferi*

A basic genetic system requires the ability to inactivate and restore genes in order to analyze the effects of defined mutations on particular functions or phenotypes. Mutants are typically recovered by targeted insertion of a selectable marker. We initially utilized a mutated form of an endogenous gene, *gyrB*^{r}, which conferred resistance to the antibiotic coumermycin A1 [1, 2], as a selectable marker for gene inactivation by allelic exchange [3, 4, 5, 6, 7, 8]. However, while the *gyrB*^{r} marker was key to developing a method for transformation of *B. burgdorferi* [1], using it to generate gene inactivation mutants was a very inefficient process because allelic exchange most frequently occurred with the endogenous *gyrB* gene rather than at the targeted locus [3, 9]. This necessitated a time-consuming screen of transformants in order to identify the desired mutants. To circumvent this shortcoming, we subsequently utilized two separate strategies to develop selectable markers that lacked this undesirable trait.

12.2.1 Efficient Selectable Markers

We initially observed that a foreign antibiotic resistance cassette from another bacterium did not confer a selectable phenotype when stably introduced into *B. burgdorferi* [10]. However, close analysis of these transformed bacteria indicated that this foreign gene was poorly expressed, suggesting that enhanced expression by fusion to a strong native

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promoter might yield a useful selectable marker [10, 11]. This approach proved successful and we used it to create two new markers, termed the *kan* and *gent* cassettes, which confer resistance to the antibiotics kanamycin and gentamicin, respectively [11, 12]. We have used these markers to inactivate genes in *B. burgdorferi* and have obtained the desired mutations without the high background of irrelevant transformants previously obtained with similar constructs carrying *gyrB*^{r} [11, 13, 19]. The development of these cassettes also gave an early indication that promoter structure in *B. burgdorferi* likely differed from that of other bacteria in which the original promoters of these antibiotic resistance genes were active.

We utilized a different strategy to generate a more useful marker from *gyrB*^{r}, which we knew conferred a strong selectable phenotype but was limited in its utility. To reduce or eliminate recombination with the endogenous *gyrB* locus, (the source of its inefficiency as a tool for targeted gene inactivation), we designed a synthetic gene that had the same promoter and encoded the same protein as *gyrB*^{r}, but had a substantially different nucleotide sequence in the open reading frame (ORF) of the gene [12]. This was accomplished by modifying the codons at the wobble position wherever possible, while maintaining the same amino acid sequence and not straying too far from the codon bias of *B. burgdorferi* [12].

The resulting antibiotic resistance cassette, termed *gyrB*$_{syn}$, conferred a similar level of resistance to coumermycinA1 as *gyrB*^{r}, but it did not recombine with the endogenous *gyrB* gene, thus eliminating the high background of irrelevant transformants and yielding another useful selectable marker [12]. The elimination of recombination between the divergent *gyrB* genes also provided insight into parameters governing homologous recombination in *B. burgdorferi*.

12.2.2 Shuttle Vectors

Shuttle vectors are engineered plasmids capable of autonomous replication in at least two bacterial species. Typically, one of the bacteria grows quickly and is relatively easy to genetically manipulate, like *Escherichia coli*. The other bacterium is generally the object of investigation, and it typically grows more slowly and is more difficult to transform, like *B. burgdorferi*. Shuttle vectors are useful tools for delivering foreign or modified DNA or for complementing mutations. Initial attempts to develop a shuttle vector for *B. burgdorferi* were hindered by the shortcomings of the available *gyrB*^{r} marker (as described above). The availability of the *kan* cassette greatly facilitated the development of a shuttle vector for *B. burgdorferi*.

We have developed a small, stable, and useful shuttle vector that currently has widespread application in the *Borrelia* field. In its original form, termed pBSV1, the shuttle vector was a fusion of a small 10 kbp circular plasmid of *B. burgdorferi* with the *kan* cassette and a standard *E. coli* cloning vector [20]. Subsequent refinement of the shuttle vector eliminated all but a small ~3kbp fragment of the original *B. burgdorferi* plasmid and introduced a multiple cloning site, yielding pBSV2 [20]. Deletion of irrelevant sequences of the *E. coli* vector and substitution of the *gent* cassette as a selectable marker resulted in pBSV2G, a shuttle vector that has been particularly valuable in the complementation of mutations generated with the *kan* cassette in an infectious clone [12, 17]. In addition to their usefulness as genetic tools, the development of shuttle vectors derived from endogenous *B. burgdorferi* plasmids has also defined the minimum element required for autonomous replication in *B. burgdorferi*, as described below in Section 1.3.2.

12.2.3 Genetic Manipulation of an Infectious Clone

One of the primary reasons to inactivate a particular gene is to see what effect this mutation has, by studying the phenotype of the resulting mutant. Some phenotypic traits can be readily evaluated during *in vitro* growth, such as morphology, growth characteristics, gene expression, biochemical composition, or enzymatic activity. Other traits, however, can only be assessed *in vivo*, in the context of the infectious cycle. These traits include the ability to colonize relevant sites in the tick vector and mammalian host, maintain a persistent infection in both environments, and ultimately be efficiently transmitted between them. In order to study the contribution of a particular gene to any *in vivo* aspect of infection, the ability to genetically manipulate an infectious clone is required. This obvious and seemingly trivial point represented a significant technical hurdle and continues to present a challenge to genetic studies in *B. burgdorferi*.

For a number of reasons, not all of which are fully understood, *B. burgdorferi* becomes easier to grow and manipulate as it is passaged *in vitro*, with a corresponding loss of infectivity in mice and ticks. Our initial efforts at transforming *B. burgdorferi*, inactivating genes and developing a shuttle vector all utilized high passage, attenuated, non-clonal *B. burgdorferi*. A number of years ago, we undertook an analysis of an uncloned but infectious population of the type strain B31 and derived a clone with a defined plasmid content that was infectious, transformable by electroporation, grew at an optimal rate in liquid media, and formed colonies with high efficiency in solid media [14]. This clone, termed B31-A3,

has become the mainstay of our *in vivo* genetic investigations and is widely used by other researchers in the field.

The segmented genome of *B. burgdorferi* represents one of its most problematic features for genetic studies. Shortly after the recognition that *B. burgdorferi* contained a number of plasmids, it also became apparent that plasmid content varied significantly between strains and with *in vitro* passage [21, 22, 23]. Since plasmids make up approximately one third of the *B. burgdorferi* genome and encode many of the abundant lipoproteins on the spirochete's surface, they have been the focus of intense interest. Many plasmids vary not only in their presence among strains but also have undergone significant re-assortment of sequences [24, 25]. Fortunately this latter trait, while prominent when comparing plasmids among strains, has not been observed during *in vitro* cultivation. However, the instability of this critical component of the genome demands that the plasmid content of a clone is completely defined before it can be used in a genetic study, as well as subsequently monitored throughout an experiment. This requires the complete and compiled sequence of the chromosome and all plasmids of the *B. burgdorferi* strain from which the clone is derived, which is currently only available for the type strain B31[25, 26]. We have carefully analyzed plasmid loss during *in vitro* cultivation of an infectious B31 clone and concluded that although diligence is required to monitor plasmid content, rigorous genetic studies with isogenic clones are feasible in *B. burgdorferi* [27].

12.2.4 Transposon Mutagenesis

Transposon mutagenesis represents a convenient way to randomly mutagenize an entire genome by introducing a single, identifiable mutation per cell. When coupled with an appropriate genetic screen, the genes contributing to a particular trait can be readily identified through transposon mutagenesis. This strategy represents the opposite approach to targeted gene inactivation, in which a particular mutation is introduced and then the phenotype is analyzed. We have added transposon mutagenesis to our genetic toolbox by adapting the Himar1 transposon of the *mariner* family for function in *B. burgdorferi* [28]. This required introducing the *gent* cassette for selection in *B. burgdorferi* and expressing the *himar1* transposase gene from a *Borrelia* promoter. The resulting transposon, termed pMarGent, was used to generate a saturated library of transposon mutants whose insertion sites were easily identified. Mutations in genes putatively involved in cell division, chemotaxis and cell structure, as well as genes of unknown function, were identified by a simple screen of the transposon library for mutants with altered growth rates [28]. The pMarGent transposon of *B. burgdorferi* has been used either directly or as a blueprint for the development of a mariner-based transposon mutagenesis system in several other bacteria for which genetic tools are just emerging, including *Coxiella burnettii*, *Leptospira interrogans* and *Treponema denticola* [29, 30, 31].

12.3 Plasmid Structure and Function

The unusual form of the *Borrelia* genome poses basic molecular questions about chromosome and plasmid dynamics. The multiplicity and shape of the DNA also raise questions about genomic organization and biological function. Are linear and circular DNAs in *B. burgdorferi* replicated by the same mechanism and does their shape influence their stability and tendency to recombine? Does the location of a gene have an impact on its role? These are some of the questions that we would like to address, utilizing and building upon the genetic tools available for *B. burgdorferi*.

12.3.1 Essential Elements of Plasmid Replication

The development of the shuttle vector pBSV2 by our lab defined a minimal element necessary for autonomous plasmid replication in *B. burgdorferi* [20]. This plasmid region encompassed three ORFs that belonged to large paralogous gene families (PGFs) whose divergent members are present on every plasmid. These genes were previously hypothesized to provide plasmid replication functions based on their common plasmid location and weak similarity of one ORF to a gene involved in plasmid partitioning in other bacteria [25, 26, 32, 33, 34]. We subsequently confirmed that related regions from additional *B. burgdorferi* plasmids, both circular and linear, also conferred autonomous replication in *B. burgdorferi* of DNA with which they were associated. We demonstrated that the same region from a linear plasmid could support replication of a circular plasmid and vice-versa [35, 36]. This indicated that all plasmids in *B. burgdorferi* likely shared a similar mechanism of replication, regardless of their shape. Importantly, introduction of a shuttle vector into *B. burgdorferi* resulted in displacement of the endogenous plasmid from which it was derived, indicating that the region necessary for plasmid replication also mediated incompatibility. This finding has allowed us to experimentally manipulate the plasmid content of *B. burgdorferi*, as described more fully in the following Sections.

12.3.2 Experimental Assessment of Plasmid Function

A number of studies have attempted to correlate plasmid content with the *in vivo* infectious phenotype of *B. burgdorferi*. Through this type of analysis, two linear plasmid of *B. burgdorferi*, lp25 and lp28-1, were proposed to contribute to mouse infectivity *(37,38)*. We have confirmed and extended these findings through an experimental approach that entails selectively displacing or restoring entire plasmids, and then comparing the phenotypes of the resulting isogenic clones in an experimental mouse-tick infectious cycle *(19,39,40)*. This was accomplished by introducing shuttle vectors derived from lp25 or lp28-1 in order to displace the endogenous plasmid and by re-introducing a modified version of the missing plasmid bearing a selectable marker [36, 39, 40]. These studies confirmed the feasibility of experimentally manipulating the plasmid content of *B. burgdorferi* and formally proved the requirements for lp25 and lp28-1 in the infectious cycle. They also demonstrated for the first time the disparate requirement for these two important plasmids in the tick vector [40]. In collaboration with Dr. Justin Radolf's laboratory at the University of Connecticut, we also broadened and extended these findings to a strain with an undetermined genomic sequence and incompletely defined plasmid content [39]. This approach will be useful in addressing the roles of other plasmids in strain B31 whose contributions to the infectious cycle are unknown and in determining the biological roles of related plasmids in other uncharacterized strains.

12.3.3 Linear Plasmid lp36

We subsequently used a similar approach to investigate the role of a previously uncharacterized 36 kbp linear plasmid (lp36) of strain B31. We compared the *in vivo* phenotype of a variant lacking lp36 to one in which lp36 had been restored [19]. We found that lp36 encoded functions critical for *B. burgdorferi* survival in the mouse, but was dispensable for colonization of the tick vector, similar to lp28-1 [19, 40]. Further analysis indicated that a single gene on lp36, *bbk17*, was sufficient to restore mouse infectivity to an lp36-minus *B. burgdorferi* variant. The *bbk17* gene encodes a homolog of the purine salvage enzyme adenine deaminase and we demonstrated that BBK17 has this enzymatic activity, in collaboration with Dr. Frank Gherardini's laboratory at the NIAID's Rocky Mountain Laboratories (RML) [19]. Our studies do not rule out possible contributions of other lp36 genes to the infectious cycle of *B. burgdorferi,* but they demonstrate that only one gene on this plasmid, *bbk17*, is essential [19].

12.3.4 Definition of a Functional Telomere

The linear chromosome and linear plasmids represent a hallmark of the *B. burgdorferi* genome. The telomeres, or ends of these linear DNAs, were previously determined to be covalently closed hairpin structures [41, 42]. Dr. George Chaconas, while on sabbatical in our laboratory, investigated telomere structure and resolution by introducing a DNA fragment into *B. burgdorferi* that represented a putative replicated telomere [35]. This synthetic sequence was recognized, cleaved, and rejoined in *B. burgdorferi* by a telomere resolvase activity, which generated two covalently closed hairpin ends that were identical in structure to the native *B. burgdorferi* telomeres [35]. Introduction of the synthetic replicated telomere into a circular plasmid in *B. burgdorferi* resulted in its stable conversion to a linear replicon with typical ends [35]. In addition to the definition of a functional telomere, these results further confirmed that linear and circular plasmids in *B. burgdorferi* utilize a similar mechanism of replication. This work directly enabled the subsequent identification and characterization of the telomere resolvase of *B. burgdorferi* by the Chaconas laboratory [43].

12.3.5 Ubiquitous and Stable Plasmid

Many plasmids are shed by *B. burgdorferi* during *in vitro* passage or are widely divergent in occurrence or gene content among isolates. One plasmid represents a striking exception to this description because it is present in all *B. burgdorferi* strains, it has a highly conserved gene order and content, and it persists in even the most highly attenuated lab strains. This 26 kbp circular plasmid, cp26, remains a focus of our interest for several reasons. As described in subsequent sections, we have found that cp26 carries certain genes whose functions are critical at discrete points of the infectious cycle, although dispensable or even detrimental at other times [15, 17, 44, 45]. However, we have also demonstrated that cp26 encodes functions that are required for bacterial survival under all conditions, and thus cp26 represents an essential genetic element or a mini-chromosome [13, 18].

We have identified three essential genes on cp26 that account for its stable and ubiquitous nature. We have accomplished this with a two-pronged approach whereby essential genes are defined as those that cannot be inactivated, but which allow displacement of cp26 when provided together *in trans* on an incompatible shuttle vector [13, 18]. We found that the three essential genes on cp26 genes are *resT, bbb26,* and *bbb27*. The single copy *resT* gene encodes the telomere

resolvase, which is required for the replication of all linear DNA, including the chromosome [43]. The unique cp26 genes *bbb26* and *bbb27* are of unknown function and lack homologs in other bacteria. They encode membrane-associated proteins and are an ongoing topic of investigation in our laboratory [18].

The strategic advantage to *B. burgdorferi* of maintaining an extrachromosomal location for these essential genes is obscure, but it is perhaps related to the selective pressures that resulted in the segmented *Borrelia* genome. The co-linkage on cp26 of *resT, bbb26,* and *bbb27* with *ospC*, a gene encoding an immunogenic surface protein that we have shown to be critical for bacterial survival at a single discrete point in the infectious cycle (described in section 1.4.1), ensures the stable retention of this virulence factor throughout the infectious cycle, even when the presence of OspC is irrelevant or confers a selective disadvantage. The strict conservation of cp26 gene content suggests that the other genes on cp26 are also important for *B. burgdorferi* survival or fitness in some environmental condition encountered during the natural infectious cycle, but perhaps these genes do not confer adequate selective advantage to guarantee their stable retention if not present on an essential replicon.

12.4 Molecular Mechanisms of Adaptation and Variation

As described above, in order to meet the distinct challenges presented by these two environments, the requirement by *B. burgdorferi* for individual plasmids varies significantly between the tick vector and mammalian host. This is clearly reflected by a dramatic switch in the abundant, plasmid-encoded outer surface proteins (Osp) at different stages of the infectious cycle (Fig. 12.1) [46, 47, 48]. The Osp profile of *B. burgdorferi* reflects changes in gene expression, which in turn are controlled by regulatory pathways that respond to pertinent environmental signals. We have begun by studying these most obvious components of the adaptive response because they form the critical interface between the pathogen and its host and vector. However, other more subtle, but equally important adaptations, are no doubt also taking place to enable the efficient transmission and persistence of *B. burgdorferi* throughout all stages of the infectious cycle. These less obvious changes remain topics of future investigation by our lab and others in the borrelia field.

OspA/B and OspC are plasmid-encoded lipoproteins that were first described as abundant components of *in vitro*

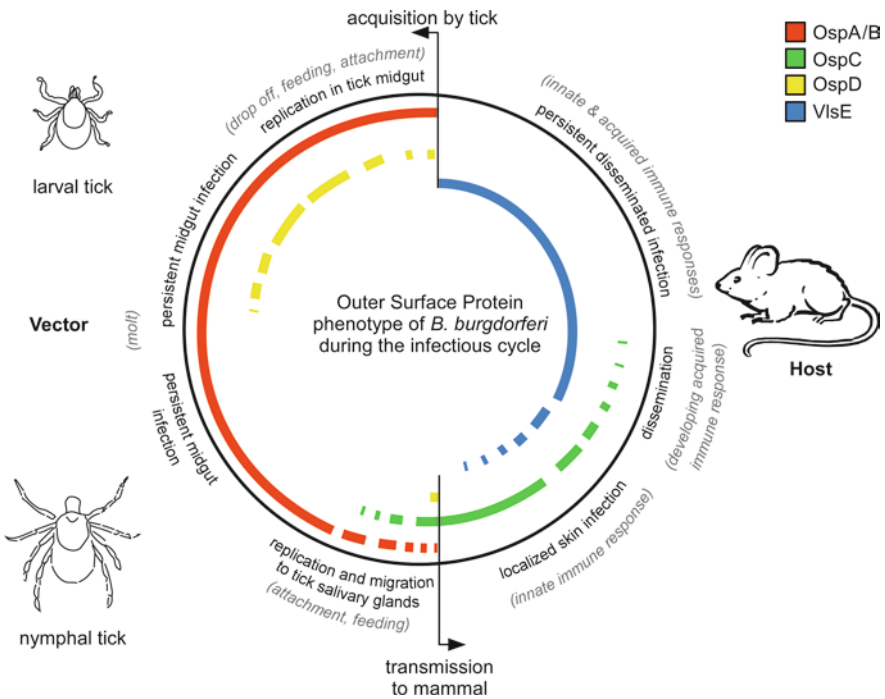

Fig. 12.1 Molecular adaptations of *B. burgdorferi*. The surface composition of *B. burgdorferi* changes dramatically within and between the tick vector and mammalian host. Prime examples of this phenotypic variation, and depicted as swaths of different colors, are the expression patterns of four abundant surface lipoproteins, OspA/B, OspC, OspD and VlsE (identified by the key in the upper right corner). Text encircling the diagram describes pertinent aspects of the spirochete (dark font) or host/vector (light font) at different stages of infection

grown spirochetes, typically with a reciprocal pattern of expression [49, 50, 51, 52]. The *ospA/B* operon is carried by a 54 kbp linear plasmid, lp54 [53], whereas the *ospC* locus is encoded by cp26 [54, 55]. During transmission from an infected tick to the mammalian host, OspC replaces OspA/B as the major protein on the spirochete's outer membrane [46, 48] (Fig. 12.1). This observation led to the hypothesis that OspC mediates spirochete migration from the tick midgut to the salivary gland during tick feeding, which is a prerequisite for transmission [47, 56, 57, 58]. Alternatively, in order to facilitate subsequent infection in the mammalian host, OspC may be induced in the tick preceding transmission [46, 47]. We have utilized a genetic approach to test these hypotheses through a comparison of wild type, *ospC* mutant and complemented *B. burgdorferi* clones in an experimental mouse-tick infectious cycle [15, 17, 44, 45]. The expertise and advice provided by Dr. Tom Schwan's laboratory at RML in establishing and analyzing tick and mouse infections was instrumental in our ability to pursue these *in vivo* studies.

12.4.1 OspC – an Essential Virulence Factor

We constructed two different *ospC* mutants in *B. burgdorferi*, representing either a simple gene disruption or a complete gene deletion, and complemented both mutations, either *in cis* on cp26 or *in trans* on a shuttle vector, respectively [15, 17]. Both *ospC* mutants efficiently colonized the tick midgut and migrated to the salivary gland during tick feeding, but neither mutant could infect mice, either by tick bite or needle inoculation. Complementation of both mutants restored mouse infectivity, confirming that the defective phenotype *in vivo* resulted from the *ospC* mutation [15, 17]. These results demonstrated that upregulation of OspC in response to signals received in the feeding tick represents a critical adaptive response that anticipates and prepares the spirochete for colonization of the mammalian host.

12.4.2 Early Requirement for OspC

We observed that *ospC* mutant spirochetes were cleared from immunocompetent hosts preceding an acquired immune response and were likewise unable to infect SCID mice [15]. These results suggest an early role for OspC in colonization of the mammalian host by *B. burgdorferi*. However, we also found that *ospC* mutant spirochetes could replicate in the mammalian host if sequestered in an immune-privileged site provided by a dialysis membrane chamber (DMC) implant [44]. The DMC model was previously developed by other investigators to study the host-adaptive response of *B. burgdorferi* and to distinguish between spirochetal defects in growth or immune evasion [59, 60]. We observed that *ospC* mutant spirochetes underwent host adaptation when propagated in DMCs, but spirochetes from this source remained non-infectious for mice [44]. We concluded that OspC likely provides the spirochete with protection from some element of the host innate immune response, rather than fulfilling a physiological function.

Although these data suggest that OspC shields *B. burgdorferi* from the host's innate immune response, we have been unable to identify which component of innate defense this represents. In collaboration with Dr. Janis Weis's laboratory at the University of Utah, we have found that *ospC* mutant spirochetes cannot infect MyD88-deficient mice, which lack a branch of the innate immune response that was previously shown to be effective in controlling spirochete replication [44, 61, 62]. Through a series of experiments, most of which remain unpublished, we have found that spirochetes with or without OspC are equally resistant to killing by macrophages, neutrophils, natural killer cells, complement, and antimicrobial peptides. Hence, the critical contribution of OspC early during mammalian infection remains elusive.

12.4.3 Transient Role of OspC

We have conducted additional experiments to address more precisely the time frame during which OspC is required for spirochete survival in the mammalian host. A time course experiment demonstrated that spirochetes lacking OspC were cleared within the first 24 to 48 hours after inoculation into mice, which was well before dissemination of the OspC-plus spirochetes at approximately one week [45]. Paradoxically, although OspC is absolutely essential to initiate infection by spirochetes following tick bite or needle injection [15], the continued presence of OspC limits persistent spirochetal infection. This can be surmised from the downregulation of *ospC* gene expression that takes place during chronic infection [63, 64, 65], as well as the experimental demonstration that constitutive *ospC* expression leads to spirochete clearance by an immune host [66]. Consistent with these observations, we found that complemented *ospC* mutant spirochetes in chronically infected immunocompetent mice had lost the shuttle vector carrying the wildtype copy of *ospC*, whereas the same spirochetes in persistently infected SCID mice retained the shuttle vector, presumably because OspC did not present a selective disadvantage in the absence of acquired immunity [17]. These data demonstrate that OspC is absolutely required during the initial stage of infection but is dispensable thereafter.

Various models can be proposed to explain these data. One is that OspC fulfills an essential role that the spirochete

must meet only once during the initial phase of infection and never again. Another model invokes an essential role for OspC that confronts the spirochete throughout infection, but one that another protein subsequently fulfills in place of OspC. This second model requires that the putative OspC surrogate be absent from *in vitro* grown or tick-derived spirochetes, as well as possess a mechanism for immune evasion that OspC lacks. We favor this latter model because the spirochete must colonize the same skin site during persistent infection that it encounters immediately after inoculation, yet it does so at this later stage without OspC [17]. We have proposed a potential surrogate protein, VlsE, which satisfies these criteria, and have obtained preliminary data to support this model, but additional experiments are required to rigorously test it.

12.4.4 OspD – A Highly Regulated Non-Essential Protein

A similar logic to that which argued a potentially important role for OspC also motivated us to investigate the requirement for another outer surface lipoprotein of *B. burgdorferi* during the infectious cycle. OspD represents another abundant, plasmid-encoded, temperature-regulated surface component of the spirochete, but one with an expression pattern quite distinct from that of OspA/B or OspC. Unlike OspC, OspD synthesis and gene expression can be induced by lowering the *in vitro* growth temperature, a condition that mimics the tick vector rather than the mammalian host [67, 68, 69, 70]. We investigated the expression of OspD *in vivo* and found that, during the period following feeding and detachment from the mammalian host, it was abundant on spirochetes in the tick midgut (Fig. 12.1), but it was not detectable on spirochetes following transmission to the mammalian host [71]. This timeframe of expression coincides with lysis and digestion of the blood meal and represents a period of rapid spirochete replication, preceding the transition into stationary phase that accompanies the tick molt [72]. The tick midgut and the period during and after tick feeding represent the only place and point during the infectious cycle that spirochetes achieve a high local density (Fig. 12.2). Spirochete levels remain constant or decline at other stages in the colonized tick [72] and never reach levels that are detectable by direct microscopic examination in infected host tissues or blood. The lysis of red blood cells during digestion of the blood meal in the tick midgut may provide critical nutrients that are limiting for spirochete growth in other stages or sites during the infectious cycle.

Because the expression pattern of OspD was consistent with a contribution during this critical period of growth, we inactivated the *ospD* gene to investigate whether loss of OspD impaired the spirochete's ability to colonize the tick midgut [71]. As anticipated by its lack of expression in the mammalian host, *ospD* inactivation did not impede mouse infectivity. Quite surprising, however, was the finding that *ospD* mutant spirochetes were indistinguishable from

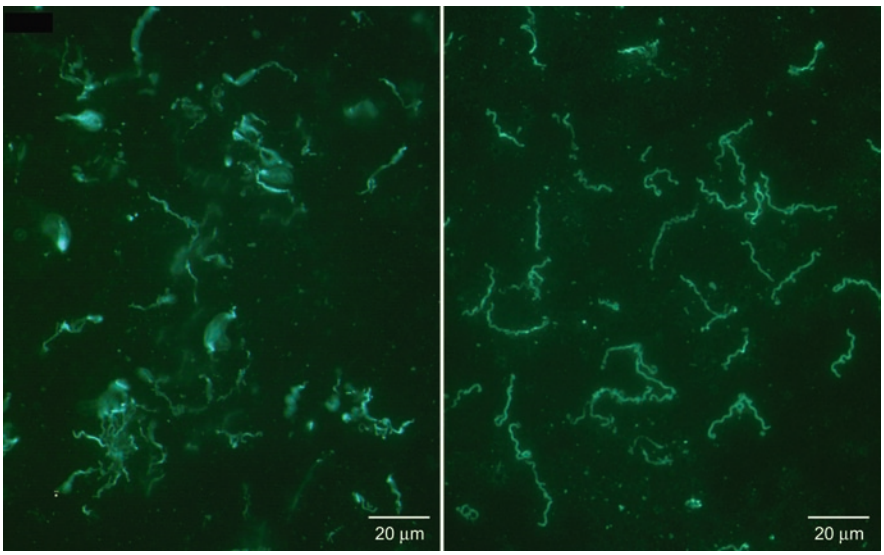

Fig. 12.2 Infected tick midgut containing *B. burgdorferi*. A midgut of an infected tick was dissected 5 days after the nymphal blood meal and incubated with polyclonal anti-*B. burgdorferi* antiserum, followed by an FITC-labelled secondary antibody. Spirochetes were visualized with a fluorescent microscope at 600X magnification. *B. burgdorferi* can be detected associated with tick midgut as multicellular aggregates (left panel) or as individual spirochetes (right panel). Spirochetes achieve their highest density in the tick midgut at this point and abundantly express OspA/B, OspC and OspD, as depicted in Fig. 1

wild type organisms in the tick vector at all stages of infection [71]. This unanticipated result indicated that although tightly regulated and at times highly expressed, OspD plays no obvious role at any stage of the infectious cycle. This represents a seemingly wasteful scenario and we conclude that it is more likely that our experimental infectious cycle does not capture some aspect of spirochete fitness that is important for survival in nature and to which OspD contributes.

12.5 Conclusion

The transmission of the Lyme disease spirochete between ticks and mammals presents a facile system in which to investigate the interplay between a bacterial pathogen and its vector and host environments. The infectious cycle can be fully reproduced in the laboratory, rendering it amenable to experimental study. The objectives of our research – to develop a genetic system for *B. burgdorferi*, to understand the basic molecular biology of the segmented genome, to discern how the spirochete survives in disparate environments – overlap productively at many levels. We hope that these studies will contribute to a broader understanding of related topics beyond our specific endeavors.

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Chapter 13
Modeling of Acute Respiratory Melioidosis and Glanders

Jonathan M. Warawa and Frank C. Gherardini

Burkholderia pseudomallei and *Burkholderia mallei* are the etiological agents of the diseases melioidosis and glanders, respectively, and they are related Gram-negative bacterial pathogens affecting both human and animal hosts. *B. pseudomallei* is an environmental bacteria that frequently opportunistically infects a very wide range of animal species, including mammals, birds, and shellfish. Conversely, the natural host range of *B. mallei* is limited to solipeds; however, humans, when in frequent contact with infected animals, appear to be incidental hosts. *B. pseudomallei* and *B. mallei* are infectious through both subcutaneous inoculation and inhalation routes of entry, and both pathogens can elicit a highly fatal septicemia.

Since *B. pseudomallei* and *B. mallei* are pathogens that could be used in widespread biological attacks, resulting in potentially high mortality, they have been designated as Category B Biological Agents by the Centers for Disease Control (CDC) (Rotz et al., 2002). In the case of these specific pathogens, widespread dissemination would require an aerosolized delivery for maximum impact, and therefore, understanding the pulmonary forms of glanders and melioidosis is a much greater concern than is the subcutaneous forms of these diseases. As such, defining the bacteria/host interactions that lead to the pulmonary forms of melioidosis and glanders has become a major focus of this laboratory.

To this end, our laboratory has begun to identify virulence determinants of both *B. pseudomallei* and *B. mallei*, using genetic tools that are available to rapidly generate allelic exchange mutants in both species. Since both have been shown to survive within both professional and non-professional phagocytes, *B. pseudomallei* and *B. mallei* are thought to be facultative intracellular pathogens (Pruksachartvuthi et al., 1990; Jones et al., 1996; Harley et al.. 1998). Thus mutants are screened using an intracellular survival assay as a way to identify genetic determinants for further characterization. While these assays do identify some virulence determinants required for the intracellular lifecycle of these pathogens, there is growing evidence that *B. pseudomallei* also requires key factors for initial colonization and dissemination. Taken together, these observations suggest that initial, acute infection is distinct. Therefore, studies of critical virulence determinants required for protection from host immune responses requires the use of animal models, for which no good *in vitro* model exists.

Several animal models have been used for the study of *B. pseudomallei* and *B. mallei*. The first studies identifying the susceptibility of animals to melioidosis and glanders included guinea pig, hamster, mouse, rat, rabbit, ferret, and monkey models (Duval and White 1907; Whitmore and Krishnaswami 1912; Miller et al., 1948), each with varying degrees of susceptibility affected by the route of infection. Recently, horses have also been tested as an experimental model for glanders (Lopez et al., 2003), and while being a biologically relevant glanders animal model, the facilities required for these studies are not available to most researchers. The current animal model used for modeling both melioidosis and glanders disease by laboratories world wide is the murine model. Since the mouse can be used to model both chronic and acute forms of disease, since it is cost effective, and since it has excellent genetic tools available, it is ideal for dissecting critical aspects of host response to disease.

A focus of this laboratory has been the development of acute murine respiratory melioidosis and glanders models of infection. One important question to address is the identification of the key sites of infection following the introduction of these pathogens into the respiratory system of mice. Previous studies have identified lung, liver, spleen, and blood as common sites of *B. pseudomallei* colonization by various routes on inoculation, and a recent study demonstrated lung, spleen, and blood as sites of colonization during murine respiratory melioidosis (Wiersinga et al., 2008). Our laboratory has conducted a histological analysis of BALB/c mice, terminally infected with wild

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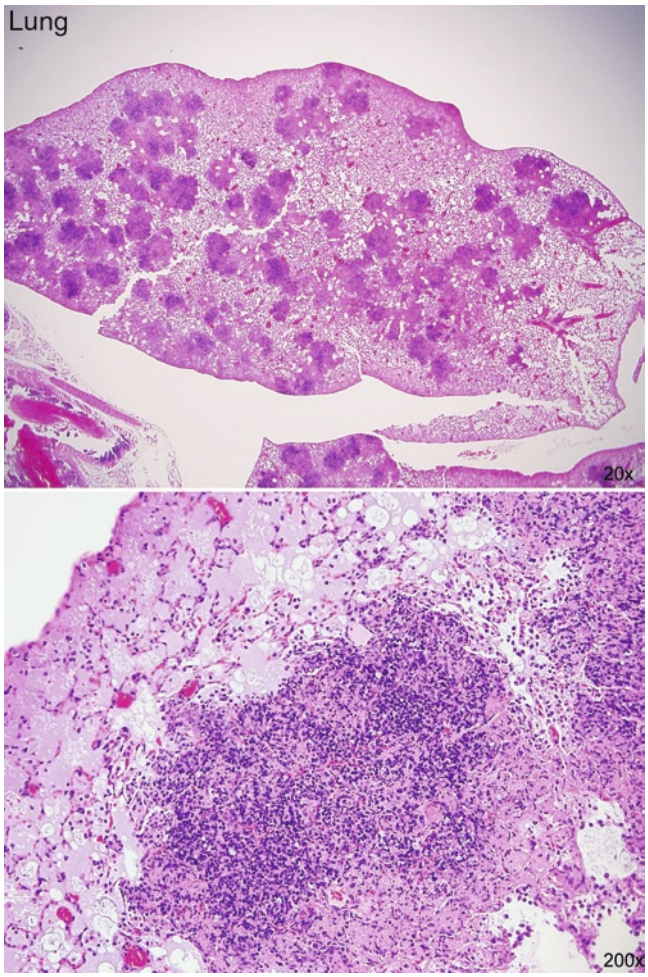

Fig. 13.1 Photomicrograph of *B. pseudomallei*-infected lung tissue. Formalin-fixed/paraffinized lung tissue was hematoxlin and eosin (H&E) stained and imaged at 20x (Top panel) or 200x (Bottom panel)

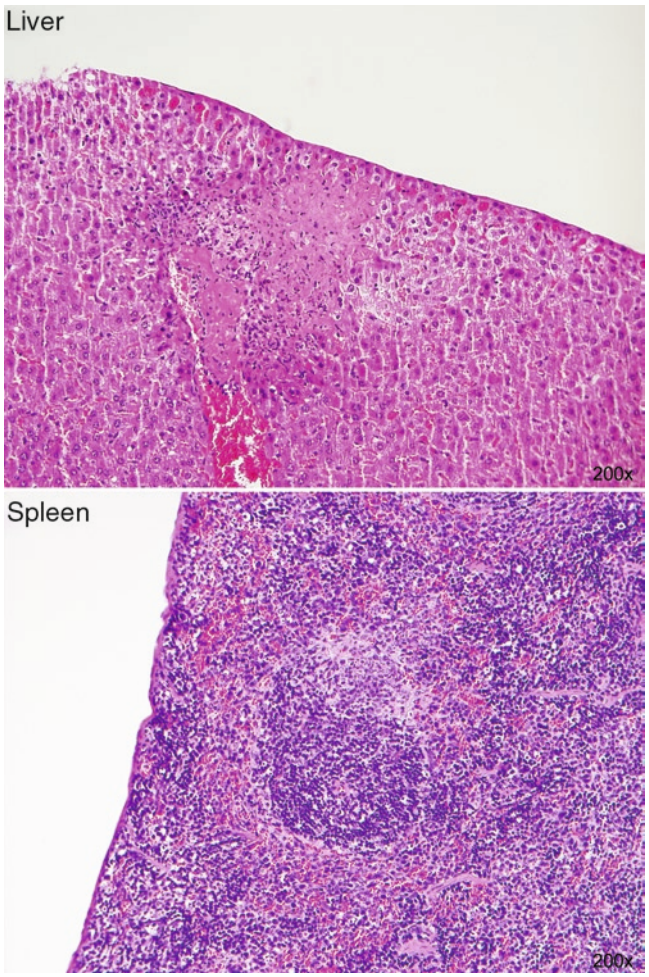

Fig. 13.2 Photomicrograph of *B. pseudomallei*-infected liver and spleen. Forma lin-fixed/paraffinized liver (Top panel) and spleen (Bottom panel) tissue were H&E stained and imaged at 200x

type *B. pseudomallei*, to identify additional sites of infection not identified by culturing techniques.

Mice infected by the intranasal route of infection develop multifocal lesions of the lung (Fig. 13.1), consistent with high rates of lung colonization that was previously reported (Wiersinga, de Vos et al., 2008). Infected lung tissue had few air filled alveoli, and instead, most were filled with inflammatory cells, necrotic cell debris, and fibrin. Similarly, liver and spleen, key sites of infection in rodent melioidosis models, showed sporadic lesion formation at both sites (Fig. 13.2) with the majority of the splenic lesions associated with the lymphoid nodules. These results were consistent with those reported for these sites of infection in various murine models of melioidosis (Atkins et al., 2002; Liu et al., 2002).

As a result of intranasal delivery, the trachea of *B. pseudomallei*-infected mice becomes infected, with inflammation and sloughing of the epithelial lining as well as inflammation of the tracheobronchial lymph node (Fig. 13.3). Additional tissue damage was observed in the upper respiratory tract where rhinitis was a common presentation of the intranasally-infected mice (Fig. 13.4, Top panel) with mild to severe congestion of the upper nasal passages. It was not unexpected that the middle ear was also frequently infected, presumably from drainage from the respiratory tract through the Eustachian tube where otitis media was another common presentation (Fig. 13.4, Bottom panel). Less frequently, the colonization of the upper respiratory tract led to meningitis where *B. pseudomallei* cause the inflammation of the olfactory bulbs and breach of the

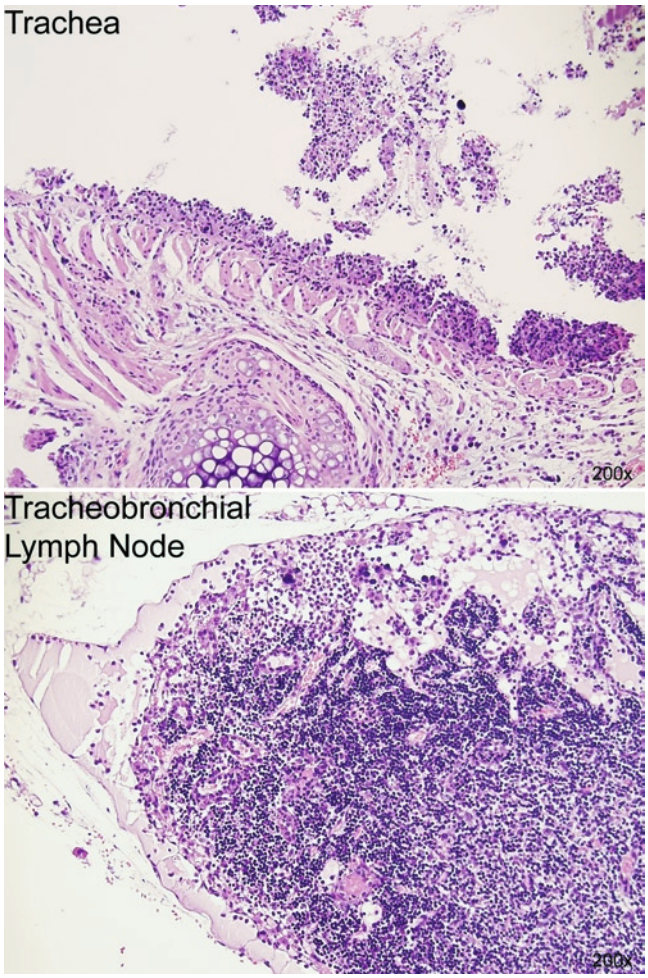

Fig. 13.3 Photomicrograph of *B. pseudomallei*-infected trachea. Formalin-fixed/paraffinized trachea (Top panel), and associated tracheobronchial lymph node (Bottom panel) were H&E stained and imaged at 200x

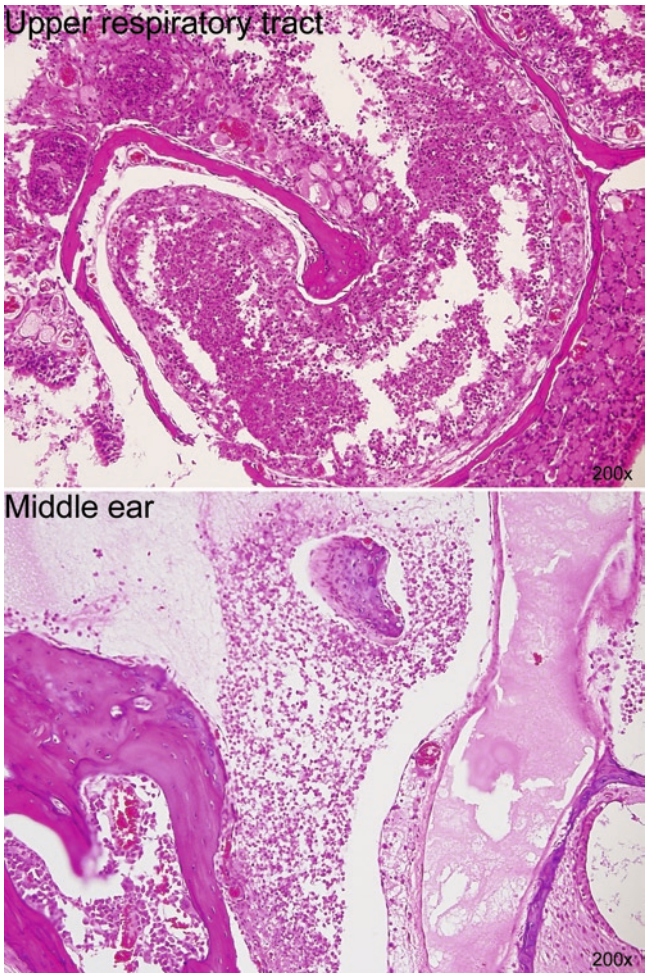

Fig. 13.4 Photomicrograph of *B. pseudomallei*-infected respiratory tract and middle ear. Formalin-fixed skull was decalcified and paraffinized before H&E staining. Sections containing the upper respiratory tract (Top panel) and the middle ear (Bottom panel) were imaged at 200x

blood-brain barrier (Fig. 13.5). Meningitis has been a recognized presentation of melioidosis in humans for several years (Woods et al., 1992); in our mouse model, neurological symptoms associated with meningitis are a common complication necessitating the euthanization of animals that had otherwise survived acute respiratory melioidosis.

Immunohistochemical analyses are also being conducted in this laboratory in order to identify specific host cell types infected with *B. pseudomallei*. Polyclonal antibodies were produced against paraformaldehyde-fixed bacteria, and these antibodies have been successfully used to detect *B. pseudomallei* in murine lung tissue (Fig. 13.6). Extracellular *B. pseudomallei* are found in close association with the epithelial cells of bronchioles with infrequent infection of epithelial cells (Fig. 13.6, Bottom panel). Epithelial cell invasion may serve as the primary route of entry into the lung, from which *B. pseudomallei* infect the entire lung, with elevated levels of bacteria found in association with lesions (Fig. 13.6, Top panel). The elevated association of *B. pseudomallei* with pulmonary lesions has also been observed in human tissues (Wong et al., 1996).

These findings indicate that the murine respiratory infection model represents an excellent method for modeling clinical symptoms of melioidosis and glanders, which will facilitate future research to study host-pathogen interactions and therapeutics.

Acknowledgements We would like to thank Don Gardner, Dan Long, Rebecca Rosenke, and Juraj Kabat for their excellent support with the histology studies.

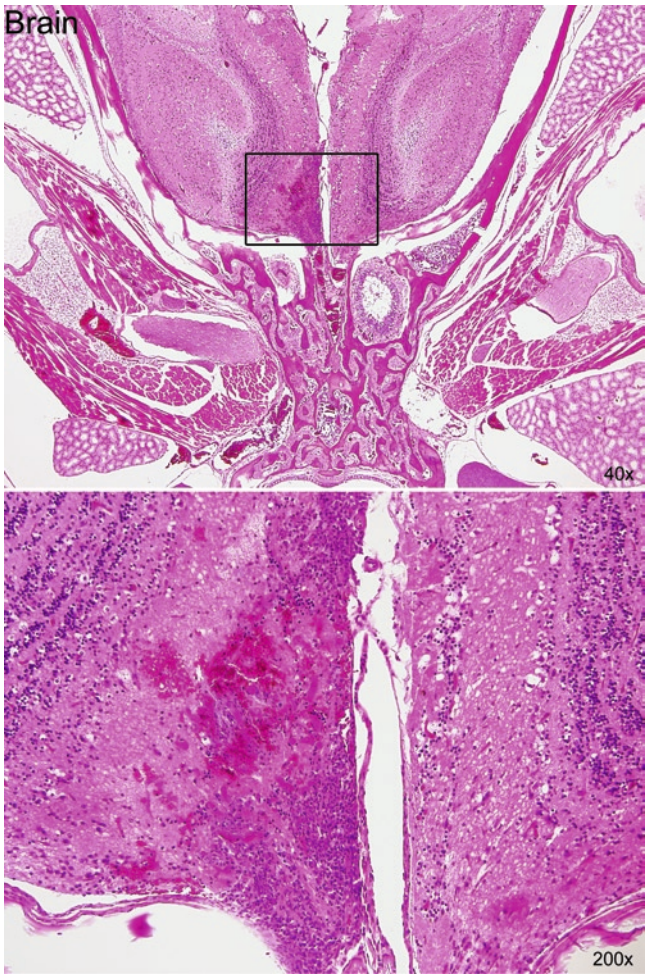

Fig. 13.5 Photomicrograph of *B. pseudomallei*-infected olfactory bulbs. Formalin-fixed skull was decalcified and paraffinized before H&E staining. Sections containing the olfactory bulb were imaged at 40x (Top panel) or 200x (Bottom panel)

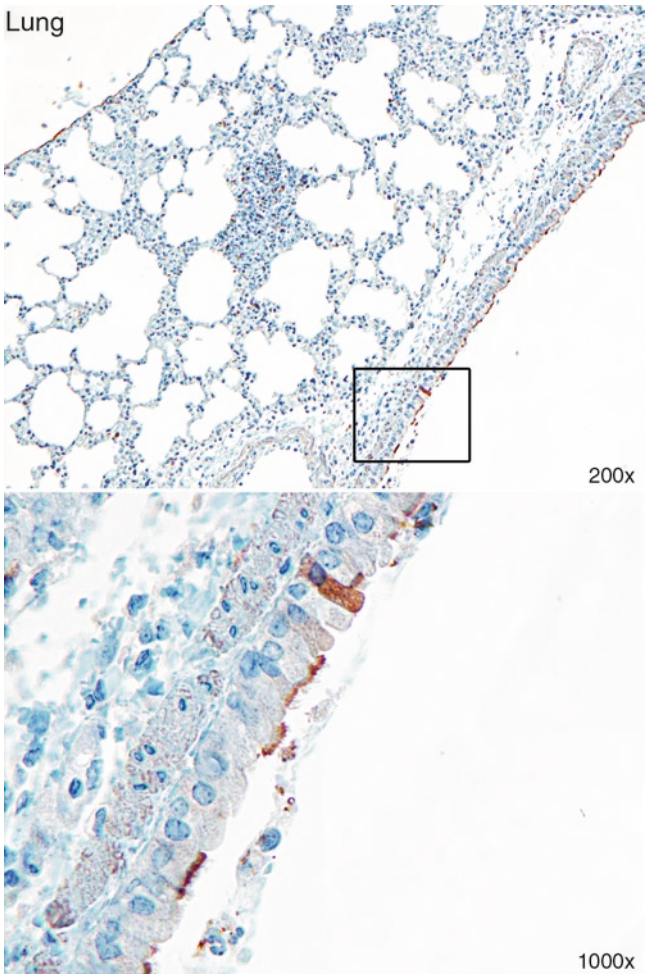

Fig. 13.6 Immunohistochemistry of *B. pseudomallei*-infected lung tissue. Formalin-fixed/paraffinized lung tissue was stained with hematoxlin and a rabbit α-*B. pseudomallei* polyclonal antibody, followed by secondary antibody and DAB detection. Samples were imaged at 200x (Top panel) or 1000x (Bottom panel)

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Part IV
Microbiology: Mycology

Chapter 14
Cryptococcosis: From Discovering the Natural Reservoir of its Etiology to the Genetic Manipulation of *Cryptococcus neoformans*: Milestones in Cryptococcal Research by Intramural Investigators at NIAID

K.J. Kwon-Chung and John E. Bennett

14.1 Introduction

Cryptococcosis is one of the most serious fungal infection encountered worldwide. The etiologic agent is *Cryptococcus neoformans,* an environmental yeast species which initiates the infection after inhalation of the organism by the host. Although *C. neoformans* can cause lesions in almost every organ, the most common clinical manifestation, as well as the most common cause of death, is meningoencephalitis, an infection of the brain. Untreated cryptococcal meningoencephalitis is fatal, and death can result in as little as two weeks from the onset of symptoms or the symptoms may last for up to 13 years before death [1]. Despite the most advanced medical treatment, the fatality rate for cryptococcosis is close to 25% [1]. While cryptococcosis sporadically occurs in normal populations without any underlying condition, the disease primarily affects immunocompromised individuals, especially those with T-lymphocyte depletion or dysfunction such as is the case with HIV-1 patients. In fact, cryptococcosis was designated as the AIDS-defining opportunistic infection, as the HIV-1 epidemic became widely known since the mid 1980s. Infections with *C. neoformans* have significantly declined in the developed world as a result of antiretroviral therapy that has reduced the reservoir of immunosuppressed HIV-1 infected patient. However, due to an explosion of AIDS-associated cryptococcosis cases in developing countries, the disease still causes 780,000 deaths in the world annually [2].

Cryptococcosis was first diagnosed in Germany, in 1894, by Busse and Bushke. However, the environmental source of the pathogen remained unknown until 1951 when Dr. Chester Emmons, the Head of the Medical Mycology Section at the National Institute of Allergy and Infectious Diseases in Bethesda, isolated the fungus from soil [3] and also found it to be enriched in pigeon guanos [4]. Since Dr. Emmons' seminal discovery of the environmental source of *C. neoformans*, milestone discoveries, on various aspects of the disease as well as on the pathobiology of the causative agent, were made by the NIAID intramural researchers, which assisted the field of cryptococcal research to transition into the 21st century.

In this paper, from among the many seminal discoveries made in the field of Medical Mycology by the NIAID intramural research team, we have focused on the landmark achievements regarding cryptococcosis, and its etiologic agent.

14.2 Identification of the *Cryptococcus neoformans* Ecological Niche

Cryptococcosis was first diagnosed in 1894 in Germany [5] by a pathologist; in the same year, the first isolation of its etiologic agent, *C. neoformans*, was also reported in Italy by Sanfelice, a mycologist, who cultured the fungus from peach juice [6]. The natural source of *C. neoformans,* however, was unknown, since the isolation of a *C. neoformans* strain from peach juice in Italy was an isolated incident that could not be reproduced. Nearly sixty years later, the late Dr. Chester Emmons, an intramural medical mycologist at NIAID, discovered the primary ecological niche of *C. neoformans.*

In 1936, Dr. Chester W. Emmons (Fig. 14.1) started his service as a senior mycologist at the National Institutes of Health before the NIAID was established within the NIH. As the first medical mycologist at NIH, he began to seek out the site of natural occurrence of pathogenic fungi and determine the role of saprobic activity in the epidemiology of mycosis. By the time he became Chief of the Medical Mycology Section, Laboratory of Infectious Diseases, NIAID in 1961, he was successful in identifying the environmental sources of many etiologic agents of systemic as well as superficial mycoses. His first success in identifying the saprobic source among the agents of systemic mycosis was the isolation of *Histoplasma capsulatum,* the etiologic agent of histoplasmosis, from the soil in 1949 [7], and subsequently he found the association of *H. capsulatum* with bats in 1958 [8]. In 1951, he identified the soil as the environmental source of *Cryptococcus neoformans* [3]. Unlike the other systemic mycosis agents, he noted the close association of *C. neoformans* with pigeon guanos [4, 9]. Since then, the pigeon droppings and soil contaminated

V.St. Georgiev (ed.), *National Institute of Allergy and Infectious Diseases, NIH: Volume 3: Intramural Research*, Infectious Disease,
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Fig. 14.1 Chester W. Emmons (1900–1985). He was the first medical mycologist at NIH and Head of the Medical Mycology Section at NIAID. Dr. Emmons discovered the environmental niche of *Cryptoccocus neformans* in 1951

with the avian guanos became known as the primary ecological niche of the fungus throughout the world [1, 10]. Presently, it is widely recognized that the fungus can also be isolated from old hollows of various species of trees, especially in the geographic regions with a mild climate such as South East Asia, South America, and Vancouver Island [11, 13].

14.3 Treatment of Cryptococcosis

Discovery of nystatin, which prompted the discovery of the amphotericin B, permanently changed the diagnosis and treatment of cryptococcosis. Much of the early work on amphotericin B was done in NIAID, initially called the Laboratory of Clinical Investigation (LCI), under the direction of Vernon Knight, M.D., who was both laboratory chief and Clinical Director, and John P. Utz, chief of the Infectious Disease Section of LCI. Initial studies with amphotericin B were not very encouraging because early formulations gave low systemic exposure. A report in 1958 describing 10 cryptococcosis patients treated with oral amphotericin B and particulate suspension given intravenously was discouraging [14]. However, oral absorption was found to be poor and intravenous particulate suspensions, then called "crystalline" drug, provided neither toxicity nor efficacy, due to rapid clearance from the blood stream [15, 16]. Colloidal amphotericin B, introduced into clinical trials in 1957, became the first effective treatment of cryptococcosis. By 1964, Butler and colleagues in NIAID were able to report that 31 of 36 patients with cryptococcal meningitis had responded favorably and 55% were considered cured at one year post therapy [17]. Colloidal amphotericin B proved to be effective but so toxic that many clinicians were afraid to use it [18]. In dogs, amphotericin B caused significant renal tubular damage and, with rapid infusion, released potassium from tissues into the circulation, leading to fatal ventricular fibrillation [19, 20, 21]. This cautionary experience in dogs was followed by an analysis of 81 amphotericin B-treated patients, showing for the first time that temporary azotemia during therapy could be managed and did not predict the residual renal damage, which correlated with total dose [22]. Acute febrile reactions were common and found in a double-blind randomized clinical trial to be diminished by coadministration of hydrocortisone 25 mg [23]. Use of hydrocortisone became the standard of practice for patients with chills and fever post infusion. Hypokalemia and normocytic anemia were also usual during prolonged therapy [21, 24]. Anemia from amphotericin B was found to be due to suppression of erythropoietin production by the kidney [25]. Slow resolution of amphotericin B toxicity in patients was found to be explicable by slow build up of drug in the body tissue and slow release of drug at the end of therapy, with a terminal elimination half life from the bloodstream of 15 days [26, 27].

By an analysis of 111 patients in LCI, elucidating the prognostic factors that determined outcome, clinicians were advised what to expect from amphotericin B treatment of cryptococcal meningitis [28]. Keeping in mind that a substantial number of patients responded slowly or incompletely to treatment, efforts were made to understand failures and to improve outcome. Ability of *C. neoformans* to become more resistant to amphotericin B, during prolonged, but unsuccessful, therapy of cryptococcosis was shown for the first time, using a sensitive photometric assay and isolates from 15 patients with positive cultures during treatment [29]. Studies of amphotericin B pharmacology found that the drug did not penetrate into the cerebrospinal fluid. Instillation of amphotericin B into the lumbar subarachnoid space was used but the chemical arachnoiditis caused excrutiating pain and leg weakness in some patients [21]. Working with the neurosurgeon, Dr. Ayoub Ommaya, a subcutaneous reservoir was devised that permitted intraventricular administration of amphotericin B. Use was eventually terminated when analysis showed that incorrect insertion occurred in 9 of 23 patients, contributing to death in 3 patients and neurologic deterioration in another four. Nine patients encountered 10 bacterial infections of the device, leading to removal in 5 [30]. Intraventricular therapy was considered successful in only 3 of 15 patients with cryptococcal meningitis but 3 of the 4 patients with coccidioidal meningitis. Subsequently, this device continued in use for coccidioidal but not cryptococcal meningitis. The reservoir was studied by the National Cancer Institute and found useful for intaventricular chemotherapy of leukemic meningitis.

Studies at LCI then turned to a recently discovered drug, flucytosine. This drug exhibited modest efficacy in cryptococcal meningitis and penetrated well into the CSF [31, 32]. Used alone, however, flucytosine was less effective than amphotericin B and drug resistance arose during treatment of some patients [33, 34]. Several studies, including those in LCI, prompted a clinical trial of flucytosine in combination with amphotericin B.

The combination of flucytosine and amphotericin B was found to be at least additive in a murine model of disseminated cryptococcosis, in a novel design that combined fractional amounts of the PD_{50} (dose protecting 50% of the animals) of each drug to determine whether the total equaled or exceeded the PD_{50} (35). A multicenter randomized open clinical trial found that low dose amphotericin B plus flucytosine was less toxic and at least as effective in cryptococcal meningitis as higher dose intravenous amphotericin B [36]. Clearance of cryptococci from the CSF was dramatically more rapid with the combination, a finding that has been duplicated in subsequent studies. The investigators conducting this trial became the NIAID-funded Mycoses Study Group, a clinical trial group that over the next two decades continued to perform clinical trials of cryptococcal meningitis [37].

Despite the advantages of adding flucytosine to amphotericin B therapy for cryptococcal meningitis, the drug could cause serious bone marrow suppression, colitis, and hepatotoxicity [36]. A bioassay and eventually HPLC and chemical assays [38, 39] for flucytosine were developed at NIAID, showing that bone marrow suppression and colitis were related to high flucytosine blood levels and that monitoring these levels could largely prevent this toxicity. Conversion of flucytosine to fluorouracil, a potent antimetabolite, by colonic bacteria seemed to explain the toxicity [40].

Referral of patients to LCI for treatment of cryptococcal meningitis resulted in a broad experience with that disease and clinical descriptions which expanded the clinical literature. Chronic endobronchial colonization with *C. neoformans* without clinical disease was documented for the first time [41]. Clinical description and management options were reported for bilateral blindness occurring in patients with increased intracerebral pressure [42]. Clinical and imaging differences could distinguish between patients having increased pressure and those with hydrocephalus. Those with hydrocephalus improved with CSF shunting [43]. When MRI was introduced for brain imaging, intracerebral cryptococcal lesions were causing concern because of their persistence during otherwise successful therapy. Analysis of the LCI experience allayed that concern by showing the benign nature of persistent lesions [44].

14.4 Cryptococcal Polysaccharide (CPS) and Its Role in Host Defense

Diagnosis of cryptococcosis changed from an autopsy curiosity to a necessity when effective therapy became available. Early studies in NIAID used complement fixation as a diagnostic test to detect cryptococcal polysaccharide in patients [45, 46]. The test was also used to measure CPS in the CSF and blood after injection of CPS into the cisterna magna of rabbits. CPS rapidly passed out of the CSF into the blood, where elimination required six weeks to occur [45]. This observation explained why serum CPS titers fell much more gradually in blood than they did in CSF during treatment of cryptococcal meningitis. Ability of intravenously injected CPS to increase mortality in mice with experimentally induced cryptococcosis was reported [47]. but unexplained until later studies found that CPS injection depleted serum complement [48, 49]. Depletion followed activation, largely by the alternative complement pathway [48]. Complement was found to play an essential role in host defense against cryptococcosis outside the CNS [50]; cryptococcal sepsis in patients and guinea pigs markedly decreased serum complement, the presence of which was required for opsonization and oxidative killing by neutrophils and monocytes [49, 51]. Human macrophages obtained by culturing monocytes differed from neutrophils and fresh monocytes in that complement was not required for phagocytosis and the cryptococci were not killed after ingestion, but rather they grew and killed the macrophages [52]. The role of complement was confined to areas of the body which blood and neutrophils were abundant but not in the central nervous system. Macrophages in their resting state had no demonstrable ability to arrest the growth of cryptococci.

While CPS appeared in patient body fluids, antibody against CPS did not appear during recovery, even using very sensitive methods [53]. Infection caused immune tolerance, in that 8 patients cured of their cryptococcosis made very little antibody following injection with CPS, although 10 healthy volunteers had brisk responses [53]. Failure of antibody secretion following immunization could not only be demonstrated in the blood, but it also could be demonstrated in B cells isolated from peripheral blood [54]. Failure of antibody production could represent an important weakness of host defense. Although purified CPS itself is weakly immunogenic in mice, studies in collaboration with Drs. John Robbins and Rachel Schneerson showed that high titer antibody could be produced in mice by a CPS-tetanus toxoid conjugate [55]. In unpublished studies, antibodies produced in mice or human volunteers by the CPS-tetanus toxoid conjugate protected mice from experimental infection. Further experiments went on to show that HIV-infected volunteers also responded to this conjugate, raising the possibility of protecting HIV patients from cryptococcosis by immunization, particularly in areas where highly active antiretroviral therapy is not available.

14.5 Serotyping Cryptococci

The discovery by E. E. Evans of three capsular serotype in *C. neoformans* prompted serotyping of isolates collected by LCI. A fourth serotype, designated D, and a double AD serotype,

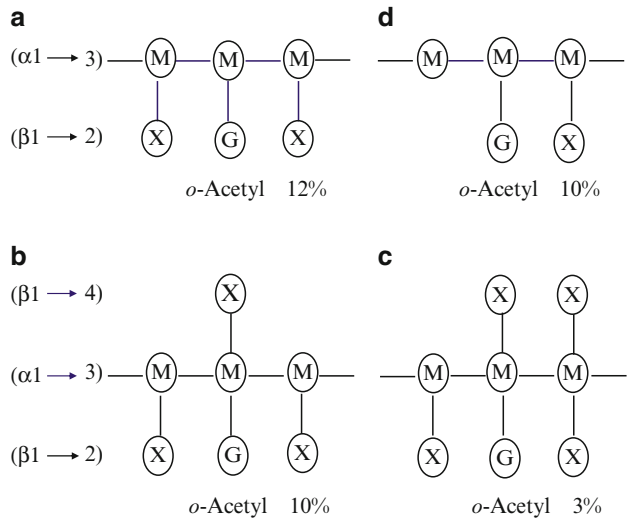

Fig. 14.2 Chemical structures of capsular polysaccharides isolated from *C. neoformans* strains of four serotypes

were described using cross absorbed rabbit antibody in an agglutinin reactions [56, 57]. In 89 isolates, 5 were designated serotype D and another 4 A-D. The structures of the four polysaccharides were described in work done with chemists elsewhere in NIH [58] (Fig. 14.2). Serotyping lead to discovery of biochemical differences and geographic differences between serotypes, as described elsewhere in this paper, as is the correlation between serotype and varietal status [59, 60].

14.6 Delayed Hypersensitivity to Cryptococcosis

A skin test antigen was developed with the hope of using delayed hypersensitivity to detect subclinical exposure to *C. neoformans* as well as to test hypersensitivity in patients with active or cured cryptococcosis. Impaired delayed hypersensitivity to cryptococcal antigen was shown using lymphocyte proliferative responses in peripheral blood lymphocytes and skin testing with an experimental antigen [61, 62, 63]. The skin test antigen, called cryptococcin and prepared at LCI, had a 10 kDa protein as the major immunogenic component [64]. CPS did not elicit delayed hypersensitivity. The skin test reaction to cryptococcin 1:100 was positive in 7 of 25 pigeon fanciers who had *C. neoformans* isolated from their pigeon coops, although none had clinical cryptococcosis. Only one of 38 volunteers from the same town had a positive skin test [65]. In a group of 21 college volunteers, none had a positive skin test. In patients with cryptococcosis, 5 of 12 with active disease and 13 of 26 cured patients had a positive skin test [62]. As measured by these assays, exposure to *C. neoforman* in the environment can elicit delayed hypersensitivity but cryptococcal meningitis elicits a poor delayed hypersensitivity response and the response, when present, is not to CPS but to cryptococcal protein. In keeping with this interpretation, an unpublished study in guinea pigs sensitized by cryptococci in complete Freund's adjuvant and tested with cryptococcin from all four serotypes found no distinction between serotypes in skin test hypersensitivity reactions. Further development of this antigen for epidemiologic studies was halted because of evidence of cross reactivity with other mycoses [61, 62].

14.7 Discovery of Sexual Life Cycle in *C. neoformans*: Opening the Road for Genetic Analysis

For over 80 years since the discovery of cryptococcosis and *Cryptococcus neoformans* in 1894, the fungus was considered to be a haploid yeast that reproduced only by asexual budding [66]. The lack of sexual life cycle in the fungus did not allow the conclusive establishment of its systematic position in the Kingdom FUNGI since the classification of the three major phyla, Zygomycota, Ascomycota, and Basidiomycota was based on the morphology of their sexual states up until the phylogenetic classification based on DNA sequence became available. In 1975, Kwon-Chung studied a large number of *C. neoformans* strains collected and serotyped by Bennett and discovered that *C. neoformans* was a bipolar heterothallic sexual fungus that occurred either of the two mating types *MAT*α or *MATa* (Fig 14.3; A. Life Cycle) [67, 68].

The discovery of an obligate mating system and the sexual life cycle of *C. neoformans* allowed placement of the fungus in its correct taxonomic position in the Kingdom FUNGI for the first time. The sexual fruiting bodies (Fig 14.3) produced by the agent of cryptococcosis unequivocally placed the fungus in the Phylum Basidiomycota closely with jelly fungi. Furthermore, the sexual state revealed two distinct species among different strains of cryptococcosis agent that are antigenically distinguishable as strains of serotype A and D in one species and strains of serotype B and C for another species [69]. The first species was named *Filobasidiella neoformans* [67], and the second species was named *F. bacillispora* [69], which denoted the bacillary shape of the sexual spores that distinguishes it from *F. neoformans* which produces globose to oval shaped spores [67].

The most significant outcome relative to discovery of the heterothallic sexual cycle was the opening of the path to recombinational analysis which enabled classical genetic research. Among the first breakthrough of such an

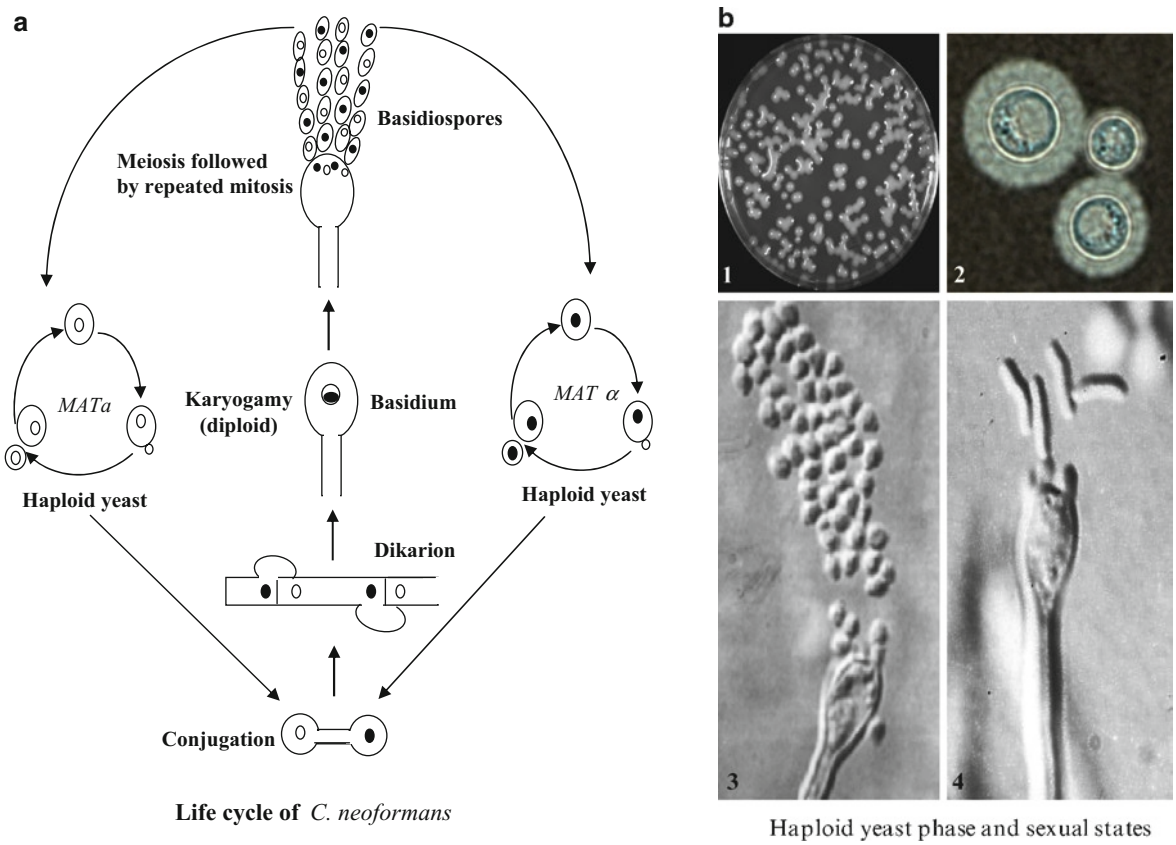

Fig.14.3 Life cycle of *C. neoformans*. *A*: Drawing of the life cycle showing the asexual yeast state and sexual filamentous state which is initiated by the conjugation of *MATa* and *MATα* yeast cells. *B-1*: Mucoid colonies of *C. neoformans* grown on agar media. *B-2*: India-ink stained *C. neoformans* yeast cells showing the thick extracellular polysaccharide capsule. *B-3* and *B-4*: sexual spores produced by mating between *MATa* and *MATα* cells of *C. neoformans* (serotype A,D) and of *C. gattii* (serotype B,C) respectively [copyright: *FEMS Yeast Res.*, **6**, 574–587, 2006]

analysis, has been the determination that capsule and melanin formation are major virulence determinants of *C. neoformans* (Fig. 14.4) [70, 71]. Molecular cloning of the laccase and capsule genes was accomplished soon after we developed the DNA-mediated transformation system in 1990s and confirmed the importance of these genes at the molecular level [72]. Since then, melanin and capsule have become one of the most active subjects of molecular pathobiological research in the cryptococcal field [73, 74, 75, 76, 77, 78].

Discovery of the sexual state in *C. neoformans* also allowed the determination of the role of mating type in the epidemiology of cryptococcosis. Unlike most other heterothallic fungi, strains of the *MATα* type drastically outnumber those of the *MATa* type in nature resulting in a majority of cryptococcosis cases to be caused by *MATα* strains [79]. Contrary to the skewed ratio between the two mating types among environmental and clinical strains, the *MATa* and *MATα* progeny, that results from the mating between two sexually compatible strains are 1:1 in ratio with no differences in survival under various laboratory growth conditions [80]. This suggested that *MATα* strains are better fit to survive in nature than the strains of the *MATa* type. In order to study the role of mating type with respect to the virulence of *C. neoformans*, an isogenic set of *MATα* and *MATa* strains was constructed for serotype D strain in collaboration with Jeff Edman at the University of California in San Francisco [81]. Analysis of the virulence among the *MATa* and *MATα* isogenic strains as well as five each of their *MATa* and *MATα* progeny showed significantly higher virulence in the *MATα* strains [81]. This study fostered mapping of the *MAT* loci and a molecular dissection of the genes located at the *MAT* locus which revealed a striking difference from the other known fungal mating type loci. Unlike the *MAT* loci of other fungal species, the *MATα* and *MATa* loci of *C. neoformans* contained mating type specific mitogen-activated protein kinase cascade homologs in addition to pheromone and pheromone receptor genes [82]. Further sequencing of the loci revealed that the *MAT* locus spans >100 kb and contains more than 20 genes, some of which function in sexual development and virulence while others have no clear link to either. The *MATα* and *MATa* alleles

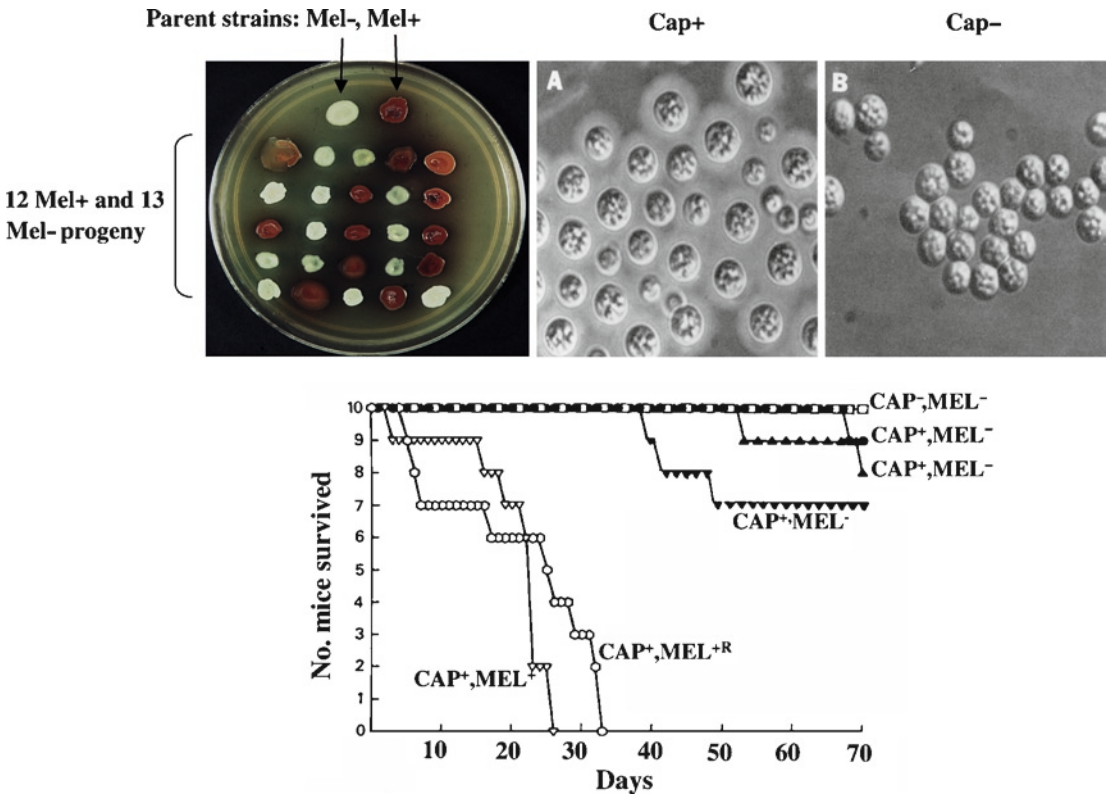

Fig.14.4 The first set of *C. neoformans* virulence factors, melanin and capsule, identified by recombinational analysis in mid 1980s [copyright: American Society for Microbiology, *Infect Immun*, **51**, 218–223, (1986)]. Upper left panel shows 12 melanin positive and 13 melanin negative progeny produced by a cross between a melanin negative mutant and a melanin positive wild type strain. Panels A and B show yeast cells of CAP^+ and CAP^- strains. Lower panel shows the survival of mice infected with progeny exhibiting different capsule and melanin phenotypes produced by a cross between a CAP^+, MEL^+ and a CAP^-, MEL^- strain. MEL^+R denotes a MEL^- strain reverted to MEL^+ phenotype

were found to have been rearranged extensively which suppressed recombination across the entire region [83]. It was also found that the *MAT* loci are flanked by recombination hotspots that could have driven several key events during the assembly and evolution of the *MAT* loci (84). The unique arrangement of the cryptococcal *MAT* locus has fostered an in-depth comparison of the mating type loci of fungi and algae which have revealed features that are shared with mammalian X and Y chromosomes. Evolution of a fungal sex chromosome has been suggested based on these studies [84, 85].

The construction of an isogenic set served as a molecular and genetic landmark for the progress made in the identification of virulence derterminants and pathobiology of *C. neoformans* [81]. Not only was the initial research on molecular genetic characterization of virulence determinants carried out using this set by almost every cryptococcal laboratory, but the *MATα* strain (JEC-21) of this set along with its ancestral strain B-3501A were also chosen to be sequenced as the representative of the *C. neoformans* genome. Availability of these *C. neoformans* genome sequences since 2004 [86] to laboratories worldwide has catapulted the cryptococcal field into the state of the art research of the 21st century.

14.8 Epidemiological Differences Between the Strains of A,D Versus B,C Serotypes

The antigenic heterogeneity among the strains of *C. neoformans* represented by four serotypes, A to D, had received relatively little attention during the first 20 years since Evans' report in 1949 [87]. Discovery of the two different sexual forms among the four serotypes in mid 1970s, however, raised significant interest in the biological differences between the strains of serotype A, D, and those of serotype B and C. The distinct morphology of *F. neoformans*, the sexual state produced by strains of serotype A, D and *F. bacillispora* produced by strains of serotype B and C suggested that their difference was not limited to the shape of sexual spores and chemical compositions of the polysaccharides produced in their haploid states. Soon after the discovery of sexual life cycle, we began comparative studies of the

two serogroups with respect to their biochemistry, ecology, epidemiology and genetics. The studies showed that strains of the two serogroups did not even co-habit in nature. While strains of serotype A and D are found primarily in pigeon droppings [1], the ecological niche of serotype B and C strains remained unknown until 1990 [11]. It has been shown that the patterns of carbon source and creatinine utilization also differed between the two groups [60]. Based on their biochemical difference, we proposed to classify the two groups as two varieties of *C. neoformans*: *C. neoformans* var. *neoformans* for the strains of serotypes A, D and *C. neoformans* var. *gattii* for the strains of serotype B and C [88]. This new classification scheme was widely accepted soon after our proposal. Further analysis revealed that the two varieties were also different in their epidemiology: All the clinical strains of *C. neoformans* var. *gattii* studied had originated from tropical to subtropical regions such as Africa, South East Asia, Central and South America and South Western United States while none were from geographical regions that were temperate or extremely cold. Occurrence of the *C. neoformans* var. *neoformans* strains, however, showed no relationship with climate [59, 89,90,91]. These studies have stimulated investigators in endemic regions of the *C. neoformans* var. *gattii* to compare clinical features of the patients infected by the strains of two varieties. Analysis of the clinical features in a large population of patients infected by strains of serotypes B in Australia revealed that all cases of infection due to *C. neoformans* var. *gattii* occurred in healthy hosts while the majority of *C. neoformans* var. *neoformans* infection occurred in immunocompromised hosts [92]. The predilection of *C. neoformans* var. *gattii* with immunocompetent patients was also recognized in other parts of the world where strains of *C. neoformans* var. *gattii* are endemic [89].

14.9 Formulation of a Single-Step Diagnostic Media to Distinguish between the Two Sero-Groups (A,D/B,C) of *C. neoformans* and Its Impact on Epidemiological and Ecological Studies

Genetic differences between *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* were confirmed by the lack of recombinants among the progenies of the mating between strains of the two varieties. Although about 30% of the progeny obtained by crossing the strains of serotype B with serotype D were viable, all were either diploid or of the parental D type [88, 93]. Based on these differences, strains of the two varieties were eventually classified into two species: *C. neoformans* for the strains of serotype A and D and *C. gattii*

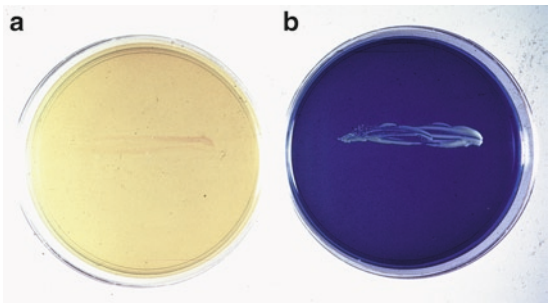

Fig.14.5 Canavanine-glucose-bromthymol blue agar, a one-step diagnostic media for the identification of **a**, *C. neoformans* (A and D serotypes) and **b**, *C. gattii* (B and C serotypes) strains [copyright: *FEMS Yeast Res*, **6**, 574–587, (2006)]

for the strains of serotype B and C [93, 94]. Subsequent multilocus sequence typing of the strains belonging to the two species supported the new taxonomic classification [95]. Since identification of the two species by immunological or genetic methods is labor intensive and not cost effective, there was an urgent need for a simple one-step diagnostic method to differentiate between the two species. We had formulated a few one-step diagnostic media based on the major biochemical differences between the strains of the two species. Of those, the canavanine glycine bromthymol blue (CGB) agar media (Fig. 14.5) was the most reliable and reproducible with no false positive or negative reactions [96]. The CGB agar formulation allowed two milestone discoveries in cryptococcal field. First, utilization of CGB agar allowed the screening of large numbers of environmental strains of *Cryptococcus.* Utilization of CGB agar led to the discovery of the ecological niche of *C. gattii,* which had been an enigma for 95 years, since the first human case of infection caused by the yeast, now known as *C. gattii,* was reported by Curtis in 1895 [97]. In 1990, Australian investigators discovered *C. gattii* to be associated with *Eucalyptus* trees in nature [11]. Association of *C. gattii* with *Eucalyptus* and other trees have been confirmed in many parts of the world. Second, CGB screening of cryptococcal strains isolated from AIDS patients residing in the American continent and Europe revealed that *C. gattii* rarely caused cryptococcosis in HIV-1 infected patients, even in areas where *C. gattii* is endemic [89]. This observation concurs with the predilection of *C. gattii* with otherwise healthy individuals.

14.10 Establishment of DNA-mediated Transformation in *C. neoformans*

Classic approaches for identifying candidate virulence genes often started with random mutagenesis and the selection of mutants based on desired phenotypes. Whenever possible, sexual or parasexual recombinational analysis was then

attempted to confirm co-segregation of low virulence and the mutant phenotype. Since the discovery of the heterothallic sexual life cycle in *C. neoformans*, classic approaches were taken to identify virulence factors such as capsule and melanin formation [70, 71]. However, it was practically impossible to characterize the genes encoding the polysaccharide capsule or laccase (phenoloxidase) with respect to the number of loci involved, the precise mutations in these genes and their role in giving rise to the capsule or melanin deficient phenotype by using classic recombinational analysis. Conclusive identification and characterization of virulence determinants required the introduction of defined, nonreversible mutations, specifically into the genes of interest and reintroduction of intact genes of interest into the mutants. Such research required a DNA-mediated transformation system. A transformation system had already been established in *Saccharomyces cerevisiae* before 1980 and also in other model fungi such as *Aspergillus nidulans* and *Neurospora crassa* by the mid 1980s. However, the methods used in these model fungi were not applicable to *C. neoformans,* which is phylogenetically distant. In 1990, we successfully transformed *C. neoformans* by using the electroporation system in collaboration with Dr. Jeff Edman at the University of California in San Francisco [98]. The electroporative transformation system had been successful in transforming only in plant cells and had not yet been applied to fungal cells. This landmark achievement of DNA-mediated transformation allowed the reverse genetic approaches based on gene disruption and homologous recombination with transforming DNA. The molecular genetic confirmation of the major virulence determinants such as polysaccharide capsule and melanin formation soon followed [72, 73, 74, 75]. Such achievement in recombinant DNA technology ushered the cryptococcal research field from the era of classical studies to the molecular era. Undoubtedly, the cryptococcal research field has since progressed rapidly and is now at comparable levels to those of other model fungi with respect to molecular genetic studies.

14.11 The First Fulfillment of Koch's Molecular Postulates for Fungal Virulence Factors: Molecular Genetic Proof of Polysaccharide Capsule as the Major Virulence Factor of *C. neoformans*

Cryptococcus neoformans cells are covered with a thick polysaccharide capsule composed mainly of glucoxylomannan (GXM) (Fig. 14.2) and the capsule had been suspected to be the major virulence determinant. Classical studies had shown that mice infected with acapsular mutants of *C. neoformans* created by using chemical or radiational mutagens failed to produce fatal infection [71]. Since *C. neoformans* is the only pathogenic yeast with polysaccharide capsule, probes or primers constructed based on the genomic sequences and proteins of model fungi were not applicable for the cryptococcal capsule study. Although some molecular tools utilized in the study of the bacterial capsule had been available since 1980s, these were not useful for studying the cryptococcal capsule due to fundamental differences in the capsule structure between bacteria and *Cryptococcus.* In light of these circumstances, we proceeded with complementational cloning of the capsule genes. As soon as the electroporative transformation system was established in 1990, we attempted to verify the capsule's role in virulence. A capsular mutants that had been used for classical virulence studies, were complemented with capsule genes. The avirulent mutant, *cap59*, devoid of capsule, was successfully complemented with a genomic library constructed with DNA isolated from a serotype D strain of *C. neoformans*. The transformed clones were screened for the presence of capsule and were found to contain a plasmid harboring DNA inserts essential for capsule formation (Fig. 14.6). This approach was successful in identifying the *CAP59* gene. The cloned *CAP59* gene was then used to transform the *cap59* mutant which not only produced encapsulated cells but also produced fatal infection in mice equivalent to wild type strain. It is important to note that our molecular work on the *CAP59* gene was the first fulfillment of Koch's molecular postulates for a fungal virulence factor [73].

14.12 Contribution to the Genomic Sequencing of *C. neoformans*

Saccharomyces cerevisiae was the first species in the Kingdom FUNGI to have its genome sequenced by 1996 [99]. The access to complete genomic sequences of the yeast has liberated researchers from the tedious task of conventional determinations of DNA sequences. It has further facilitated genome wide approaches for gene deletions as well as development of the DNA array technology. These developments undoubtedly have revolutionized the biological study of the organism by providing all the raw molecular information required to understand the genes, their regulation and the blueprints of life. The public genome projects in fungi soon expanded to other species of Ascomycetes such as *Aspergillus nidulans, Neurospora crassa* and the pathogenic yeast *Candida albicans*. Since *C. neoformans* is phylogenetically distant from these fungi and its pathobiology as well as life cycle are quite distinct from the ascomycetous yeasts, the need for cryptococcal genome sequencing was evident. In order to prepare a

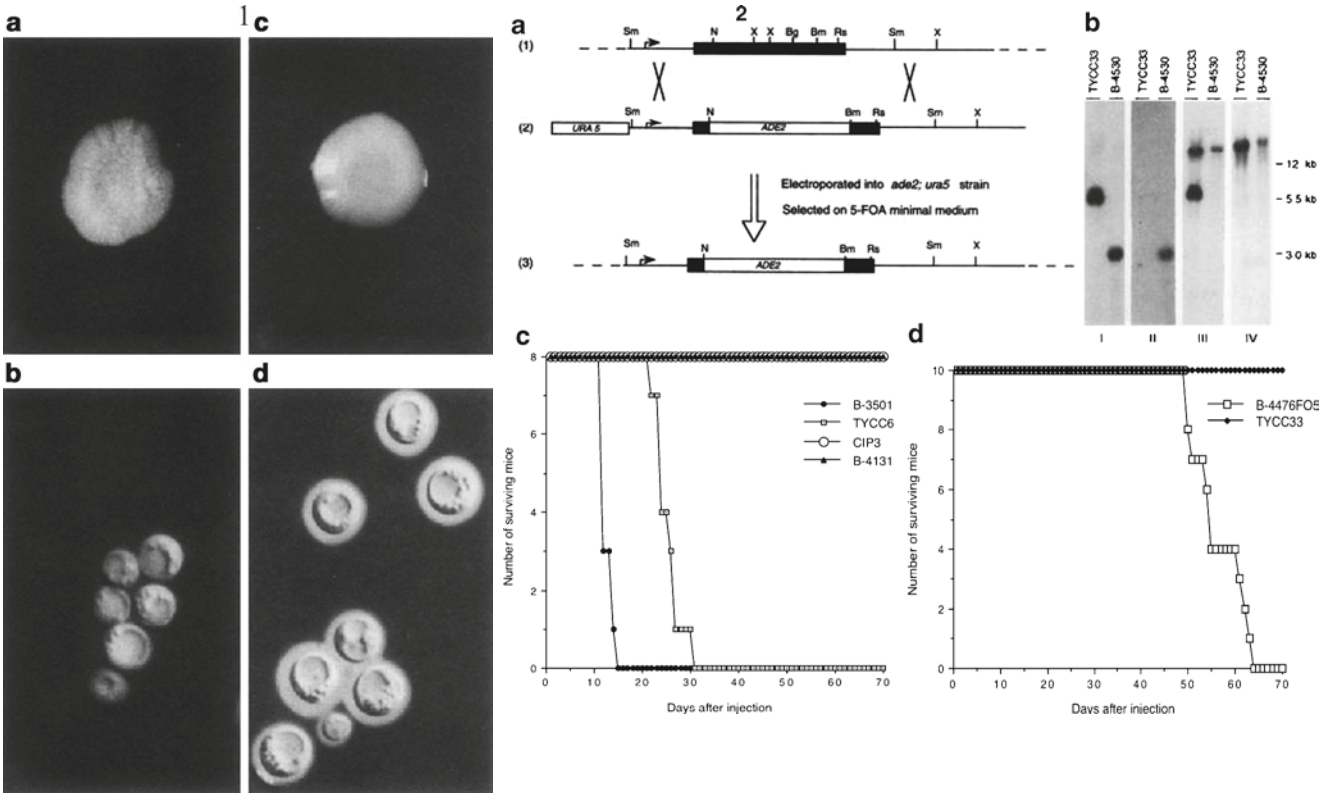

Fig.14.6 Molecular genetic manipulations of *C. neoformans* that enabled fulfillment of the Koch's molecular postulate for fungal virulence factors

1. Phenotype of an acapsular mutant and a capsule positive transformant. *1-A* and *1-B*: a dry colony and acapsular yeast cells of an acapsular mutant created by chemical mutagenesis and its acapsular yeast cells. *1-C* and *1-D*, a smooth colony and capsulated yeast cells of the acapsular mutant transformed with a genomic library.
2. Gene deletion of *CAP59* and the effect of *cap59Δ* in virulence. *2-A*: the strategy for *CAP59* deletion by gene replacement. *2-B*: confirmation of gene replacement by Southern analysis. TYCC33 and B-4530, a *cap59Δ* deleted strain and a capsule containing transformant respectively.

Panel I: hybridization of restriction enzyme digested DNAs with the SmaI-SmaI fragment shown in A(1). *Panel II*: hybridization with NcoI-BamHI fragment shown in A(2). *Panel III* and *Panel IV*: hybridization with ADE2 and URA5 gene probes respectively. The genomic library was constructed in a plasmid with *URA5* gene as a selectable marker and the transformed acapsular mutant was a *ura5* auxotroph. C and D, virulence test in a murine model. C, all mice infected with the original acapsular mutant (B-4131) and the mutant transformed with vector survived while all mice infected with the wild type (B-3501) and capsule positive transformant (TYCC6) died within 31 days after infection. D, mice infected with the capsulated strain (B-4476FO5) succumbed to fatal cryptococcosis while the *cap59*Δ strain of B-4476FOA (TYCC33) remained healthy [copyright: American Society for Microbiology, *Mol Cell Biol*, **14**, 4912–4919, (1994)].

public project on genomic sequencing of *C. neoformans,* the international cryptococcal research community met in St. Louis, MO in February 1999 and deliberated about the choice of a *C. neoformans* strain for genomic sequencing. The strain chosen for sequencing by a consensus of the meeting participants was JEC-21, a serotype D *MAT*α strain from an isogenic set constructed in our laboratory (see above). Since it had been well characterized for its mating efficiency, animal virulence, and proven suitability for recombinant DNA studies, the strain was chosen.. Sequencing was performed at The Institute for Genomic Research (presently, The J. Craig Venter Institute) and the completed genome sequence was published in Science in 2005 [86].

14.13 Establishment of the International Conference on Cryptococcus and Cryptococcosis

Investigators in the field of cryptococcosis from all over the world meet every three years to present their research data, discuss the current state of cryptococcosis therapy as well as new discoveries in basic pathobiology of *C. neoformans*. The *International Conference on Cryptococcus and Cryptococcosis* (ICCC) was founded and organized in 1989 in Jerusalem by Dr. I. Polacheck, mentored by J. Kwon-Chung, with her help along with Professor Fritz Staib in

Koch's Institute in Berlin. There was an urgent need for the investigators in the field of cryptococcosis to meet together as cryptococcosis became one of the AIDS defining opportunistic infection in mid 1980s when the HIV-1 epidemic was spreading rapidly. Since then, the ICCC has functioned as the single most important forum where cryptococcologists could interact and share their research data without any distraction from other subjects. The ICCC has been organized and held in various parts of the world including the Middle East, Europe, South East Asia, United States, and the Far East. The foundation of the ICCC has served as one of the intramural researchers' extracurricular activities contributing significantly to the progress and development in the field of cryptococcosis.

14.14 Conclusion

Through astute study, careful observation and diligent effort, the late Dr. Chester Emmons (the first medical mycologist at NIAID) discovered the natural sites of many of the pathogenic fungi that cause life-threatening systemic diseases and determined the role of saprophytic activity in the epidemiology of these pathogens. One of such fungi was *Cryptococcus neoformans*, the major agent of fungal infection of the brain world-wide. His pioneering work on *Cryptococcus* and cryptococcosis fostered the next generation of researchers at NIAID achieving landmark contributions in the progress of cryptococcal research for the past 35 years. Pioneering intramural research in the field of genetics, biochemistry, taxonomy, serology, epidemiology and the molecular biology of *Cryptococcus* and therapy of cryptococcosis described in this paper helped to bring cryptococcosis research to the present stature as a model fungus for pathobiological study.

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Part V
Microbiology: Parasitology

Chapter 15
Lessons from Parasites on CD4+ T-Cell Subset Differentiation and Function

Alan Sher and Dragana Jankovic

15.1 Introduction

Parasites are highly complex eukaryotic pathogens that represent a major cause of morbidity and mortality in humans and livestock. Endoparasites (those that invade the host) exist in two phylogenetic groups: unicellular protozoa and helminths. The interaction of parasites with the immune system has several distinguishing features that are of special interest to fundamental immunologists. Most parasitic pathogens are able to survive the initial host response and produce long-lasting chronic infections designed to promote transmission. The development of chronicity depends not only on the ability of the parasite to escape protective immune responses (*immune evasion*) but also on the generation of finely tuned mechanisms of *immunoregulation* that serve both to prevent parasite elimination and suppress host immunopathology. The study of these immunomodulatory pathways in both human and experimental parasitic infections has yielded important insights concerning the mechanisms by which T lymphocytes and cytokines control immune effector functions *in vivo*. In addition, studies in host-parasite systems have played a major role in elucidating the pathways by which CD4+ T-cells with different effector activities are triggered during antigenic encounter.

CD4+ T-helper (Th) lymphocytes are now known to consist of 3 major subsets: Th1, Th2, and Th17 that display different effector functions in both host defense and autoimmunity (Fig. 15.1). Although it is known that the cytokine environment in which naïve T cells first encounter antigen presented by antigen presenting cells (APC) is a key regulator of the polarization process, the exact in vivo mechanism by which naïve CD4+ T-cells differentiate into either Th1, Th2, and Th17 effector cells is still a matter of some controversy [1, 2]. Specifically, important questions remain to be answered as to the relative contribution that certain cell types and cytokines make in shaping the phenotype of developing T cell responses. The delineation of the signals that trigger differentiation of these CD4 subpopulations is important for understanding mechanisms of host resistance, for understanding disease pathogenesis, and for the development of interventions based on the selective induction or ablation of specific Th responses. A major approach for studying the basis of Th effector choice has been the analysis of pathogen-induced immune responses that polarize toward Th1, Th2, or Th17 dominated phenotypes [3, 4]. Such polarization is particularly striking in parasitic infection where intracellular protozoa and helminths trigger opposing and highly polarized Th1 and Th2 responses, respectively [3]. Th17 cells, which were recently defined as a unique Th subset, appear to be selectively induced by extracellular bacteria and fungi; however, this relationship is not as clear cut as the association of intracellular protozoa and helminths with Th1 and Th2 responses.

In our work, we have studied the highly polarized Th1 and Th2 responses elicited by two distinct parasites: *Toxoplasma gondii*, an intracellular apicomplexan protozoan and *Schistosoma mansoni,* a trematode helminth. Hosts infected with *T. gondii* display potent CD4+ and CD8+ T-cell derived IFN-γ production, which is necessary for control of infection and the maintenance of the parasite in a latent state [5]. Thus, IFN-γ–deficient mice are highly susceptible to infection as are mice deficient in IL-12, a cytokine that regulates IFN-γ induction and thereby is an important determinant of Th1 effector choice [6, 7]. In direct contrast, hosts infected with *Schistosoma mansoni* develop potent Th2 responses which are stimulated by parasite eggs deposited in tissues [8]. These Th2 cells help protect the host by participating in granuloma formation, a process which walls off the parasite eggs preventing tissue damage by egg toxins and by inhibiting septic-like responses triggered when eggs transit from the blood stream into the intestinal lumen. Thus, mice deficient in IL-4R signaling show decreased granuloma formation and fibrosis and in some cases succumb with cachexia-like symptoms associated with enhanced proinflammatory cytokine production [9, 10, 11].

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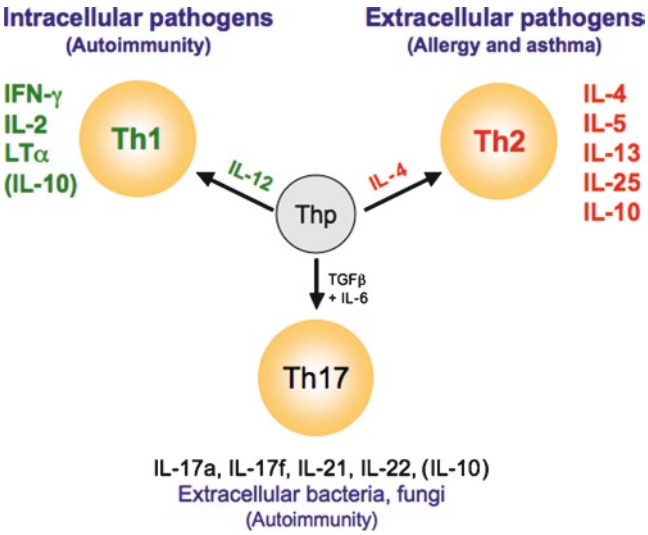

Fig. 15.1 Pathogen Induced Polarization of CD4⁺T Helper Subsets T helper (Th) differentiation is a multistep process in which naïve CD4⁺ T-lymphocytes upon activation by antigen acquire the ability to synthesize a distinct set of cytokines essential for their effector functions. There are 3 major populations of Th effectors, Th1, Th2 and Th17 which are identified by the expression of their signature cytokines IFN-γ, IL-4 and IL-17, respectively. As presented in this scheme, different types of pathogens favor the selective induction of Th1, Th2 or Th17 dominated responses. Among the many factors that play an important role in influencing Th polarization, the presence of specific polarizing cytokines (indicated next to each arrow) at the time of CD4⁺ T-cell priming is considered to be of major importance based on extensive *in vitro* studies. Nevertheless, the extent to which polarizing cytokines dictate the development of particular Th phenotypes *in vivo* is still poorly understood

15.2 Role of Cytokines in Th1 and Th2 Differentiation in Response to Parasitic Infections

Early *in vitro* studies had established key roles for the cytokines IL-12 and IL-4 in the differentiation of Th1 and Th2 cells, respectively (Fig. 15.1). Using parasitic infection of mice deficient in these cytokines or their signaling pathways, we were able to test the relevance of these functions *in vivo*. Surprisingly mice deficient in IL-4, IL-4R or STAT-6 (required for IL-4R signal transduction) when infected with *S. mansoni* developed highly significant although reduced Th2 responses [9, 12]. Similarly, mice deficient in IL-12 when infected with attenuated *T. gondii* (to avoid death of the animals) or injected with STAg (a soluble tachyzoite extract) still displayed residual Th1 responses [13]. These findings suggested that while determining the magnitude of parasite induced Th1 and Th2 cytokine production, IL-12 and IL-4 are not required for Th1 or Th2 effector choice.

15.3 Role of Antigen-Presenting Cells in Parasite Induced Th Polarization

One hypothesis of Th1/Th2 differentiation is that upon pathogen encounter APC such as dendritic cells are triggered through pattern recognition receptors (e.g., Toll-like receptors (TLR)) to deliver distinct signals to CD4⁺ T-cell precursors that determine their Th1 versus Th2 development. Strong support for this concept was obtained in *in vitro* studies employing *T. gondii* or *S. mansoni* as polarizing agents. In the system employed, we assay the effects of either STAg or SEA (a water soluble extract of *S. mansoni* eggs) to influence Th1 versus Th2 cytokine production by ovalbumin (OVA)-specific transgenic CD4⁺ T-lymphocytes following stimulation with an OVA peptide in the presence of different APC populations [14]. We found that as expected the addition of STAg to the cultures promoted a strong Th1 response (evidenced by intracellular cytokine staining (ICS) for IFN-γ) while SEA addition led to polarized Th2 cytokine production (evidenced by IL-4 expression in ICS) (Fig. 15.2). Importantly, the presence of DC was shown to be required both for T-cell polarization in this *in vitro* system [14]. In the case of the *T. gondii* induced Th1 response, DC belonging to only one subpopulation, the CD8α⁺ subset were able to trigger polarization while DC of multiple subsets could promote SEA induced polarization toward Th2 subset (unpublished data). Consistent with the *in vivo* findings discussed above, DC deficient in either IL-12 or IL-4 triggered highly reduced Th1 or Th2 polarization respectively; although in each case residual responses were observed supporting the hypothesis that these cytokines while stabilizing Th subset differentiation are not its sole determinant [14]. In additional experiments, it was shown that DC pre-incubated with STAg or SEA were also able to mediate Th polarization indicating that the DC rather than the T cells in the cultures are the major cellular targets acted upon by these extracts [14].

When the responses of purified DC to STAg or SEA was analyzed a major dichotomy was observed. Thus, while STAg exposed DC expressed highly levels of pro-inflammatory cytokines and cell surface co-stimulatory molecules [14, 15], SEA exposed DC failed to mount significant responses for the same proteins [14] and when examined by microarray analysis showed only minor differences in gene expression compared to unstimulated cells [16]. Moreover, pre-exposure to SEA actually inhibited the induction of cytokines and DC by the TLR agonist LPS [14, 16]. These findings argued that whereas Th1 differentiation by *T. gondii* requires strong stimulation of DC, Th2 polarization in direct contrast involves weak signaling in DC or even direct inhibition of DC activation.

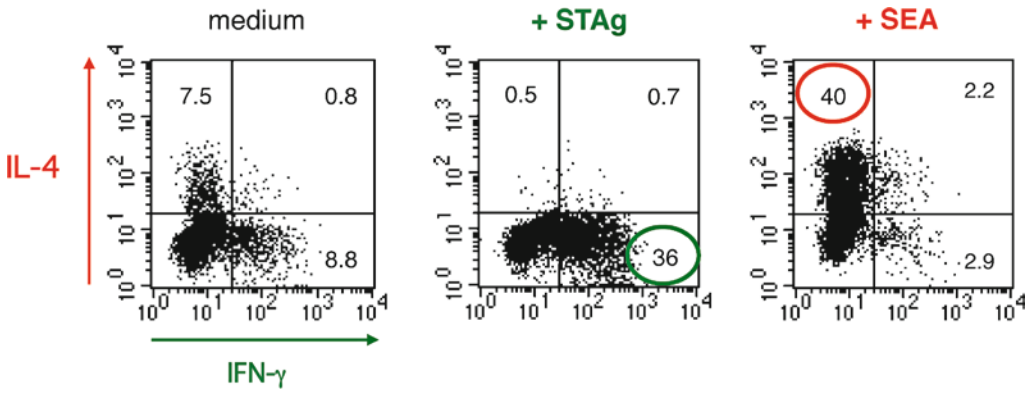

Fig. 15.2 Exposure of Dendritic Cells to Parasite Extracts Results in Induction of Polarized Th Responses to an Unrelated Ag Naive OVA-specific Tg CD4+ T-cells were stimulated with FACS-purified syngeneic splenic DC and nominal peptide in the presence of medium, STAg (a soluble *T. gondii* extract) or SEA (a soluble *S. mansoni* egg extract). Single-cell analysis of IFN-γ and IL-4 producing CD4+ T-lymphocytes in each culture was performed by intracellular cytokine staining on day 7. The FACS dot plots shown are gated on CD4+ T-cells. IFN-γ producing CD4+ T-lymphocytes are selectively induced in the presence of STAg, while SEA promotes differentiation of IL-4+ CD4+ T cells. Adapted from Jankovic, et al., [14]

15.4 Identification of Major Agonists for Th Polarization In *T. gondii* and *S. mansoni*

A long–term effort for our group has involved the identification of the agonists in STAg and SEA that trigger Th1/Th2 polarization. Because of the important role of IL-12 in Th1 induction by *T. gondii* and early studies indicating that STAg exposed DC produced high levels of this cytokine both *in vivo* and *in vitro*, we utilized IL-12 induction in splenic DC as our major assay for identifying the Th1 polarizing agonist in STAg. Although initial studies identified a CCR5 binding parasite ligand, cyclophilin, as an agonist of this response [17], the activity of the purified protein could not account stoichometrically for that of the full extract. The discovery that both host resistance to *T. gondii* and STAg induced IL-12 production by DC are dependent on the TLR adaptor molecule MyD88 suggested the existence of a major TLR agonist in STAg that might better explain its activity. Further purification resulted in the identification of such a molecule, profilin, which is a parasite protein that plays a role in parasite motility by stabilizing actin polymerization [18]. Purified native and recombinant *T. gondii* profilin potently stimulated DC IL-12 production by an MyD88 dependent signaling pathway [19]. Studies with recently developed knock–out mice for TLR 11 definitely identified this TLR as the major receptor on DC mediating profilin induced cytokine production in vitro as well as *in vivo* [19]. As expected, this signaling pathway was also shown to play a major role in Th1 polarization by STAg in the OVA TCR transgenic T cell bystander assay described above (Fig. 15.3). Together then these studies argued that Th1 differentiation in *T. gondii* infection is driven in large part by the potent stimulation of DC through TLR11 signaling resulting in both IL-12 production and costimulatory molecule expression.

In related studies, we also identified the major agonist in SEA responsible for Th2 polarization. Here, because of the lack of a more convenient and reliable read-out, it was necessary to utilize the OVA transgenic T-cell polarization in vitro assay itself for the purification of the relevant molecule. We identified the Th2-inducing component in both a water-soluble egg extract (SEA) and in secreted egg products as a 31 kD ribonuclease identical to the previously described *S. mansoni* T2 ribonuclease, omega-1 [20]. The Th2 promoting activity of this glycoprotein was sensitive to ribonuclease inhibition and did not require MyD88 or TRIF (the second major TLR adaptor) signaling in DC for its function. In common with unfractioned SEA, the purified native protein suppressed LPS-induced DC activation, but unlike SEA failed to trigger IL-4 production from basophils, another previously described Th2 associated activity of the extract. When exposed to purified omega-1, DC displayed decreased conjugate formation with CD4 T cells *in vitro,* a property associated with both reduced adherence to plastic and pronounced morphological changes, particularly in the intracellular distribution of actin. Together these observations identified omega-1 as the major egg-derived product in *S. mansoni* eggs that triggers Th2 polarizing activity in DC and provided evidence arguing that the molecule may do so by inhibiting the interaction of these APC with CD4 T-cells.

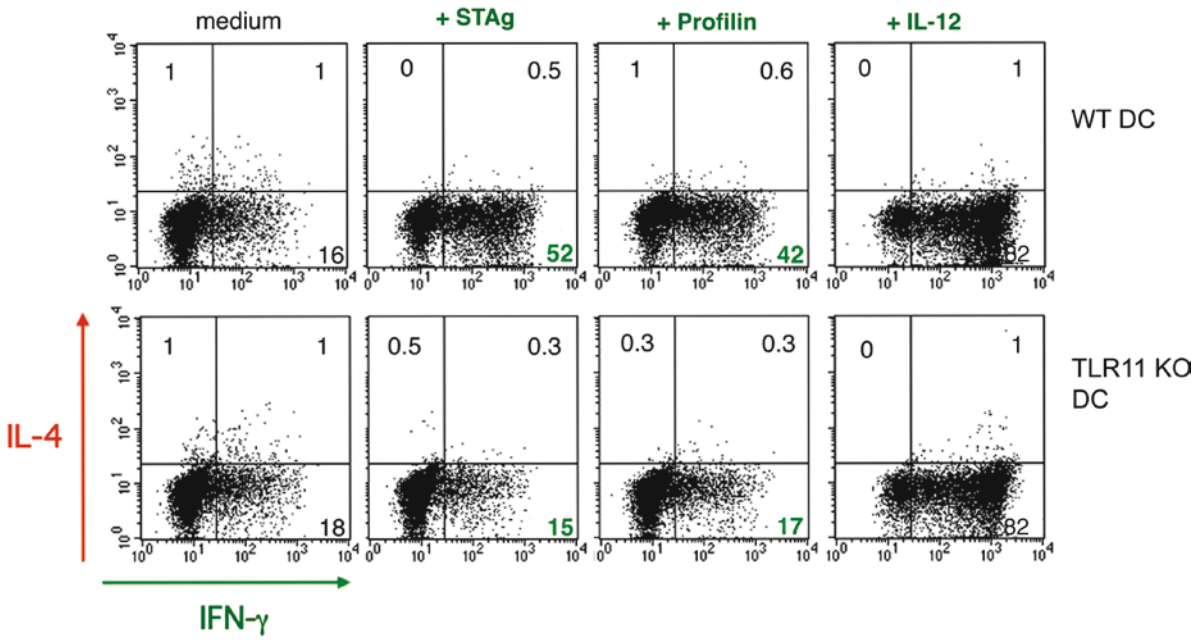

Fig. 15.3 The Th1 Polarizing Effect of both STAg and Profilin Depend on Expression of TLR11 on Dendritic Cells. FACS-purified splenic DC isolated from either wild type or TLR11-deficient mice were used to stimulate OVA-specific Tg $CD4^+$ T-lymphocytes in the presence of medium, STAg, profilin (a *T. gondii* structural protein known to be a TLR11 ligand) or IL-12. The frequency of IFN-γ^+ $CD4^+$ T-cells was determined as described in Fig. 2. The FACS dot plots shown are gated on $CD4^+$ T-cells. In the presence of exogenous IL-12 both types of DC are equally efficient in promoting Th1 differentiation. In contrast, the Th1-polarizing effects of both STAg and profilin are completely ablated when TLR11-deficient dendritic cells are substituted for wild type dendritic cells. These results demonstrate that TLR11 interaction is critical for the Th1-promoting activity of profilin in vitro and that the latter parasite protein represents the major Th1-polarizing component in STAg

15.5 General Implications of Findings for Th1/Th2 Effector Choice

The conclusion that parasite triggered Th1 differentation requires strong signalling by APC while Th2 induction involves an actual lowering of T cell-APC interaction (Fig. 15.4) is consistent with previous studies involving defined protein Ag, which agrues that the strength of signal delivered to the T cell is a major determinant of Th1/Th2 development [21]. In particular in this work, low dose antigen was found to favor Th2 development, a finding which correlated with the lower TCR signal triggered under these conditions [22]. The latter situation [specify what situation in particular you are refering to] is likely to occur in the dampened DC-T cell encounter occuring during SEA/omega -1 exposure and indeed when the responding $CD4^+$ T-lymphocytes were examined in SEA containing cultures they were found to exhibit a transient delay in cell cycling [14]. Previously, the strong induction of Th2 responses during helminth infection was thought to be inconsistent with the concept of lowered signal strength, since worm infestations result in the release of high levels of Ag which should lead instead to increased T-cell signaling. The discovery that omega-1 decrease T cell-APC encounter independent of the level of Ag exposure provides an explanation for this paradox and predicts that other helminth infections as well as common allergens may favor Th2 development by a similar mechanism.

15.6 Implications for Vaccination and Immunotherapy

The central conclusion of our studies that pathogen derived signals delivered to dendritic cells can influence Th1/Th2 polarization is highly relevant to the design of immunological interventions based on the selective induction or deviation of Th responses. For example, effective vaccination against intracellular bacteria or protozoa and most viruses is thought to depend on the priming of a highly robust Th1-type cytokine response in both $CD4^+$ and $CD8^+$ T-cells and the induction of a similar response profile can be used to prevent or treat allergic disease. In the opposing direction, biasing responses toward Th2 could promote immunity to helminths or block the induction of infection or autoimmune induced immunopathologies that rely on Th1 or Th17 responses. Our findings in the *T. gondii* and *S. mansoni* parasite models support the use of agents that target DC function as strategies

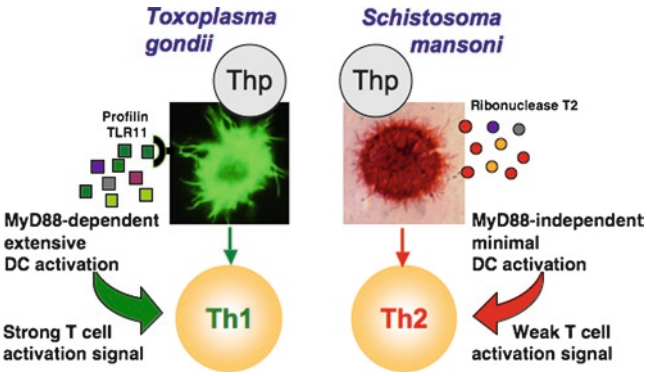

Fig. 15.4 Proposed Model for DC- Dependent Th1 vs Th2 Polarization by *T. gondii* and *S. mansoni* Eggs. Intracellular pathogens such as *T. gondii* and/or their soluble factors strongly activate DC as measured by the induction of cytokine and chemokine expression as well as the upregulation of co-stimulatory molecules. They do so through the interaction of pathogen molecules with pattern recognition receptors such as TLR that require MyD88 or TRIF dependent signalling. This stimulation results in high signal strength activation of CD4+ T-cells leading toTh1 polarization. In direct contrast, helminths and/or their products (example *S. mansoni* eggs) trigger in an MyD88/TRIF-independent fashion minimal changes in gene expression in dendritic cells. They nevertheless, induce pronounced morphological changes which result in diminished T cell contact and the delivery of a signal of lowered strength to these cells. As a consequence of being conditioned by the two contrasting microbial stimuli described, DC promote either IFN-γ or IL-4 production in Th precursors and thus, play a critical role in determining their Th1/Th2 effector phenotype. We propose that this scheme elucidated for *T. gondii* and *S. mansoni* may have general relevance for other microbial agents as well the induction of autoimmunity and allergy

for achieving these outcomes; they do so by corroborating the potent influence of TLR stimulation of DC on driving Th1 induction while at the same time revealing the potential of agents like omega-1 that act on DC by suppressing T-cell interaction for promoting Th2 responses. Moreover, if pathogenic Th2 responses induced by allergens can be shown to function through a similar mechanism, then the development of strategies specifically designed to overcoming this suppression could offer a new approach to the prevention and treatment of allergic disease. Thus, in these as well as numerous other studies carried out in the field of immunoparasitology, the manipulation of the immune system by parasitic organisms has provided important lessons for how immunologists themselves can control and direct the immune system to benefit the host.

15.7 Summary

CD4+ T-lymphocytes play a major role in host defense against pathogens and in the regulation of disease states. The functions of these cells are diverse, and they are now known to be determined by defined subsets called Th1, Th2, and Th17 based on their secretion of mediators called cytokines. Interestingly, parasitic infections often induce CD4+ T-cell responses that are highly polarized in terms of their Th1/Th2 cytokine profiles. This polarization phenomenon is particularly striking in the case of helminths, which in contrast to nearly all other pathogens routinely trigger strong Th2 responses leading to high IgE levels, eosinophilia and mastocytosis. At the opposite pole, many intracellular protozoa (in common with their bacterial counterparts) induce CD4+ T-cell responses with Th1 dominated cytokine patterns. A major goal of our work has been to decipher the functions of these T-cell subsets in parasitic disease and to understand the pathogen signals that determine their selective differentiation in helminth versus protozoan infection. Our findings are of general relevance in understanding how the immune system chooses specific effector mechanisms to respond to different external stimuli and for the design of immune interventions based on the induction of these specific pathways.

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Chapter 16
Molecular Aspects of Parasite – Vector Interactions In Leishmaniasis

David Sacks

16.1 Introduction

Leishmania are pathogenic protozoa of the order Kinetoplastida and the family Trypanosomatidae, and they are responsible for diseases affecting more than 12 million people a year throughout tropical and sub-tropical regions. *Leishmania* have a dimorphic life cycle consisting of extracellular promastigotes that multiply and develop within the alimentary tract of their sand fly vectors and intracellular amastigotes that reside and multiply within the phagolysosomal vacuoles of their host macrophages. Depending mainly upon the species of *Leishmania*, human infection can display a spectrum of clinical manifestation from localized cutaneous involvement to late destruction of mucous membranes to generalized systemic disease with fatal outcome. Distribution of the more than 20 species and sub-species of *Leishmania* and the diseases they produce are determined by the availability of competent vectors. Phlebotomine sand flies are the only known natural vectors of *Leishmania,* and of the more than 400 phlebotomine species described, fewer than fifty are known to be involved in the transmission cycle of these parasites. Furthermore, some vectors species are highly restricted to the species of *Leishmania* that they transmit in nature. This chapter reviews our vector biological studies, which over the last 20 yrs have sought to better understand the natural habitat of *Leishmania* parasites during their transformation, growth, differentiation, and migration in the alimentary tract of their competent sand fly vectors and the barriers to survival that are encountered in refractory flies. The review emphasizes those of our studies that have sought to define the parasite-derived molecules that permit the development of transmissible infections to proceed. The work will hopefully serve to better inform the search of the *Leishmania* and sand fly genomes so that a fuller accounting of the molecules controlling parasite-vector interactions can be achieved, with a longer view toward the development of a successful transmission blocking vaccine(s).

16.2 The Life-Cycle of *Leishmania* in their Natural Vectors

The three general stages of parasite development in the vector, extensively reviewed [1], are depicted in Fig. 16.1:

(i) The infective blood meal containing parasitized macrophages is passed into the abdominal midgut where water is removed and the blood is retained inside a well-structured peritrophic membrane (PM). The transformation of amastigotes to promastigotes occurs within 12–18 hr. These initially transformed promastigotes are termed procyclics, and remain short, ovoid, and only slightly motile. Intense multiplication of these forms begins at approximately 18h–24 h. During 36–72 hr, rapid multiplication within the digesting blood meal continues, accompanied by the transformation of promastigotes to a long, slender, highly motile form termed nectomonad;

(ii) by 3–4 days, degeneration of the PM is apparent, and tremendous numbers of nectomonads are found packed in the anterior portion of the abdominal midgut, with many attached via their flagella to the epithelial cell microvilli;

(iii) by day 7, the passage of the digested blood meal is complete, and the anterior migration of promastigotes to the region of the cardia (thoracic midgut) and stomodeal valve proceeds until a massive accumulation of parasites behind the valve is achieved. This migration is associated with transformation of nectomonads into short, broad forms termed haptomonads, which are occasionally seen in division, and into metacyclic promastigotes, which are short, slender, highly active forms with a flagellum at least twice the length of the cell body and which are never seen in division. Some parasites, mainly metacyclics, migrate beyond the stomodeal valve into the esophagus, pharynx, and proboscis.

Our studies were the first to demonstrate that the metacyclic promastigotes that develop in the anterior midgut are

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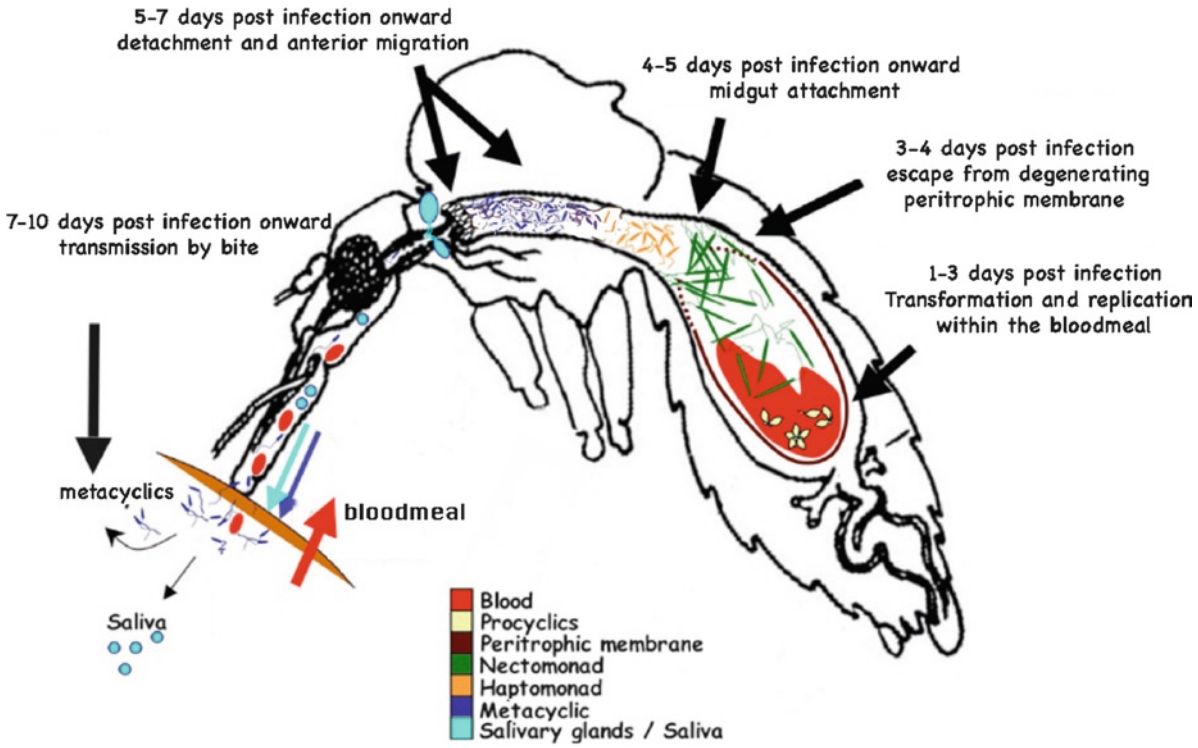

Fig. 16.1 The life cycle of *Leishmania* in the digestive tract of a competent vector sand fly

uniquely adapted to life in the vertebrate host, and they are indeed the forms that initiate infection following transmission by bite [2, 3]. In these studies, the infectivity of promastigotes taken from late stage, anterior midgut infections in the fly were found to greatly exceed those taken from earlier stages of colonization in the posterior midgut, and similar developmental changes were found to accompany the growth of promastigotes in culture [4]. The differentiation of promastigotes into the infectious, metacyclic stage coincident with the vector's search for another bloodmeal is a key factor in *Leishmania* transmission.

16.3 The Role of Lipophosphoglycan in *Leishmania* – Sand Fly Interactions

The ability to grow and purify infectious stage promastigotes from axenic culture permitted the first description of developmentally regulated molecules associated with increased virulence. The finding that the metacyclics of *L. major* could be purified by their loss of agglutination with the lectin peanut agglutinin (PNA) indicated that metacyclogenesis is accompanied by changes in surfaces carbohydrates, and in particular, the loss of terminally exposed galactose residues [4]. By immunizing with purified metacyclics, a monoclonal antibody was raised that recognized a surface and released glycoconjugate [5] that was identified as a developmentally modified form of the major surface glycoconjugate found on all extracellular promastigotes of all *Leishmania* species, the lipophosphoglycan (LPG). LPG is expressed on the entire cell surface, including the flagellum, and is organized as a densely packed filamentous glycocalyx. Its tripartite structure has been determined by others [6] to consist of a backbone of multiple repeat phosphodisaccharide units of -6Galβ1,4Manα1-PO_4- that are either unsubstituted or variably substituted with a variety of sidechain oligosaccharides. As discussed below, the phosphoglycan repeats demonstrate remarkable variation both between different species and substrains of *Leishmania* and between different developmental stages of the same strain. The phosphoglycan chain is linked via a hexasaccharide glycan core to a 1-*O*–alkyl-2-*lyso*-phosphatidylinositol lipid anchor, and it is capped by a non-phosphorylated oligosaccharide. The generic structure of LPG, common to all *Leishmania* species and promastigote developmental forms, is represented in Fig. 16.2.

The LPG of *L. major* undergoes two general modifications during the development of promastigotes into an infectious stage: (i) an elongation of the molecule due to an approximate doubling in the number of phosphorylated oligosaccharide repeats [7], resulting in an approximate 10nm thickening of the glycocalyx [8, 9]; and (ii) a down regulation in the number of side chain substitutions expressing a terminal galactose residue in favor of substitutions containing a terminal arabinopyranose [10]. The biological consequences of LPG elongation are believed to explain the

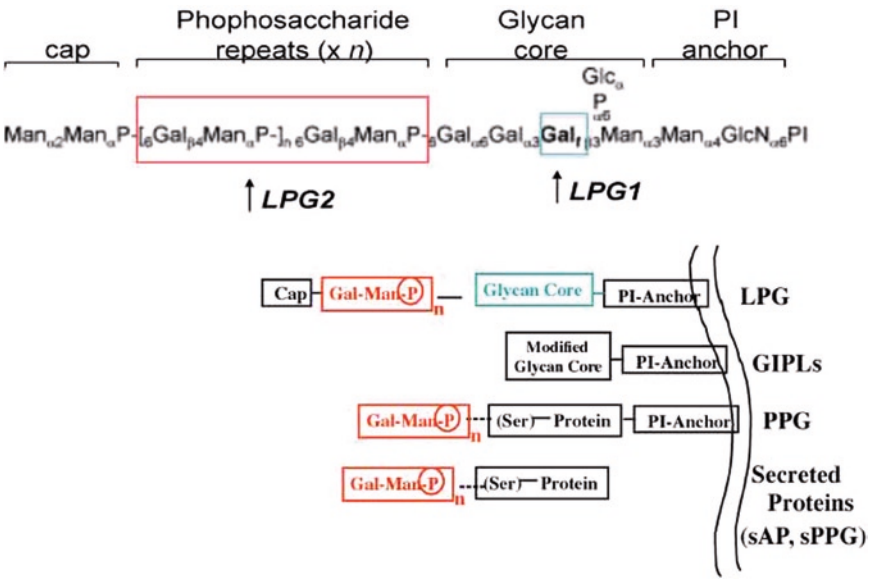

Fig. 16.2 The structural domains of LPG, and the targeted deletions in LPG biosynthetic genes affecting related surface and secreted phosphoglycan-containing molecules

increased serum resistance of metacyclic promastigotes. Metacyclics from culture or from the sand fly were found to display a dramatically enhanced resistance to complement mediated lysis [11], which would be expected for a vector transmitted organism immediately exposed in the mammalian host to the potentially lethal effects of fresh serum, due to activation of the alternative and/or classical complement pathways. Despite their resistance to complement mediated damage, metacyclics were surprisingly found to activate complement as efficiently as the serum sensitive procyclic forms, as determined by C3 deposition studies [12]. The elongated LPG on metacyclics appears to behave as an effective barrier to membrane insertion and pore formation by the membrane attack complex, C5b-9 [13]. However, because the LPG activates complement so efficiently, resulting in extensive opsonization with C3 cleavage fragments, it serves to promote parasite attachment to and uptake by host phagocytic cells. C3 opsonization was shown to be necessary for efficient uptake of *L. major* metacyclics by human monocyte-derived macrophages *in vitro*, and the use of complement receptors CR1 and CR3 to facilitate parasite entry promoted their subsequent intracellular survival by failing to trigger a respiratory burst in these host cells [14]. This non-lethal encounter with the complement cascade may thus be a key event that establishes *Leishmania* as an intracellular parasite.

As mentioned, the second developmentally regulated change in LPG structure during *L. major* metacyclogenesis concerns the display of terminally exposed galactose residues, which are down regulated in favor of arabinose. The loss of polygalactose epitopes available for binding provided a clue as to the nature of the parasite ligands that mediate attachment to the midgut wall, a step necessary to prevent the loss of infection during excretion with the blood meal remnants. In contrast to procyclics, nectomonads, and haptomonads, metacyclic promastigotes have never been seen in attachment but remain free in the lumen to migrate anteriorly and to be egested by the fly during transmission by bite. We were able to explain this behavior, in large part, by their loss of intrinsic binding potential. In *in vitro* binding assays using dissected *P. papatasi* midguts, logarithmic phase *L. major* promastigotes from culture displayed an inherent capacity to attach to midgut epithelial cells whereas metacyclic promastigotes purified from stationary phase culture no longer bound [15]. The binding of procyclic promastigotes could be completely inhibited by procyclic LPG but not by metacyclic LPG. Similar stage-specific LPG-mediated binding to midgut epithelial cells was demonstrated for other natural *Leshmania* – sand fly combinations, including *L. donovani* – *P. argentipes*, and *L. tropica* – *P. sergenti* [16, 17]. Additional evidence that LPG is the key ligand for midgut attachment included immuno-labeling of infected midguts revealing direct binding of procyclic LPG to the microvillar lining [15], and most compellingly, the demonstration that *L. major* mutants deficient in β-linked galactose side-chain biosynthesis [18], or mutants deficient solely in LPG expression as a consequence of targeted deletion of *LPG1* (Fig. 16.2), a gene required for the biosynthesis of the hexasaccharide core domain, failed to attach to the midgut *in vitro* or to persist in the sand fly following blood meal excretion *in vivo* [19].

Another remarkable aspect of LPG structure as it relates to its function is that the extensive inter-species polymorphisms in the repeating phoshoglycan domains of LPG can in most cases fully account for species- specific vector

competence. Based on extensive field investigations, there appears to be a close evolutionary fit between a *Leishmania* species and the sand fly species that transmits it in nature (i.e., certain sand flies are able to transmit only certain species of *Leishmania*). There is, for example, no evidence that *P. papatasi* is involved in the natural transmission of any species other than *L. major*, despite the fact that this sand fly has a wide distribution in regions endemic for other species of *Leishmania*. Similarly, *P. sergenti* is a proven vector of only *L. tropica*, again despite the fact that it is found in biotopes containing other *Leishmania* species. We have been able to reproduce these specific associations in the laboratory [16, 20]. The ability of *P. papatasi* to transmit only *L. major* sp. was directly attributed to the unique, highly substituted nature of *L. major* LPG that provides for multiple, terminally exposed β-linked galactose residues available for binding. Thus *Leishmania* species that bear unsubstituted LPGs (i.e., *L. donovani* strains from Sudan), partially substituted or even fully substituted LPGs bearing side-chain oligosaccharides that do not contain β-linked galactose (e.g., *L. mexicana, L. tropica*) fail to bind to *P. papatasi* midguts or to persist in the gut following blood meal excretion [20]. Furthermore, of the LPGs purified from these respective species, only *L. major* LPG stained *P. papatasi* midguts *in vitro*. By contrast, *P. sergenti* midguts were intensely stained following incubation with purified LPG from *L. tropica* but not LPG from *L. major* or *L. donovani* [16]. The LPG that controls the specificity of *P. sergenti -L. tropica* interactions is characterized by highly branched side-chains substitutions terminating in arabinose and glucose, distinct from the β-linked galactose side-chains that are characteristic of most *L. major* strains. The data strongly suggested that at least some phlebotomine vectors differ with respect to the parasite recognition sites that they express and that midgut adhesion is a sufficiently critical aspect of vector competence as to provide the evolutionary drive for LPG structural polymorphisms. The remarkable species variability of the LPG phosphoglycan repeats is summarized in Fig. 16.3.

The variable oligosaccharide domains expressed by the promastigote surface LPG, shown to mediate species-specific binding, implicated gut lectins or lectin-like molecules as parasite attachment sites and that these molecules should themselves be variably expressed by different vector species. This prediction has essentially been borne out by the identification of a galectin homologue, termed PpGalec, that is expressed only on the midguts of natural vectors of *L. major* (i.e., *P. papatasi* and *P. duboscqi*) [21]. The binding of recombinant PpGalec was restricted to *L. major* promastigotes

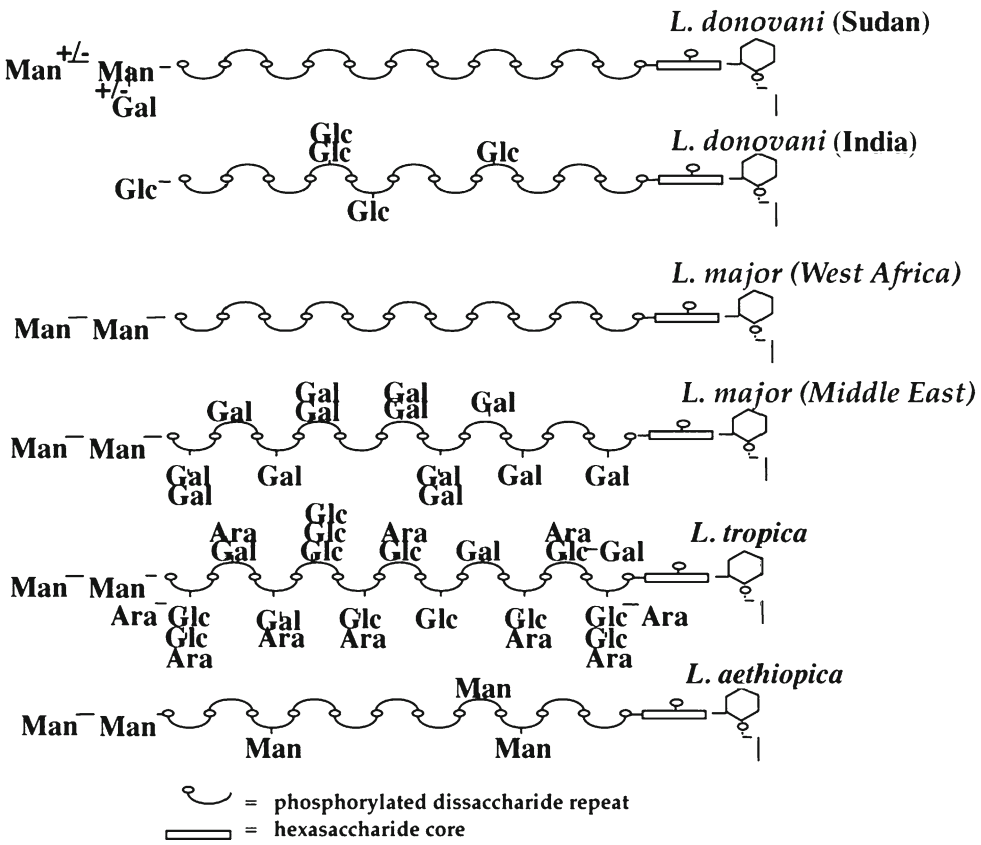

Fig. 16.3 Polymorphic structures of procyclic LPG expressed by Old World species of *Leishmania*

bearing poly-Gal (β1–3) side chains on their LPG. Moreover, anti-PpGalec antibodies inhibited *in vitro* midgut binding of *L. major* LPG as well as live parasites. Finally, PpGalec antibodies fed to *P. papatasi* severely impaired parasite development and survival in the insect midgut, indicating that interaction of *L. major* promastigotes with PpGalec is an essential condition for the development of transmissible infections.

The downregulation of terminal galactose residues during metacyclogenesis of *L. major* promastigotes in culture, which as discussed, is reflected by the loss of parasite agglutination with peanut agglutinin and other galactose-binding plant lectins, has a similar consequence in terms of the loss of binding to PpGalec [21]. Stage-specific mAb have been used to document identical modification of the LPG during metacyclogenesis of *L. major* promastigotes *in vivo*, beginning on day 5 when most parasites are still in the abdominal midgut [22]. Metacyclic LPG was detected on the majority of promastigotes by day 7, with most of these corresponding to anterior metacyclic forms by morphological criteria. Finally, in forced-feeding experiments using 10–14 day infected sand flies, the parasites emerging from the proboscis were all identifiable both morphologically and antigenically as metacyclics. The *in vivo* data thus support a molecular mechanism of parasite attachment and release controlled by developmental modifications of the LPG. The stage-specific polymorphisms in *L. major* LPG structure, and the stage-specific associations with PpGalec that are central to the development of transmissible infections, are depicted in Fig. 16.4.

The inter-species polymorphisms in LPG structure will necessarily require that fundamentally different modifications of LPG accompany metacyclogenesis of promastigotes bearing, for instance, unsubstituted or glucosylated backbone repeats. During metacyclogenesis of a Sudanese strain of *L. donovani*, the salient structural feature of LPG is conserved, involving expression of a phosphoglycan chain made up of unsubstituted disaccharide-phosphate repeats [17]. As with *L. major*, the size of the molecule is substantially increased, due to a 2-fold increase in the number of phosphorylated disaccharide repeat units expressed. In addition, the display of terminally exposed neutral capping sugars is affected, resulting in the loss of lectin-binding domains, including those involved in mediating binding to midgut epithelial cells of a competent vector, *P. argentipes*. Since the neutral capping sugars expressed by metacyclic LPG still contain both βGal- and αMan- terminating structures shown to mediate binding of the procyclic promastigotes, it was suggested that these epitopes become cryptic as a possible consequence of the elongation and clustering of the phosphoglycan chains. For the LPG of Indian strains of *L. donovani*, which are partially substituted with glucose side-chains and a cap structure that also terminates with β1,3-linked glucose, metacyclogenesis is associated with both chain elongation and the down-regulation of glucose side chain biosynthesis, again resulting in loss of LPG-mediated binding to *P. argentipes* midguts [23]. For *L. tropica* LPG, the sugar modifications that accompany metacyclogenesis are not yet known in detail, yet the consequences of these changes in terms of loss of binding to *P. sergenti* midguts has been confirmed [24]. Collectively, these developmental modifications in LPG are the basis for identifying and purifying metacyclic promastigotes from culture using stage-specific lectins and mAb.

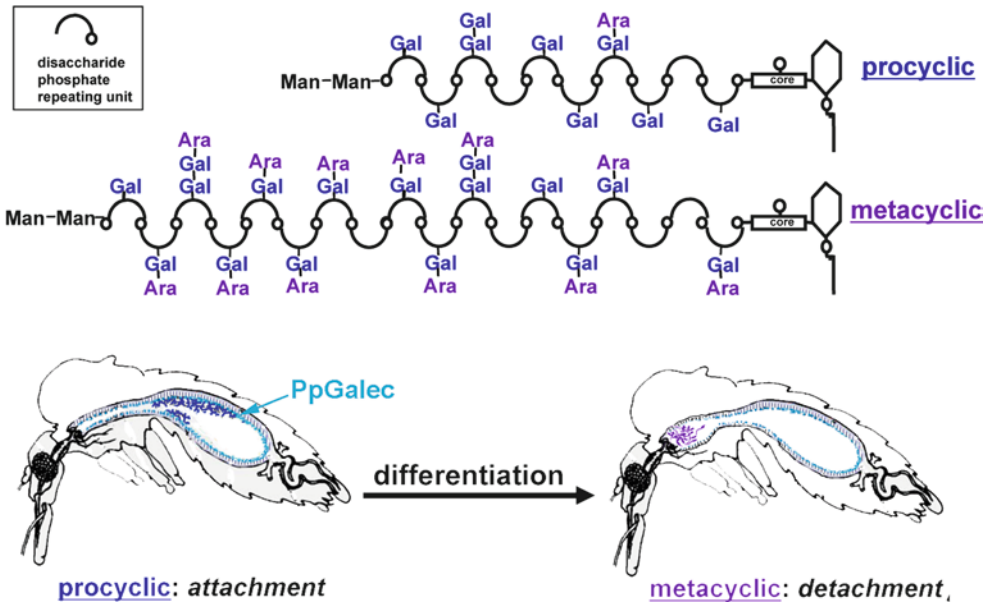

Fig. 16.4 Developmentally regulated modifications in LPG structure controlling stage-specific attachment to the sand fly midgut

16.4 The Role of Other Phosphoglycan – Containing Molecules

In addition to the threat posed by blood meal excretion to the loss of infections from the fly, the early blood-fed midgut presents an important barrier to parasite survival. Following engorgement, a number of physiological changes take place within the gut, including the release of proteases for digestion of blood meal proteins. Adler, in 1938 [28], was the first to suggest that serum-induced proteolytic factors might prevent the development of an unnatural strain of *Leishmania* in *P. papatasi;* his claim was based on his observation that lowering the rabbit serum concentration enhanced parasite survival. We conducted experiments with soybean trypsin inhibitor added to the blood meal and found enhanced survival of even a natural strain of *L. major* [26]. These studies also detailed a role for the peritrophic membrane (PM) in reducing the rate of diffusion of digestive enzymes into the blood meal and delaying the exposure of blood cells and parasites to the digestive process. The rapid diffusion of enzymes in chitinase-treated flies with poorly formed PMs was thought to account for the early mortality of parasites during their transition to extracellular promastigotes.

The clearest indication that the early blood-fed midgut is a potentially lethal environment for *Leishmania* was provided by the results of fly infections using targeted null mutants deficient not just in LPG but also in biosynthesis of the entire class of related molecules that have in common their expression of the Gal(β1,4)Man(α1-PO4)]–derived repeating units. The repeating units can be linked directly to the cell surface via a glycosylphosphatidylinositol (GPI) anchor, as described above for the abundant surface LPG, or attached to the Ser/Thr-rich regions on a large protein backbone, as found in the surface and secreted proteophosphoglycan (PPG) and secretory acid phosphatase (sAP). The repeating units are synthesized in the Golgi lumen by the sequential transfer of mannose 1-phosphate and galactose from their respective nucleotide donors. Mutants deficient in the Golgi nucleotide-sugar transporter GDP-Man, encoded by *LPG2*, have been generated by homologous gene replacement that are devoid of all phosphoglycan-containing molecules (Fig. 16.2). For both *L. donovani* and *L. major*, the targeted *LPG2* null mutants were killed in the blood meals of their respective, normally permissive vectors within the first 4 days, prior to blood meal excretion [19, 27]. Transfection of the *LPG2*$^{-/-}$ mutants with *LPG2* restored their surface LPG expression and their ability to assemble other phosphoglycans, and also restored their capacity to survive the conditions in the digesting blood meal. Interestingly, mutants deficient only in LPG biosynthesis as a consequence of targeted deletion of *LPG1,* involved in biosynthesis of the LPG glycan core domain, displayed only a modest reduction in growth prior to blood meal excretion, at which time, as discussed above, they were completely lost. Taken together, the results with the *LPG1* and *LPG2* mutants suggest that one or more of the other released phosphoglycan containing structures (e.g., sAP or PPG) play important roles in protecting the parasite during its early exposure to digestive enzymes in the gut.

16.5 Concluding Comments

This chapter has reviewed the work at the NIH defining the potential barriers to parasite growth and development that exist within the midguts of phlebotomine sand flies and the molecular adaptations that the parasite has evolved to permit the development of transmissible infections to proceed. As these molecules were found in some cases (e.g., LPG) to underlie the basis of species-restricted vector competence, their characterization has permitted predictions regarding the identity of transmitting flies within endemic or emerging disease foci containing a diversity of vector species. For example, the low abundance of galactosylated side-chain repeats on LPG from West African *L. major* isolates implicates vector species other than *P. papatasi* in their transmission and strengthens the argument that *P. duboscqi*, which is permissive in the laboratory to *L. major* parasites bearing poorly substituted LPGs [28], is the natural vector of these strains. Similarly, our discovery of human isolates of *L. tropica* within the *L. donovani* endemic region of Bihar, India [29], would predict that they are transmitted by the more broadly permissive *P. argentipes* and not *P. papatasi*, although both vector species are intensively distributed in the biotope.

The consideration of parasite and vector molecules essential to transmission can of course also lead to the identification of target antigens for transmission-blocking vaccines. A number of preliminary experimental findings have been encouraging with regard to the ability of antibodies raised against sand fly or *Leishmania* molecules to inhibit normal parasite development in the vector midgut. In transmission blocking studies designed to specifically target the molecules involved in midgut attachment, *P. duboscqi* fed on monoclonal anti-LPG antibodies [30], or *P. papatasi* fed on polyclonal antibodies against PpGalec [21], were in each case compromised in their ability to support the early growth and/or persistence of *L. major* following blood meal excretion. As a practical approach to leishmaniasis control, transmission-blocking vaccines can only be applied to transmission cycles involving human or canine reservoirs of infection (e.g., *L. donovani, L. infantum*, and *L. tropica*); further studies will be needed to identify the relevant target antigens in these parasite- vector pairs. One great advantage of the approach is that, in contrast to a conventional anti-*Leishmania* vaccine

that require parasite-specific, cellular immune responses for protection in the mammalian host, and for which no safe and durable non-living vaccine exists, antibodies that confer transmission blocking immunity may be elicited by defined antigens and maintained over a long period of time. The completion of the *Leishmania* genome [31] in conjunction with the anticipated completion of the genomes of important vector species, should greatly facilitate the search for other molecules involved in parasite-sand fly interactions and their cloning as potential target antigens for transmission-blocking vaccines.

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Part VI
Microbiology: Malaria

Chapter 17
Mosquito Strategies Against *Plasmodium*: A Tale of Restrained Response and Immune Evasion

Carolina V. Barillas-Mury

17.1 Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites; it afflicts more than 300 million people and results in over one million deaths every year. The majority of lethal cases occur in young African children. The mosquito *Anopheles gambiae* is the major vector responsible for the transmission of human malaria in Africa. Our unit is interested in exploring the interactions between the immune system of the mosquito *Anopheles gambiae* and *Plasmodium* parasites to establish how they affect malaria transmission and to identify *Plasmodium* genes that allow certain parasite strains to evade the mosquito's immune system.

17.2 Mosquito Midgut Epithelial Cells Responses to *Plasmodium* Infection

Mosquitoes become infected with *Plasmodium* when they ingest gametocyte stages of the parasite from a human host. Gametocytes complete their maturation in the midgut lumen, fertilization takes place and gives rise to a zygote. Zygotes mature into a motile ookinetes that must survive the harsh environment of a blood meal undergoing digestion and invade midgut epithelial cells.

17.2.1 Cell Biology of Epithelial Cell Invasion and Repair

Our studies revealed that ookinetes inflict extensive damage when they invade the mosquito midgut, which ultimately results in cell death [1]. The damaged cell protrudes towards the midgut lumen and suffers other characteristic changes, including the induction of nitric oxide synthase (NOS) expression, a substantial loss of microvilli and genomic DNA fragmentation. The epithelium is efficiently repaired by an actin string-purse mediated restitution mechanism, which enables the epithelium to "bud off" the degenerated cell without loss of its integrity (Fig. 17.1). Based on these findings we proposed the *Time-Bomb Model* of ookinete invasion [1]. According to this model, the toxic reactions triggered during invasion (the chemical "bomb") can also damage the parasite. In order to survive, an ookinete must migrate out of the cell before high levels of toxic chemicals accumulate in the cytoplasm [1, 2].

Ookinete invasion also induces high levels of peroxidase activity in midgut cells undergoing apoptosis [3]. This induction of peroxidase activity was revealed by histochemical stainings and correlates with positive nitrotyrosine stainings. Both stainings are weak after invasion and increase in intensity as cells enter advanced stages of apoptosis. The inducible epithelial peroxidase activity can catalyze protein nitration *in vitro* using nitrite and hydrogen peroxide as substrates [3]. These findings indicate that parasite invasion triggers tyrosine nitration. This is a two-step reaction in which NOS induction is followed by increased peroxidase activity (Fig 17.1), which catalyses nitration probably by a mechanism similar to what has been described in human macrophages [4].

Most of the invasion studies in *An. gambiae* or the Indian mosquito vector *An. stephensi* were done using the mouse malaria model *Plasmodium berghei*. Comparative studies of the epithelial response of *Aedes aegypti* midgut cells to *Plasmodium gallinaceum* (chicken malaria parasite) invasion revealed that, in this mosquito species, the midgut epithelium is repaired by a novel "*Actin Cone Zipper*" mechanism in which apoptotic cells do not bud off into the midgut lumen (Fig. 17.2). Instead, a "cone-shaped" actin aggregate is formed at the base of the damaged cell, which closes sequentially, expelling the cellular contents into the midgut lumen as it brings together healthy neighboring cells [5].

We also identified a serine protease (SP30) that is highly induced in the midgut of *An. gambiae* females in response to ookinete invasion. SP30 silencing drastically reduces both *P. berghei* and *P. falciparum* infection (J. Rodrigues and C. Barillas-Mury, unpublished data). SP30 silencing abolishes the induction of SRPN6 mRNA in response to *Plasmodium* invasion, suggesting that both genes could be

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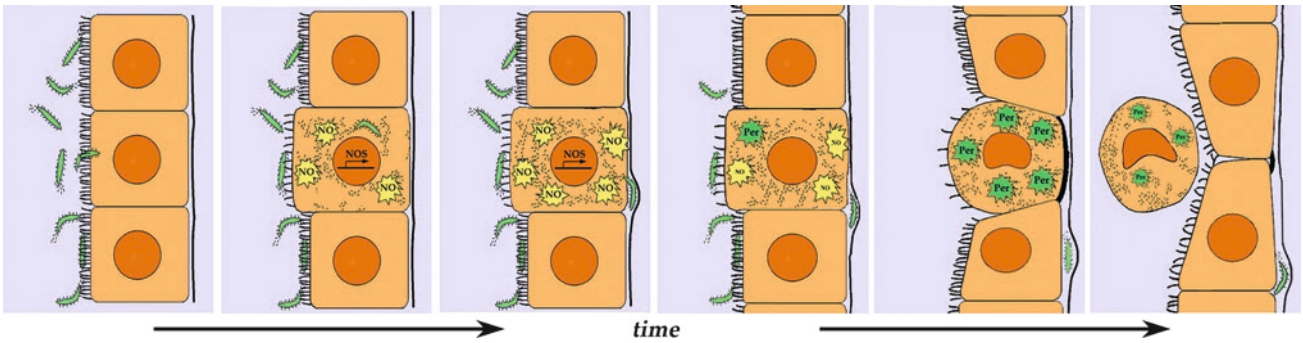

Fig. 17.1 Time Bomb Model of Ookinete midgut invasion. *Plasmodium* invasion triggers the induction of nitric oxide synthase (NOS) and an inducible midgut peroxidase (Per), which mediates nitration of damaged cells. Apoptotic cells bud off into the midgut lumen and the epithelium is repaired

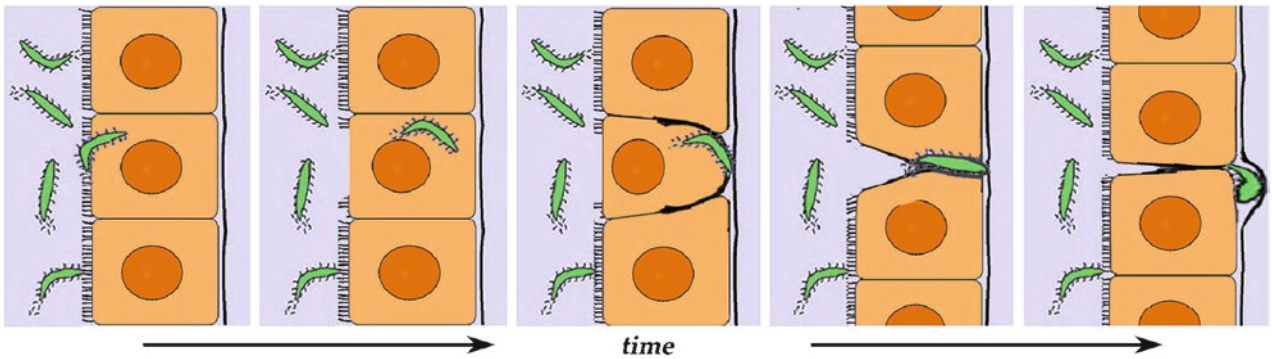

Fig. 17.2 The Actin Cone Zipper Model of epithelial repair in *P. gallinaceum*-invaded *Aedes aegypti* midguts. In this system damaged cells are not expelled into the midgut lumen, but instead a cone structure is formed, which expels the contents of the cell as it brings together healthy neighboring cells

part of the same signaling cascade, with SP30 being upstream of SRPN6. The mechanism by which SP30 silencing affects ookinete midgut invasion is under investigation. SP30 appears to be part of a general signaling cascade triggered by *Plasmodium* invasion, as SP30 expression is also highly induced in the salivary glands in response to *Plasmodium* sporozoite invasion (J. Rodrigues and C. Barillas-Mury, unpublished data).

17.2.2 Modulation of Epithelial Cell Responses to Bacteria and Plasmodium

A heme peroxidase enzyme that is secreted by midgut epithelial cell following the ingestion of a blood meal, and which modulates epithelial immune responses to microbes has been identified. We refer to it as *Immuno-Modulatory Peroxidase (IMPer)* [6]. When IMPer is present, bacteria of the gut flora proliferate without activating the expression of antibacterial genes in midgut epithelial cells. However, when expression of this enzyme is silenced, a robust immune activation that reduces the number of bacteria by ~90% is observed. Activation of this immune response does not affect the number of eggs oviposited, but decreases the number of live larvae that hatch by 24%. When the bacterial flora is eliminated by oral administration of antibiotics, the immune system is no longer activated and the decrease in viable larvae is no longer observed [6].

IMPer silencing also dramatically reduces *An. gambiae* infection with *P. berghei* or with the human malaria parasite *P. falciparum.* Ookinetes invade the midgut, but are lysed during their transit through the epithelial cells. In bacteria-free mosquitoes antibacterial genes are no longer induced when IMPer is silenced. Instead, NOS expression is induced to much higher levels in response to *Plasmodium* infection. Double silencing of NOS and IMPer reverts lethality, indicating that NOS is one of the major effector genes activated when IMPer is silenced. Our findings indicate that a physiological mechanism that prevents the activation of immune responses against the normal bacterial flora also makes *An. gambiae* much more permissive to *Plasmodium* infection [6].

17.3 Reactive Oxygen Species (ROS) and the Balance between Fecundity and Immunity in *Anopheles Gambiae*

17.3.1 ROS and Anopheles Gambiae Susceptibility to Infection

Reactive Oxygen Species (ROS) are generated as a by-product of general metabolism or as part of the immune response to pathogens. ROS are potentially toxic to the host, so they must be kept localized and rapidly neutralized by enzymes such as superoxide dismutase (SOD) and catalase, which detoxify superoxide anion and hydrogen peroxide, respectively. *An. gambiae* strains have been selected to be either refractory (R) or highly susceptible (S) to *Plasmodium* infection. In R females, ookinetes develop and invade the midgut, but are recognized by the mosquito immune system as they come in contact with hemolymph and trigger a melanization response, during which parasites are covered with melanine, a black insoluble pigment [7]. Studies from our group indicate that R females have higher systemic levels of H_2O_2 than unselected G3 females or S females [8]. This difference in ROS levels between strains becomes more pronounced 24 h post-feeding, when blood-meal digestion and *Plasmodium* invasion peak.

Microarray mRNA expression analysis and physiological studies indicate that the R strain is in a chronic state of oxidative stress that is exacerbated by blood feeding. The resulting increase in steady state levels of ROS favor melanization of parasites as well as Sephadex beads. Administration of vitamin C (antioxidant) to R females decreases H_2O_2 in hemolymph to levels similar to those of G3 and prevents the melanization response [8]. The susceptibility of the S, G3, and R *An. gambiae* strains to bacterial infection also correlates directly with their systemic levels of H_2O_2. High H_2O_2 levels in R are associated with increased survival to injection of a fixed bacterial dose. Conversely, mortality is significantly higher in the S strain, which has the lowest level of H_2O_2. Furthermore, reduction of ROS by dietary supplementation of vitamin C (strong antioxidant), dramatically decreased survival following a bacterial challenge [9].

Midgut mRNA levels of Mn superoxide dismutase (MnSOD), Cu/ZnSODs, and glutathione peroxidase (GPx) increased in mosquitoes that received either a *Plasmodium*-infected or an uninfected blood meal, indicating that blood feeding induces oxidative stress. Surprisingly, midgut catalase mRNA expression was induced in mosquitoes fed uninfected blood but not in *Plasmodium*-infected mosquitoes [9]. Midgut catalase activity, measured 24 hr after a blood meal was also lower in *Plasmodium*-infected mosquitoes. This lack of catalase induction is triggered by ookinete invasion, as a normal induction is observed when mosquitoes are infected with a mutant parasite strain in which ookinetes are unable to invade the epithelium (CTRP-strain) or when infected mosquitoes are reared at 27 °C, a non-permissive temperature for *P. berghei* development. Reduced catalase activity in invaded epithelial cells may be necessary to increase the availability of H_2O_2, a substrate required by cells undergoing peroxidase-mediated nitration. As predicted, a reduction of catalase expression by dsRNA silencing significantly decreased the number of developing oocysts, indicating that the increased levels of H_2O_2 limit *Plasmodium* infection [9].

17.3.2 Catalase Prevents ROS-Mediated Damage to Ovaries and Embryos

ROS are thought to have a negative effect on the reproductive ability in both males and females. For example, reduced female fertility has been observed in mice and *Drosophila* lacking Cu-Zn SOD [10, 11]. In *Drosophila*, lack of Cu-Zn SOD also causes nearly complete male sterility [11]. Surprisingly, mice in which the *catalase* gene is disrupted grow normally and are as fertile as wild-type mice, indicating that, in this species, catalase is not essential to protect spermatozoids or the ovaries from oxidative damage. Although flies in which the *catalase* gene is disrupted and completely lack enzymatic activity die soon after eclosion; flies with a single functional gene have normal viability and fecundity [12].

The effect of aging and oxidative stress on reproductive output of the S, G3, and R *An. gambiae* strains was analyzed [13]. The number of developing oocytes following a blood meal decreases with age in all strains, but this decline is much more pronounced in G3 (intermediate H_2O_2 levels) and R (high H_2O_2) than in the S (low H_2O_2) strain. Reduction of ROS levels in G3 and R females by administration of anitoxidants prevents this age-associated decline in fecundity. Catalase mRNA and enzymatic activity increase in ovaries as oocysts mature (Fig. 17.3) and ovary extracts from S females have higher catalase activity than those from G3. Sequencing of the coding region of the catalase gene from multiple individuals from each strain showed that the S and G3 strains are fixed for two functionally different catalase alleles, Ser/Ser (high activity) and Trp/Trp (lower activity), respectively.

Biochemical analysis of recombinant proteins revealed that the Trp is a less efficient enzyme with higher Km. The replacement of Ser for Trp appears to destabilize the active tetrameric form of the enzyme. Fecundity studies in the R strain, in which both catalase alleles are present, revealed a strong association between reproductive output and the catalase genotype of individual females. Trp/Trp females produced

Fig. 17.3 Catalase (brown staining) accumulates in the developing oocyst and protects the developing embryo from oxidative damage

significantly less viable larvae than Ser/Ser homozygotes and Trp/Ser females had an intermediate level. A systemic reduction in catalase activity by RNAi knockdown significantly reduced the reproductive output, indicating that in *An. gambiae,* catalase plays a central role in protecting the oocyte and early embryo from ROS damage [13].

17.4 Immune Responses that Limit *Plasmodium* Infection

17.4.1 The Anopheles gambiae STAT Pathway Mediates Late Phase Immunity against Plasmodium

In vertebrates, STAT-1 regulates the transcriptional activation of nitric oxide synthase (NOS) in response to interferon-gamma (IFN-γ). To prevent toxicity to the host, suppressors such as SOCS-1 (suppressor of cytokine signaling) and PIAS-1 (protein inhibitor of activated STAT) carefully regulate this pathway. SOCS-1 mRNA expression is regulated by the STAT-1 pathway, as part of a negative feedback loop [14].

In the mosquito *An. gambiae*, there are two members of the STAT family of transcription factors. The ancestral STAT gene (AgSTAT-A) duplicated through a retrotransposition event, giving rise to a second "intronless" gene (AgSTAT-B). In adult females, the new AgSTAT-B gene regulates the basal mRNA levels of the ancestral AgSTAT-A gene. AgSTAT-A, in turn,

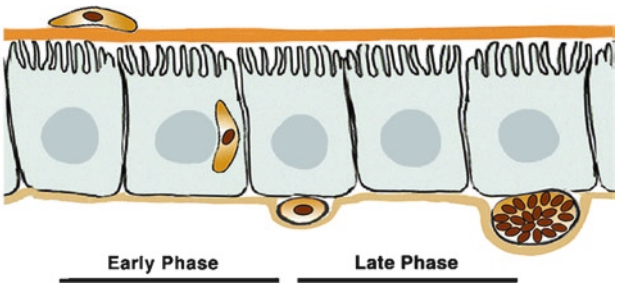

Fig. 17.4 Ookinetes come in contact with effector molecules from the mosquito immune system when they complete the invasion of the midgut epithelial cell. Several molecules participating in this early phase response, such as thioester-containing protein 1 (*TEP1*), leucine rich-repeat immune protein 1 (*LRIM1*) or *LRIM2*, have been identified. Our studies indicate that the STAT pathway mediates a late phase response that induces the expression of nitric oxide synthase and targets the oocysts stage

mediates the transcriptional activation of NOS and SOCS in response to infection with bacteria or *Plasmodium*. However, the AgSTAT-A pathways is not essential for mosquitoes to survive a systemic challenge with several different bacterial species [15]. AgSTAT-A silencing reduces the number of early *Plasmodium* oocysts by 2 days post-infection, but enhances the overall infection by increasing oocyst survival. Silencing of SOCS, a suppressor of STAT, drastically reduces *Plasmodium* infection through the induction of NOS expression (Fig. 17.4). The STAT pathway mediates a late phase *An. gambiae* immune response to *Plasmodium*, which targets the oocyst stage of the parasite [15].

17.4.2 Effect of Pre-exposure of Anopheles gambiae to Plasmodium on Subsequent Infections

Insects rely on the innate immune system, which is thought to be "hard wired" and unable of establishing an adaptive memory response, to defend themselves from microbial pathogens. However, recent work revealed that adult *Drosophila* flies pre-exposed to a sub-lethal dose of some *Streptococcus pneumoniae* remain able to survive a re-challenge with a dose that is lethal to naive individuals for the life-time of the fly [16]. We explored whether adult mosquito females could "learn" to control *Plasmodium* infection more effectively if they encounter the same parasite a second time (J. Rodrigues and C. Barillas-Mury, unpublished data). Two groups of mosquitoes were fed on the same mouse infected with *P. berghei.* One group was kept at a permissive temperature that allows ookinete development and midgut invasion; the second group was placed at a non-permissive temperature immediately after blood-feeding. The group in which midgut invasion took place had a significantly lower infection when challenged with a second *Plasmodium* infection either 1 or 2 weeks later.

The reduction in oocyst numbers is independent of the number of oocysts from the first challenge and is not due to differences in the size of the blood meal ingested. Administration of antibiotics before the first or the second challenge abolishes this difference in infectivity. We conclude that bacteria from the mosquito gut flora must be present at the time of the first exposure to establish a long-lasting enhancement in the antiparasitic response, and that they are also required at the time of the second exposure to elicit this response. We observed a similar phenomena in mosquitoes infected with *P. falciparum*. The effects on *Plasmodium* appear to be indirect and mediated by priming of an immune response to bacteria (J. Rodrigues and C. Barillas-Mury, unpublished data).

17.4.3 Identification of Mosquito Genes Required for Plasmodium Oocyst Survival

Dr. David Schneider's Laboratory carried out a forward genetic screen, using *Drosophila* as a surrogate mosquito, to identify host factors required for the growth *Plasmodium gallinaceum* (avian malaria). Eighteen presumed loss-of-function mutants that reduced infection in flies were identified [17]. We collaborated to evaluate the effect of silencing five *Anopheles gambiae* homologues of these genes on *P. berghei* infection. Reducing expression of four of these genes in the mosquito affected *Plasmodium* infection, suggesting that *Drosophila* model can be used to identify host factors relevant in the mosquito [17].

One of the genes identified in the *Drosophila* screen is a member of the tetraspanin family of proteins. Tetraspanins are proteins with four transmembrane domains that are associated extensively with one another and with other membrane proteins to form specific microdomains distinct from lipid rafts. They are involved in diverse processes from cell adhesion to signal transduction. We identified three tetraspanin genes (TSP1, TSP2 and TSP3) that are induced in the midgut in response to *Plasmodium* infection. Silencing of TSP1 enhances *P. berghei* infection, TSP2 silencing reduces infection, while TSP3 silencing has no effect. TSP1 silencing also enhances *P. falciparum* infection. The mechanism of action of TSP1 is under investigation (R. Dixit and C. Barillas-Mury).

17.4.4 Evasion of the Mosquito Immune System and Compatibility between Plasmodium Parasites and their Vectors

Several *An. gambiae* genes that limit *P. berghei* infection have been identified in functional screens based on dsRNA-mediated gene silencing. However, some of the genes identified in these screens have no effect on the human malaria parasite *P. falciparum*, raising the question of whether *P. berghei* is an appropriate system to characterize gene relevant to human malaria transmission.

The effect of silencing several *An. gambiae* genes on both *P. berghei* and *P. falciparum* infection was evaluated. Four new genes that differ in their effect on infection with these two *Plasmodium* species, two glutathione *S*-transferase of the theta class (*GSTT1* and *GSTT2*) genes, oxidation resistance gene 1 (*OXR1*) and Heat shock cognate 3 (*Hsc-3*) were identified. Silencing these four genes (or their orthologues) and two other genes reported in the literature had a similar effect on infection in two highly compatible vector-parasite combinations, *P. yoelii-An. stephensi* and *P. falciparum-An. gambiae* [18].

In *An. stephensi*, silencing the immune genes *CTL4* and *LRIM1* did not affect *P. yoelii* infection. However, in *An. gambiae* females infected with the same parasite species – a much less compatible vector parasite combination – silencing of either *CTL4* or *LRIM1* had a dramatic effect, greatly enhanced infection. Our findings suggest that, in highly compatible vector-parasite combinations, *Plasmodium* parasites evade certain immune responses of the mosquito and thus, silencing genes in these immune pathways has little effect on infection[18].

17.5 Identiffication of *Plasmodium falciparum* Gene(s) that Allow African Strains to Escape Melanization in the *Anopheles gambiae* Refractory Strain

The *An. gambiae* L35 strain was selected in 1986 to be highly refractory (R) to *P. cynomolgy* infection. However, this strain also activated a melanization response and eliminated several other *Plasmodium* species, including *P. falciparum* strains from the New World. Interestingly, parasite strains of African origin are poorly melanized and survive in the R strain (Fig. 17.5). The parental parasites from a 7G8 X GB4 genetic cross that was carried out in Dr. Wellems laboratory were phenotyped. In the R strain, the Brazilian 7G8 parasites are melanized, while those of the GB4 strain from Ghana survived (A. Molina-Cruz and C. Barillas-Mury, unpublished data).

We are currently phenotyping the progeny of the cross to identify the QTL(s) responsible for the phenotypic difference between these two *P. falciparum* strains. Silencing of either one of three mosquito genes known to mediate antiplasmodial responses, thioester-containing protein 1 (*TEP1*) [19], leucine rich-repeat immune protein 1 (*LRIM1*) [20] or *LRIM2*, also called *APL1* [21], prevents the killing of 7G8 parasites. This indicates that the Brazilian (7G8) and African (GB4) *P. falciparum* strains differ in their ability to evade the

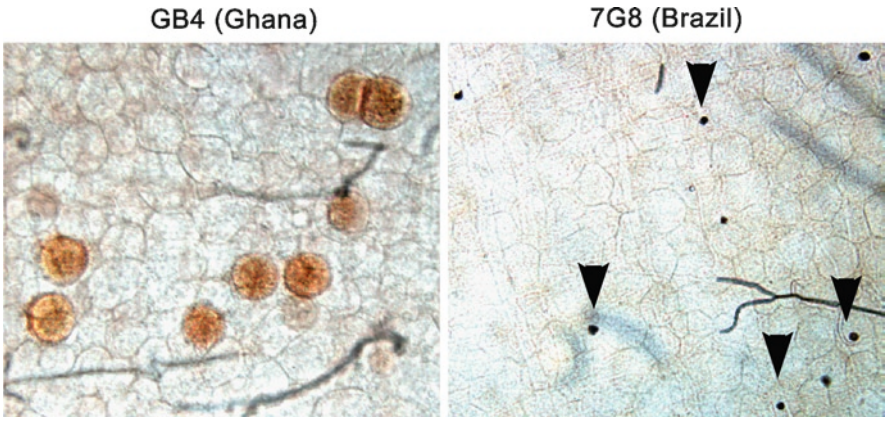

Fig. 17.5 When *An. gambiae* females that have been selected to be refractory to most *Plasmodium* species (L35 strain) are infected with *P. falciparum* strains from Africa, such as GB4 (from Ghana), parasites survive and develop into mature oocysts. In contrast, *P. falciparum* strains from the new world, such as 7G8 from Brazil, are readily recognized by the mosquito's immune system, killed and covered with melanine, an insoluble black pigment. Melanized parasites are indicated by the black arrowheads

mosquito's immune system (A. Molina-Cruz and C. Barillas-Mury, unpublished data).

17.6 Conclusions

Reducing disease transmission is a central component in preventing malaria infection to humans. However, our understanding of the biology of *Plasmodium* in the mosquito stages of the parasite is still limited. Work from our Unit and several other laboratories has shown that mosquitoes have multiple mechanisms to limit *Plasmodium* infection. Some of these responses have important fitness costs, such as reducing the fecundity of mosquito females. In nature, *Plasmodium* parasites are under strong selective pressure to evade the immune responses of their mosquito vectors. We are using a combination of genetic, cell biology, and functional genomics approaches to better understand the mosquito-parasite interaction that determine vectorial capacity. A better understanding of these responses is instrumental in developing new strategies to prevent malaria transmission.

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Chapter 18
The Plasmodial Surface Anion Channel: A Model Microbial Ion Channel and Target for Antimalarial Drug Development

Sanjay A. Desai

18.1 Introduction

Human malaria is a leading cause of morbidity and mortality worldwide; it is caused by 5 species of protozoan parasites from the genus *Plasmodium*. The most virulent of these species, *P. falciparum*, is estimated to affect more than 500 million people each year, resulting in > 1 million deaths annually. Estimates of the economic burden on developing countries are also astounding. Because there is not yet a globally useful vaccine, preventive measures such as insecticide-treated bednets [1] and treatment with drugs targeting parasite-specific activities are the mainstay of managing malaria in endemic countries. Because of evolving resistance to nearly all available antimalarials, there is a desperate need to develop new antimalarial drugs that target novel parasite activities.

My laboratory has identified and is characterizing one such parasite-specific activity that appears to be a near-ideal drug target. This target, the plasmodial surface anion channel (PSAC), is present on the host erythrocyte some hours after invasion by the parasite [2]; it is absent from uninfected erythrocytes. PSAC also has a number of unusual functional properties that warrant examination with the aim of better understanding solute transport through ion channels. This review will describe the following: (i) the identification of PSAC and our hypothesis on its physiological role; (ii) studies that have provided insights into how PSAC works; (iii) my perspectives on the genetic basis of activity; and (iv) prospects for antimalarial development targeting PSAC.

18.2 Identification of PSAC

The increased permeability of infected erythrocytes to small solutes has been known for many decades [3, 4, 5, 6, 7, 8]. Early studies were performed by various groups, using bulk transport methods such as tracer accumulation or osmotic fragility measurements on suspensions of infected cells. These studies established that infected cells exhibit dramatically increased uptake of diverse solutes and identified a number of non-specific inhibitors. They were limited to macroscopic measurements on populations of cells and were therefore unable to address a number of important questions including: Does the measured solute uptake reflect transmembrane transport or does it involve uptake of bulk extracellular solution (e.g., via endocytosis or a proposed membranous duct)? Is there a single shared mechanism utilized by all the solutes known to have increased permeability or are there multiple distinct pathways for each class of solutes? Where within the infected cell complex does the mechanism(s) localize? If the permeability reflects transmembrane transport, is it facilitated (e.g.. via an ion channel or carrier)?

To address these and other questions, my laboratory adapted patch-clamp technologies to the study of human erythrocytes and identified two unusual ion channels, one each on two separate membranes within the infected erythrocyte. These studies have led to a good working model of membrane transport between serum and the intracellular parasite (Fig. 18.1). This model suggests that sequential, facilitated transport across the erythrocyte membrane, the parasitophorous vacuolar membrane, and the parasite's plasma membrane is needed for parasite nutrient acquisition and waste removal. Subsequent studies from my group suggest this sequential pathway may also be used by some water soluble antimalarial drugs that target intracellular parasite activities.

Patch-clamp is generally reserved for larger, less deformable cells because it requires formation of a poorly understood seal between a fabricated pipette and the cell surface membrane. Our studies revealed that seal formation on erythrocytes required pipettes with smaller tip diameters than generally used for other cells [2, 9]. We also found that the pipette tip must be kept pristine to obtain high resistance seals on erythrocytes. We generally use pipettes immediately after fabrication and insist that all solutions coming in contact with the pipette be protein-free and filtered to remove debris. Finally, perfusion of the bathing solution around the sealed cell was found to mechanically damage the seal; we developed and

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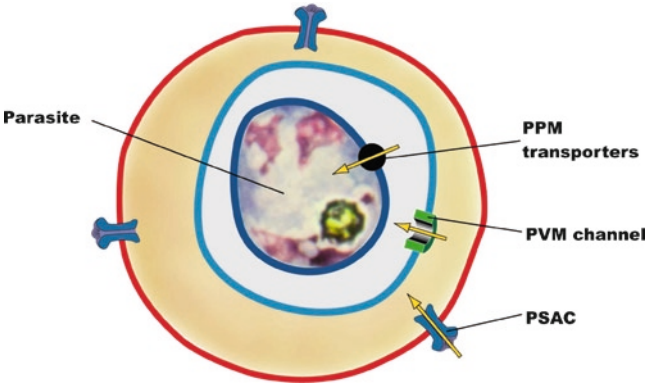

Fig. 18.1 Model for biological role of parasite ion channels. Schematic shows a trophozoites-stage infected erythrocyte and the multiple membranes that must be traversed by water-soluble nutrients in serum before utilization by the intracellular parasite. PSAC mediates uptake across the host erythrocyte membrane. Next, solutes cross the parasitophorous vacuolar membrane by passage through the PVM channel [39, 40], a channel functionally distinct from PSAC. Finally, specific carriers for solutes such as sugars and nucleobases are on the parasite plasma membrane (PPM) and can complete nutrient acquisition; metabolic waste products such as lactate may leave the infected cell by passage in the opposite direction

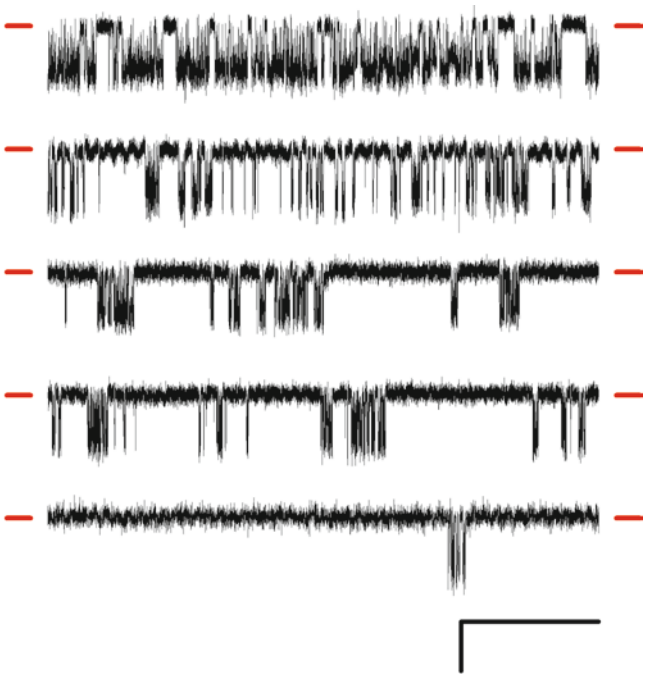

Fig. 18.2 Single channel recordings of PSAC without (top trace) or with 10 μM NPPB [41], 16 μM furosemide, 10 μM dantrolene [42], or 100 nM FQ86 (second to fifth traces from the top, respectively). FQ86 is a high affinity PSAC antagonist identified in a high-throughput screen. The NPPB trace represents a recording of the same channel molecule as the top trace, but after addition of NPPB to the bathing solution with a slow perfusion system [10]; other traces represent separate ion channel molecules recorded with inhibitor present in both bath and pipette compartments. For each inhibitor, the level of reduced PSAC gating is quantitatively similar to that seen in macroscopic flux assays. Red dashes on each side of traces represent the closed channel level; scale bar at bottom corresponds to 200 ms (horizontal)/ 2 pA (vertical)

have utilized a slower-than-gravity perfusion chamber with a geometry that permits reasonably fast solution changes without the turbulence associated with bulk perfusion [10]. With these precautions, along with state-of-the-art measures to reduce electrical noise, we achieved very high resistance seals on erythrocytes (>100G Ω and occasionally up to 1 TΩ) with associated low noise recordings (100-200 fA rms over a 5 kHz bandwidth). These values compare favorably with those from high-quality recordings on cells that are generally considered "easy" to patch [11]. As an important caveat, a number of other groups have subsequently undertaken patch-clamp of infected erythrocytes, but without adhering to such stringent guidelines. Their manuscripts report a number of ion channels that are distinct from the single ion channel we see. Interpretation of those studies has been confounded by their significantly lower seal resistances and by their lack of detailed information on recording conditions [12, 13, 14, 15]. Technical issues almost certainly contribute to the discrepant results obtained by competing groups in this field [16].

When patch-clamp of human erythrocytes was performed under the stringent conditions described above, we identified only one ion channel type on the infected cell's host membrane (Fig. 18.2). This channel, the plasmodial surface anion channel (PSAC), is absent from uninfected cells. Based on a number of quantitative observations, PSAC appears to be sufficient to account for nearly all the parasite-induced increases in solute permeabilities observed in previous studies. First, a number of biophysical markers strongly suggest that PSAC is the predominant ion channel type on the infected erythrocyte membrane [2]. Our single-channel recordings revealed voltage-dependent gating consistent with the voltage-dependent rectification of whole-cell currents. Noise analysis of our single-channel and whole-cell recordings also suggest that channels with distinct gating behavior do not contribute significantly to the whole-cell currents under our experimental conditions. Second, the pharmacological profile of PSAC is quantitatively concordant with that of macroscopic tracer flux and osmotic lysis studies carried out by various groups (Fig. 18.2). Both single PSAC and whole-cell recordings in the presence of NPPB [5-nitro-2-(3-phenylpropylamino) benzoic acid], furosemide, dantrolene, other known inhibitors, and high affinity inhibitors identified in a PSAC antagonist drug discovery project revealed that each of these agents has quantitatively similar effects on PSAC activity and on macroscopic solute flux measurements [2, 9, 17, 18, 19, 20, 21]. Third, permeability coefficients calculated from tracer flux and osmotic fragility measurements yield estimates consistent with the magnitude of our whole-cell currents [22]. Fourth, two recently identified PSAC

mutants exhibit changes in single PSAC selectivity and pharmacology consistent with macroscopic transport measurements [23, 24]. Infected cells expressing these mutant channels exhibit not only altered single-channel and whole-cell electrophysiological changes but also marked changes in permeability of organic solutes, indicating that the uptake of each of these solutes occurs via a common mechanism. Thus, PSAC appears to fully account for the increased permeability of infected erythrocytes to a range of small solutes.

These electrophysiological studies addressed many of the questions raised by tracer accumulation and osmotic fragility studies carried out in the preceding decades. However, by providing a mechanistic model for the parasite-induced permeabilities, they also raised a new collection of questions. These questions will be discussed in the following sections.

Table 18.1 Diverse collection of solutes with significant PSAC permeability

solute	vdW volume (Å^3/molecule)	net charge[a]
Cl^-	19.5[b]	-
acetate	56.8	-
glutamate	134.6	-
SCN^-	48.8	-
sulfo-NHS-LC-LC-biotin[c]	575.2	-
glycine	69.1	0
proline	108.6	0
isoleucine	138.3	0
sorbitol	165.1	0
histidine	131.7	+
phenyl-trimethyl ammonium	145.4	+
blasticidin S	380.5	+
leupeptin	442.4	+

Table 1 lists solutes with known permeability through PSAC, their estimated van der Waals molecular volume [calculated as described [43]], and net charge at physiological pH. [a]Net charge is listed as negative, neutral, or positive (-, 0, and +, respectively) without attempting to calculate partial charges for titratable groups.

[b]For Cl^- the volume was calculated as an ionic volume from established ionic radius rather than as a van der Waals volume.

[c]common name for sulfosuccinimidyl-6'-(biotinamido)-6-hexanamido hexanoate.

18.3 Mechanisms of Transport through PSAC

The various studies described above suggest that a single ion channel mediates the uptake of inorganic halide ions, uncharged solutes including sugars, amino acids, purines, some vitamins, and organic cations. This list of permeant solutes covers a broad range of solute geometries and charge (Table 18.1). Despite the wide-ranging nature of this collection, the small sodium ion [ionic volume ~ 6.5 Å^3] is largely excluded by PSAC, with estimates of its permeability relative to that of Cl^- ranging from $< 10^{-5}$ to $< 10^{-3}$. This stringent exclusion is important for parasite survival because, without it, infected cells would incur net Na^+Cl^- uptake and undergo osmotic lysis in the bloodstream. A central paradox then is how does PSAC achieve uptake of a diverse collection of nutrients while ensuring Na^+ exclusion? This combination of constraints almost certainly had a profound impact on the evolution of the channel's structure and functional properties. Two examples of how we are characterizing PSAC's unusual properties are described below.

18.3.1 PSAC's Unmatched Selectivity Profile

While there are numerous examples of ion channels permeable to similarly broad collections of solutes, these other channels do not appear to be able to exclude individual small solutes. To examine the basis for PSAC's unusual selectivity profile, we took advantage of changes in channel activity resulting from labeling with *N*-hydroxysulfosuccinimide esters, chemical modifying reagents that react specifically with primary amines. Primary amines are present at the *N*-terminus of polypeptides and the ε-amino group of lysine residues. We determined that these reagents label PSAC at its extracellular face and that the reagent side chain creates steric hindrance of the channel pore [17], leading to inhibition of transport. As predicted by this steric hindrance model, the level of transport inhibition increased with the length of the NHS ester side-chain. Then, labeling of infected cells with a combination of different NHS esters revealed that at least three separate primary amines are near the extracellular face of the channel pore. Because these primary amines are positively charged at physiological pH values, we hypothesized that they may function to fine tune the channel's selectivity profile by providing electrostatic repulsion of extracellular cations such as Na^+. We tested this hypothesis by measuring solute permeabilities at non-physiological pH values. These studies revealed that Na^+ permeability is strongly dependent on pH, increasing more than 30-fold when the pH is raised to 10.5. Because known PSAC antagonists abolished this increase and because uninfected erythrocytes did not exhibit this change in Na^+ permeability, we concluded that PSAC is responsible for this pH-dependent Na^+ permeability. Because ε-amino groups on lysine residues are typically deprotonated at this pH, these findings are consistent with electrostatic repulsion by pore-mouth amines as one mechanism of Na^+ exclusion by PSAC. Further consistent with this model, the permeability of sorbitol, an uncharged sugar alcohol, was not affected by increasing pH, excluding global changes in PSAC structure at raised pH values.

Because larger cationic solutes are not excluded by PSAC to the same extent as Na^+ (e.g., phenyl trimethyl ammonium,

Table 1), electrostatic repulsion does not fully account for how PSAC excludes Na^+. Indeed, other mechanisms almost certainly play a greater role.

18.3.2 Solute-inhibitor Interactions within the Channel Pore

Through studies of the channel's pharmacology, another unusual property of PSAC was identified by my group. We found that known antagonists exhibited differing levels of effectiveness depending on the presence of certain permeating solutes [25, 26]. Approximately half of all permeating solutes reduce the affinity of furosemide, NPPB, phloridzin, and dantrolene and its derivatives. For each of these unrelated antagonists, affinity appears to be reduced by a conserved ~ 10-fold, suggesting a common mechanism. Interestingly, a modest reduction in temperature from 37 °C to 20 °C abolishes this effect of permeating solutes. These and other biophysical observations indicate that these solute-inhibitor interactions are occurring within the confines of the channel pore. We are working on determining the mechanistic basis of these interactions, but it seems likely that they reflect complexities in the process of solute permeation and that they function in further fine-tuning the channel's selectivity properties.

Because PSAC antagonists appear to inhibit parasite growth and are being pursued in an externally funded drug discovery project, it is critical that we understand these affinity-reducing interactions. Antagonists that are not prone to them would appear to be more attractive starting points for drug development and are being sought in collaboration with both academic and pharmaceutical partners.

18.4 Approaches to Identifying PSAC's Gene(s)

Both functional characterization of PSAC and drug discovery targeting this unique activity (described below) are limited by the fact that the channel's gene has not yet been identified. Successful identification of the channel gene would also open up new areas of research: how PSAC is targeted to the host membrane, regulation of PSAC expression throughout the parasite's complex life cycle, and feedback mechanisms that alter channel expression under conditions such as malnutrition represent fundamental directions of exploration that may only be possible when the gene is definitively known.

A number of approaches have been considered and attempted by my group and others. Many have key limitations that need to be kept in mind. Protein-based biochemical approaches, such as enrichment of proteins after surface-labeling with NHS esters [17, 27] or affinity purification using known antagonists [28] followed by sequencing, are severely limited by PSAC's very low functional copy number of ~10^3/cell (see ref. 2). Such enrichment algorithms will invariably struggle with detection of this rare protein's sequence in the face of more abundant endogenous human proteins such as Band 3 and hemoglobin (~10^6 and > 10^9/cell, respectively). Unpublished studies from my group suggest it is possible to selectively label parasite proteins and to reduce detection of human proteins; we are using these selective approaches to identify yet unknown parasite activities on the host membrane.

Informatic approaches to identification of the channel's gene can now take advantage of the completed genome sequences of several species of malaria parasites; however, they are limited by the lack of clear functional orthologs of PSAC in other organisms and by the absence of a universally conserved selectivity filter in known anion channels [29]. Nevertheless, informatic approaches may start by assuming that PSAC's gene should encode a protein with several transmembrane domains, that it should be conserved in all plasmodia [21], and absent from other genera [30]. They may also restrict the list further by removing genes that lack a motif thought to be involved in trafficking parasite proteins to the erythrocyte compartment [31], though caution is required here because some proteins lacking this motif have also been known to be exported from the parasite. Unfortunately, even with application of all these criteria, there are too many candidate genes in the parasite genome to evaluate with difficult DNA transfection or heterologous expression methods.

My laboratory has taken two somewhat different approaches to identification of PSAC's gene(s).

18.4.1 PSAC Mutants Provide a Useful Molecular Handle

One approach is based on two functional mutants generated by *in vitro* selection of parasites resistant to toxins that can only reach their intracellular targets by permeation through PSAC [23, 24]. One mutant, selected for resistance to the protein synthesis inhibitor blasticidin S, exhibits markedly lower permeability to certain solutes and relatively preserved uptake of other solutes, implicating changes in the channel's selectivity filter. Single channel recordings from erythrocytes infected with either mutant parasite line exhibit marked changes in gating, pharmacology, and other biophysical properties. These mutants provide important evidence for a parasite-encoded PSAC over modified human channels

because human erythrocytes lack heritable genetic material. They also provide an important new handle for cloning PSAC's gene because the mutants were generated from a defined genetic background and, therefore, permit comparisons of gene sequences and expression levels between the mutant and a closely related wild-type parent. We have been using high-density DNA tiling microarrays and next generation whole-genome sequencing technologies with these mutants with the goal of finding the responsible mutations.

Unpublished studies using microarrays with one mutant have identified a chromosomal deletion event that is essential for generation of the mutant. Subsequent DNA transfection experiments have definitively identified the responsible gene, which appears to be an accessory protein that modulates PSAC's functional properties. As this work progresses, I anticipate it will shed new light on PSAC's biological role and may help find the channel gene.

18.4.2 Functional Polymorphisms in PSAC between Defined Laboratory I Isolates

The second approach we are taking to cloning PSAC's gene(s) is based on subtle functional polymorphisms in channel behavior induced by geographically divergent parasite isolates. We began by identifying differences in voltage-dependent gating between the Indo 1 isolate (originally taken from a patient in Indochina) and the 7G8 isolate from Brazil [9]. These differences and additional variations in PSAC behavior between isolates presumably result from heritable polymorphisms in one or more parasite genes. This reproducible and statistically significant difference in channel biophysical properties represented the first published evidence for a parasite-encoded channel over a human channel. Importantly, we have identified such differences in channel phenotype between parental isolates from each of the three available *P. falciparum* genetic crosses [32, 3, 34]. We are currently using the progeny of these genetic crosses and linkage analysis to map the channel's locus. I believe this approach has the greatest potential for definitively identifying PSAC's gene(s).

18.5 Antimalarial Drug Discovery Targeting PSAC

If the model of parasite nutrient acquisition via PSAC in Fig. 18.1 is correct, then PSAC may be a near-ideal target for antimalarial discovery because of the following: (i) inhibition of the channel should prevent both nutrient acquisition and disposal of soluble metabolic waste products; (ii) PSAC activity is unambiguously different from known human channels, suggesting that specific antagonists can be identified; (iii) PSAC's location on the surface of infected erythrocytes relaxes drug design constraints such as need for membrane permeability; and (iv) drug resistance may be less easily acquired than for drugs targeting intracellular parasite activities, where extrusion of unmodified drug from the site of action is an established resistance mechanism [35, 36]. The Medicines for Malaria Venture (MMV), a Geneva-based public-private partnership that seeks to discover and develop new antimalarial drugs [37], has recognized these advantages and is supporting a drug discovery project targeting PSAC.

The cornerstone of our discovery project is a miniaturized assay for PSAC antagonists suitable for high-throughput screening. This assay is based on osmotic lysis of infected cells in solutes permeant through PSAC; it has been adapted to screening of random compound libraries in 384-, 1536-, and 3456-well format. In each format, we have successfully optimized the assay to yield excellent separation between positive and negative controls and reproducibility of measurements. Statistical measures of assay goodness suggest a low rate of false positives and negatives [38]. We have used this assay to execute several high-throughput screens in collaboration with academic and pharmaceutical partners. These screens have identified multiple active scaffolds that inhibit PSAC activity in patch-clamp studies at nanomolar concentrations (e.g., the compound FQ86 used for patch-clamp studies in Fig. 18.2). Importantly, these compounds consistently inhibit parasite growth in culture, validating PSAC as a drug target. The next steps in our drug discovery algorithm will involve selection of scaffolds suitable for hit-to-lead chemistry, design and synthesis of derivatives, evaluation of these derivatives in functional assays and in parasite growth inhibition experiments to validate the target and to identify those compounds suitable for *in vivo* studies. Much of this work will require concerted medicinal chemistry input. MMV's approach of matching projects originating in academic laboratories with a pharmaceutical partner with experience in drug discovery and development may fulfill this requirement.

18.6 Conclusions

While intraerythrocytic growth and replication provides partial protection from the host immune response, intracellular survival requires specific adaptations not needed by free-living pathogens. We have identified two unique ion channels, PSAC and the PVM channel, that appear to permit intracellular survival by facilitating nutrient uptake and removal of soluble metabolic waste. These channels have

unique functional properties that make them useful model channels for dissecting the process of solute permeation through selective pores. They are also novel targets for anti-malarial drug discovery and development.

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Chapter 19
Genomics and Genetics of Drug Resistance and Regulation of Malaria Parasite Development

Xin-zhuan Su

19.1 Introduction

Malaria kills more than a million people each year, mostly in tropical and subtropical regions (Snow et al., 2005; Cox et al., 2007). It is caused by infection of one of the four *Plasmodium* parasites, including *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale*, and *Plasmodium malariae*. The monkey malaria parasite *Plasmodium knowlesi* has also been reported to infect humans (Singh et al., 2004). *P. falciparum* is the most deadly parasite but *P. vivax* is the most widespread species. Although *P. vivax* is generally regarded as non-lethal, it can cause severe disease in patients (Mendis et al,2001; Genton et al., 2008). Malaria can be treated and cured using antimalarial drugs, but parasites resistant to many drugs currently in use have been reported, including delayed clearance of parasites after treatment of artemisinin combination therapy (ACT) (Wongsrichanalai and Meshnick, 2008). In addition, vaccines to prevent infections of malaria parasites are still not available, and insecticide-resistant mosquitoes are spreading (Hemingway et al., 2004).

Although we have made great progresses in fighting malaria, including the completion of the genome sequences of many species of malaria parasites (Gardner et al., 2002; Carlton et al., 2002, 2008; Pain et al., 2008) and collection of genome diversity information and the recent development of various genetic maps (Su et al., 1999; Jeffares et al., 2007; Mu et al., 2007; Volkman et al., 2007; Hayton et al., 2008), we know very little about the differences in biology, physiology, and biochemistry among parasite strains. Approximately 60% of the predicted ~5,500 *P. falciparum* genes encode proteins of unknown function, which presents a formidable obstacle to our understanding of the biology of the parasite and malaria. The obstacles include difficulties in culturing the parasites (only *P. falciparum* is widely cultured, but it still requires red blood cells) and the small parasite cells that make it impossible to distinguish them morphologically.

Recent developments in genomics and other resources can be explored to facilitate our understanding of parasite biology and the underlying molecular mechanisms. All the phenotypic variations, either drug resistance or parasite development, likely have their genetic basis in DNA polymorphisms. Genetic mapping is a powerful tool that can be used to study the relationship of genetic polymorphisms and phenotypic changes. Genetic crosses have been employed to map determinants contributing to various malaria parasite phenotypes, including drug resistance, invasion, parasite development, and virulence (Walliker et al., 1971; Wellems et al., 1990; Hayton et al., 2008). Another approach is genome-wide association study, which relies on the presence of linkage disequilibrium (LD) in parasite populations and the availability of large numbers of genetic markers. LD refers to the non-random association of alleles and can be influenced by various factors such as the distance between the affected gene(s) and nearby markers, recombination rate, age of the mutations, population structures, *etc.* It is now possible to perform genome-wide association to search for genes affecting various parasite traits using high-density genetic maps saturated with single nucleotide polymorphisms (SNP) and microsatellites (MS). Genome-wide association approach will be particularly useful for mapping drug-resistant genes, because drug-resistant mutations are relatively recent (past 60 years) events, and strong drug pressure may help preserve LD surrounding the causative genes.

The Malaria Functional Genomic Section (MFGS) uses malaria parasite genome databases and develops new resources to study the mechanisms of drug resistance, gene regulation during parasite development, parasite population diversity, and parasite evolution. Our main activities and discoveries in the past few years have been on malaria parasite population structure, evolutionary genetics, development of genetic

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maps and high-throughput genotyping methods, and mechanisms of drug resistance in *P. falciparum* and *P. vivax* malaria parasites. We are applying MS and SNP maps to perform genome-wide association analyses that will provide insights into longstanding problems in malaria.

19.2 Genome-wide Analysis of Genetic Variation, Recombination, Molecular Evolution, and Genetic Association

19.2.1 Plasmodium Falciparum

One of the two major directions of our research program is genetic mapping to identify genes involved in drug resistance, parasite development, and other phenotypes. The mapping effort includes identification of genome-wide SNP and MS from malaria parasite genomes, development of high-throughput genotyping methods, phenotyping and genotyping culture-adapted field isolates or progeny of genetic crosses, and association of various phenotypes with genetic variations to detect genes of interest. After identification of candidate genes, we will study the contribution of the genes to the associated phenotypes and their underlying molecular mechanisms using genetic knockout and other methods. For effective association mapping, we first need to develop methods for high-throughput genotyping, assays that can accurately measure parasite phenotypic variations such as response to a drug and methods to calculate or analyze phenotype and genotype data.

We began our initial SNP collection from *P. falciparum* before the *P. falciparum* genome was completed in 2002, which led to the development of a SNP map for chromosome 3 (Mu et al., 2002). SNP from 204 genes on chromosome 3 were first obtained; the information was used to estimate the age of *P. falciparum.* The age and origin of *P. falciparum* has been a subject of debate. This work, supported by chromosome-wide SNP, showed that *P. falciparum* was highly diverse and came from an ancestor of ~100,000 years. A chromosome-wide genetic map integrated with SNP and MS at an average density of one marker per 2.3 kb was developed. The study also showed that low-complexity regions did not have significantly higher levels of nucleotide substitutions than the globular domains, suggesting that the low-complexity regions may be under similar functional constraints. Another issue being debated was whether there was a recent population bottleneck for the *P. falciparum* parasite. We subsequently analyzed the complete mitochondrial genome from 100 worldwide isolates and showed that there was indeed a population bottleneck in Africa ~10,000 years ago, followed by a rapid population expansion coinciding with the changes in agricultural practice and mosquito speciation at about the same time (Joy et al., 2003).

For successful genome-wide association mapping, it is critical to understand the patterns of LD and genetic recombination in the parasite genome. We further typed ~100 worldwide *P. falciparum* isolates with 183 SNP on chromosome 3. Using this data set, we established chromosome-wide haplotype maps for the parasites. We found that the parasites could be clustered into groups according to major geographic regions and that recombination rates varied greatly among parasite populations and along the chromosome with elevated recombination rates at the middle and the ends of chromosome 3 (Fig. 19.1). This study provided some important parameters for guiding our future association studies. More recently, we extended our SNP collection to all 14 chromosomes, leading to the development of a genome-wide MS and SNP map (Fig. 19.2) and used the SNP information to identify new parasite antigens (Mu et al., 2007). This study provides a foundation for developing high-throughput genotyping methods for genome-wide association studies.

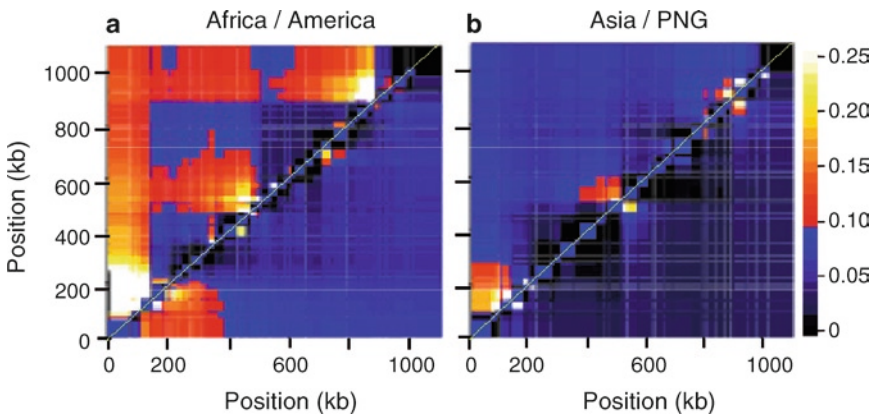

Fig. 19.1 The distribution of detectable recombination events on chromosome 3 of *P. falciparum*. Each panel shows, for two populations, a minimum number of recombination events between each pair of segregating sites, scaled by physical distance to identify regions of high and low recombination. (**a**) African (upper) and American (lower) populations. (**b**) SE Asian (upper) and PNG (lower) populations. The color bar unit is recombination event/kb. This figure was initially published in Mu et al., (2005a)

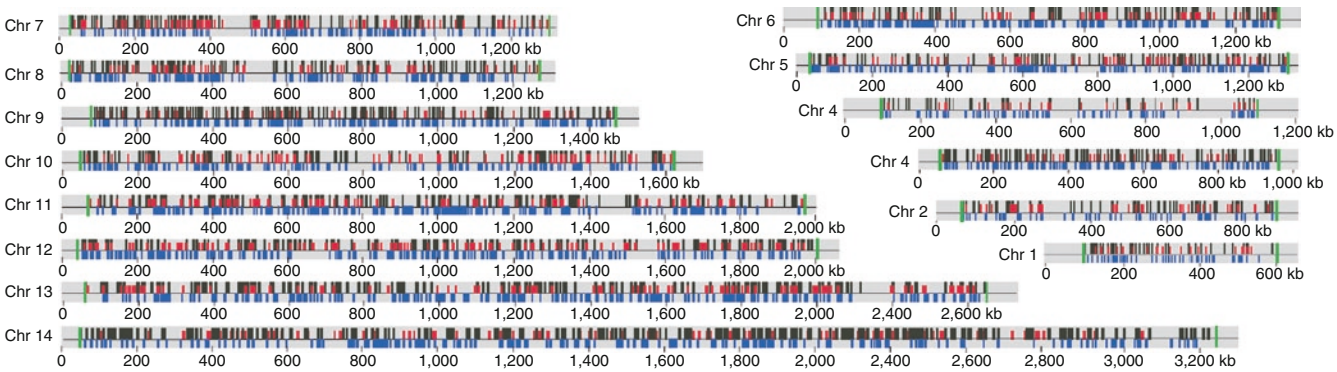

Fig. 19.2 Physical maps of polymorphic sites across 14 *P. falciparum* chromosomes. Vertical bars represent single nucleotide polymorphisms (SNP) (black, nonsynonymous substitutions; red, synonymous substitutions) or microsatellites (blue, under the horizontal lines). Only one nsSNP and sSNP (non-coding SNP were grouped with sSNP) is presented if there are more than one SNP in a gene. Most of the chromosomal ends (green vertical bars) were excluded because of gene families such as *var*, *rifin*, and *stevor*. This figure was initially published in Mu et al., (2007)

The genome-wide SNP identified from our studies have been used to develop a microarray chip based on molecular inversion probe (MIP) technology (Absalan and Ronaghi 2007) for high-throughput typing, and the array has been successfully used to genotype parasite DNA from field isolates (Mu et al. 2010). At the same time, we explored the possibility of using a high-density tiling microarray (the array was designed at Sanger Center, UK) to genotype parasite DNA (Mourier et al., 2008; Jiang et al., 2008b). More than 100,000 polymorphic sites were detected from five parasite isolates, including ~18,000 new single-feature polymorphisms. The advantage of the MIP array is that it requires as little as 100 ng of DNA for a hybridization experiment whereas the tiling array typically requires 10 μg of DNA (unless genome-wide amplification is done before hybridization). The MIP array can detect ~3,000 known SNP whereas the tiling array can detect more than 10,000 SNP in a parasite as well as unknown substitutions in new parasite isolates. Currently, both the MIP and Sanger tiling arrays are being used to genotype parasites isolated from the field and progeny from genetic crosses. We have also in the process of upgraded our MIP array to cover 78,000 SNP and, the array is available to the malaria community (distributed through MR4, www.mr4.org).

While developing high-throughput technologies for genotyping, we also started collecting parasite from patients and adapted the parasite into *in vitro* culture. Working with colleagues in LMVR (Drs. Rick Fairhurst, Robert Gwadz, and Thomas Wellems) and collaborators in Cambodia, we have established a field site in Cambodia. We have collected more than 200 parasites from Cambodia and are growing and testing the parasite responses to multiple antimalarial drugs *in vitro*. For accurate measurement of drug responses, we insisted on repeated drug tests using clonal parasites after they are stabilized in culture, because accurate phenotyping is critically important in association mapping, particularly for those controlled by multiple genes. Parasites collected directly from patients may contain populations of mixed genotypes that can greatly influence the result of a drug test (Liu et al., 2008).

Mutations and/or overexpression of various transporters are known to confer drug resistance in a variety of organisms. Based on a hypothesis that some parasite transporters may be involved in drug resistance, we searched for SNP in 49 putative transporters from 97 independent *P. falciparum* isolates. We determined the drug responses of the parasites to chloroquine (CQ) and quinine (QN) *in vitro* and performed candidate gene association analyses. Eleven putative transporters, including the gene encoding the *P. falciparum* CQ-resistant transporter (*pfcrt*) and the *P. falciparum* homolog of P-glycoprotein (*pfmdr 1)*, were found to be significantly associated with higher CQ and QN responses (Mu et al., 2003). LD between the associated genes was also detected. Our data suggests that multiple genes may contribute to a drug-resistant phenotype and that *pfcrt* may also play a role in QN resistance. Additionally, we found much lower nucleotide substitution rates in 39 housekeeping genes than those in the 49 putative transporters, supporting the view that housekeeping genes are under stronger functional constraints whereas transporter genes are under directional selection pressure; however, some of the associated SNP could be due to population structure. More studies are necessary to confirm these results. Recently, we have disrupted one of the candidate genes (homolog of multidrug resistance-associated protein or PfMRP) identified from this association study and showed that the gene indeed could contribute to CQ and QN resistances, although the changes in IC_{50} values were approximately 2–3 times (Raj et al. 2009). The results from genetic knockout were consistent with the mapping data showing weak association of the mutations in the gene and higher IC_{50} values to CQ and QN (Mu et al., 2003).

We also investigated genome-wide compensatory changes in gene expression to mutations in the *pfcrt* using an Affymetrix expression array (Jiang et al., 2008a). The *P. falciparum*

parasite responds to CQ pressure mainly through mutations in the *pfcrt* gene. The changes in the *pfcrt*, however, appear to be deleterious to the parasite, and additional changes in other genes may be necessary for the parasite to survive. We investigated genome-wide expressional changes in closely related parasite lines in their response to *pfcrt* mutations. Changes in expression level in genes involved in parasite growth, invasion, and transport and amplification of a locus containing *pfmdr-1* on chromosome 5 were observed in parasites with single amino acid substitution in *pfcrt*. Further studies of the genes and their interactions will contribute to a better understanding of the mechanisms of drug resistances in malaria parasites.

Using genome-wide MS markers, we also showed that there were more than two CQ founder mutations and that drug-resistant parasites can spread from continent to continent in relatively few generations (Wootton et al., 2002). Additionally, we demonstrated the principle of genome-wide association study for drug resistance in malaria parasites by searching for genomic regions with reduced diversity or signatures of drug selection. The chromosome 7 locus containing *pfcrt* locus was correctly identified using genome-wide MS marker association and parasite isolates collected from various regions of the world through search of signature of selection or reduced diversity (Fig. 19.3).

After developing methods for high-throughput genotyping, establishing a field site for parasite collection, and having a good understanding of population structure and patterns of LD in field populations, the final critical piece for genome-wide association study is phenotype characterization. In collaboration with scientists at NIH Chemical Genomics Center (NCGC), we have started a large-scale screening for chemical phenotypes (variation in drug responses) among field parasite isolates. Seven parasites, including the parents of the three *P. falciparum* genetic crosses (Walliker et al., 1987; Wellems et al., 1990; Hayton et al., 2008), have been tested on a panel of 1280 drugs (LOPAC) and differences in parasite responses to the compounds have been identified. These differences in chemical phenotypes are being associated with parasite genotypes to identify genes that contribute to or confer resistance to different chemical compounds (Yuan et al., 2009).

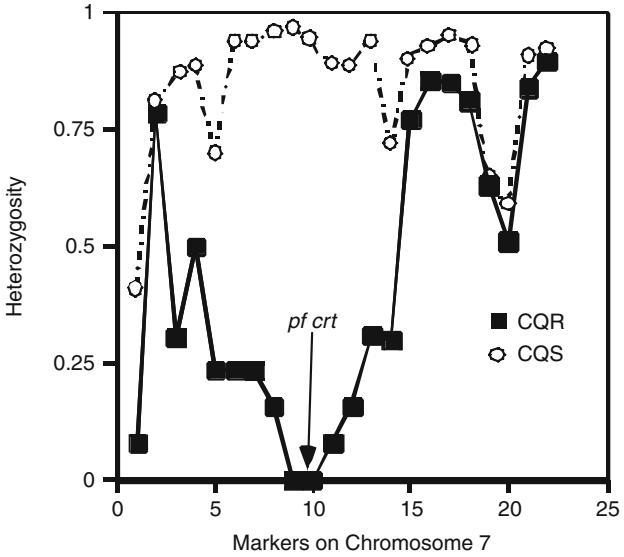

Fig. 19.3 Identification of the chromosome 7 chloroquine resistance locus in *P. falciparum* using microsatellite markers and parasite field isolates. Parasites from Southeast Asia and Africa were separated into resistant (filled square) or sensitive (open circle) groups. The heterozygosity value for each of 24 markers on chromosome 7 was calculated and plotted across the chromosome. Reduced heterozygosity for several markers flanking the *pfcrt* gene in the resistant parasites was detected, suggesting strong drug selection. The data for this figure were initially published in Su and Wootton (2004)

19.2.2 Plasmodium vivax

Another important human malaria parasite, *P. vivax*, infects more people and is distributed in a wider geographic region than *P. falciparum*. We investigated genome diversity in *P. vivax* and showed that the *P. vivax* genome is more diverse than that of *P. falciparum* based on nucleotide polymorphism in a segment of ~100 kb DNA (Feng et al., 2003). We found that polymorphic tandem repeats are abundant in the *P. vivax* genome, providing useful genetic markers for studying vivax populations. We also analyzed the complete 6-kb mitochondrial DNA from 176 world-wide *P. vivax* isolates to address the controversial issue of *P. vivax* origin. Origins of Asia, Africa, and South America have been proposed. Our data support the hypothesis that *P. vivax* originated from Asia through host switch from nonhuman primates to human ~78–265,000 years ago (Escalante et al., 2005; Mu et al., 2005b).

More recently, in collaboration with scientists in Mexico, we investigated the host specificity and population structures in parasite populations in southern Mexico (Joy et al., 2008). *P. vivax* in southern Mexico exhibits different infectivities to two local mosquito vectors, *Anopheles pseudopunctipennis* and *Anopheles albimanus.* Previous work has tied these differences in mosquito infectivity to variation in the central repeat motif of the malaria parasite circumsporozoite (*csp*) gene, but subsequent studies have questioned this view (Gonzalez-Ceron et al., 2001). We genotyped a total of 230 DNA samples with 24 MS markers and found that the parasite populations in Southern Mexico consisted of two major populations (Fig. 4) whose distributions largely mirror those of the two mosquito vectors (Joy et al., 2006). It is likely that local adaptation to different vectors plays an important role in generating population structure in Plasmodium.

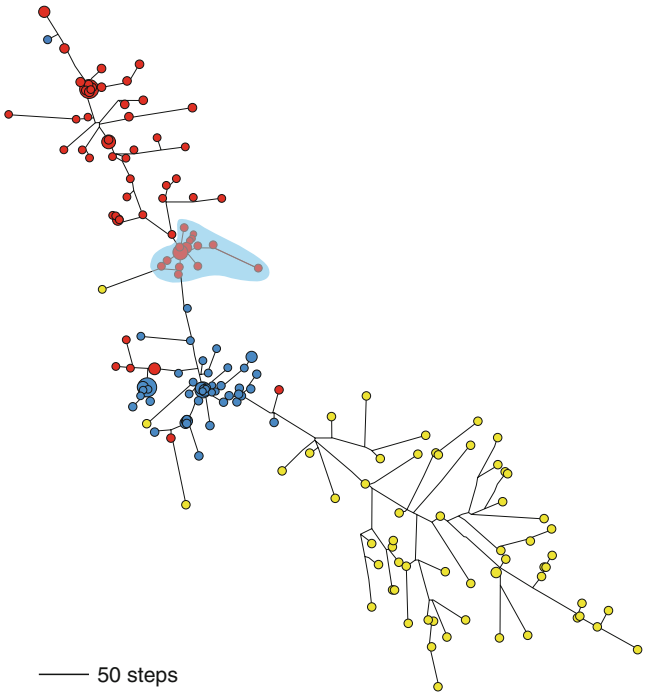

Fig. 19.4 Median-joining network of *P. vivax* isolates based on 24 microsatellite loci. Circle size is proportional to haplotype frequency in the population. Branch length is proportional to the number of mutational steps. Yellow circles, parasite population from the coastal area infecting *Anopheles albimanus*; red, parasite population infecting *Anopheles pseudopunctipennis* in the foothills; blue, parasite population from an area between the coast and foothills. The parasites with a grey background are isolates with the VK210 *csp*-variant. Individuals with mixed ancestry are shown as a single color corresponding to the population with the highest membership proportion. Data from this figure were from Joy et al., (2008), and this figure was originally generated by Dr. Deirdre Joy

19.3 Molecular Mechanism of Parasite Development and Regulation of Gene Expression

A second direction is functional characterization of sexual development of the *P. falciparum* parasite. This direction is relatively new for our section and is more functional and mechanism-oriented research. We are interested in signal transduction and gene regulation that may lead to the switch from asexual to sexual stages, including gene interaction and regulation during the cell cycle. Understanding the molecular process of parasite differentiation, particularly sexual differentiation, will provide important leads for interrupting parasite transmission.

The sexual stages are vital phases in malaria parasite transmission and are the targets of various interventions such as transmission-blocking vaccines. The molecular mechanisms underlying sexual development, however, remain poorly understood. The life cycle of the malaria parasite *P. falciparum* includes sexual reproductive and asexual division events in a mosquito host as well as multiple rounds of asexual replication in the human host. For as-yet-undetermined reasons, possibly due to stress conditions and unique signal transduction, some of the erythrocyte-stage parasites switch from asexual to sexual stages. These sexual stages are essential for mosquito infection and for the completion of the malaria parasite life cycle. A better understanding of the molecular mechanisms underlying sexual development could provide important leads for interruption of parasite transmission.

Although diploid for a short time after zygote formation in the mosquito, the malaria parasite carries a haploid genome during its oocyst and sporozoite stages in the mosquito and during its time in the human host. There are no known sex chromosomes in the parasite genome, and parasites derived from a single individual cell are capable of producing both male and female gametocytes. The switch from asexual to sexual stages is likely regulated by signal transduction and gene expression. Several studies have suggested that the switch to sexual development can be triggered using agents that may stress the parasite, because sexual stages are generally less sensitive to drugs and other harmful conditions. Examples of agents that have been reported to trigger the switch to sexual development include parasite lysates, ‘conditioned medium’, antimalarial drugs, antibodies, temperature changes, pH variation, and chemicals such as cAMP and berenil (Dyer and Day, 2000). Most of the effects, however, have been difficult to reproduce, due to a high degree of experimental variability. When (which stage) and how (what types of signals) the commitment to sexual development is made and how a sexually committed parasite determines whether to become a male or female gametocyte remain mysterious.

The ability to produce gametocytes is often diminished or completely lost after an extended period of *in vitro* culture. One example is the Dd2 clone, which was found to have a greatly reduced capacity for gametocyte production and oocyst formation relative to normal production by other parasite lines. This defect in male gamete production in Dd2 clone arose after a series of experimental manipulations including *in vitro* drug pressure and cloning from its W2 ancestor. The Dd2 parasite was one of the parents in the DdXHB3 cross (Wellems et al., 1990), and a determinant linked to *P. falciparum* male gametocyte development (*pfmdv*) phenotype was previously mapped using linkage analysis and RFLP markers to an 800-kb segment of chromosome 12 (Vaidya et al., 1995). Identification of the gene(s) underlying this defect will provide insights into how sexual development and differentiation are regulated in the *P. falciparum* parasite.

Based on previous work of mapping a determinant associated with a male gametocyte development defect to ~800 kb on chromosome 12, we have further mapped the determinant

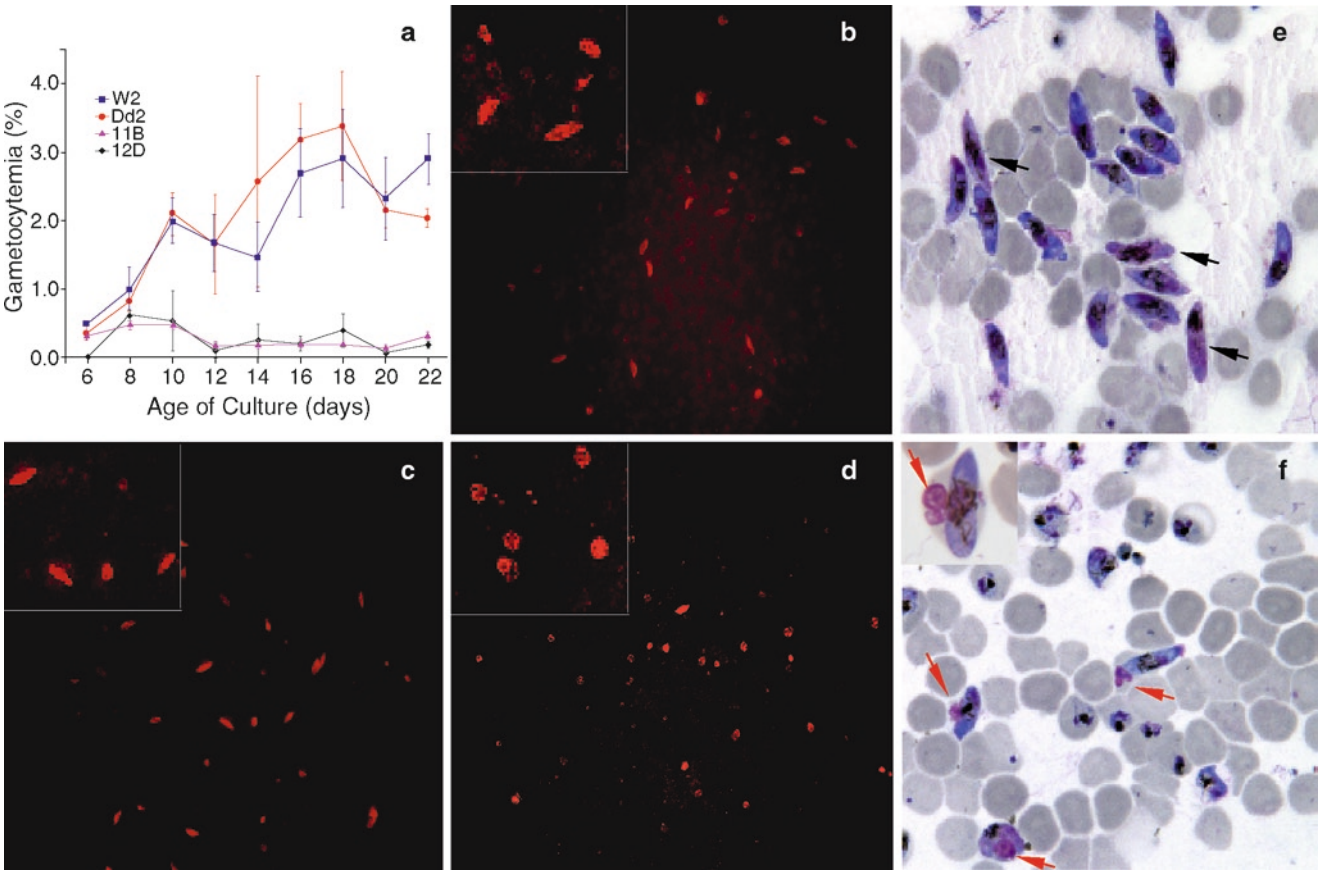

Fig. 19.5 Reduced gametocytemia and arrested gametocyte development in *pfmdv-1* KO lines. (**a**) Plots of gametocytemia show a reduction in the number of elongated stage III-V gametocytes in the *pfmdv-1* KO lines 11B and 12D compared with W2 and Dd2 after day 8. Day-14 sexual stages from W2 (**b**), Dd2 (**c**), and 11B (**d**) were stained with mouse monoclonal anti-Pfg27 antibody and goat anti-mouse Texas Red secondary antibodies. Note that the majority of stained cells in 11B are round, early-stage gametocytes, whereas those in W2 and Dd2 are elongated stage IV-V stages. N-acetyl-glucosamine-treated and percoll-sorbitol-purified day-15 gametocytes from W2 (**e**) and 11B (**f**) were Giemsa-stained. Black arrows indicate W2 male gametocytes with pink cytoplasm (**e**), and red arrows point to a residual body typically present outside a few elongated 11B gametocytes. Insets in **b**, **c**, **d**, and **f** show enlarged parasite images. This figure was initially published in Furuya et al., (2005)

to an 82-kb segment on chromosome 12 that contains 29 predicted genes. A candidate gene named *pfmdv-1* that is expressed at lower levels in Dd2 than in its progenitor W2 was identified. Disruption of *pfmdv-1* results in a dramatic reduction in mature gametocytes, especially male gametocytes, with the majority of sexually committed parasites developmentally arrested at stage-I (Fig. 19.5). The *pfmdv-1* knockout parasites show an enlarged nucleus, often with separation of the inner and outer nuclear membranes and presence of multimembrane vesicles in red blood cell cytoplasm, suggesting disturbance of Golgi-like function of the outer nuclear membrane. Mosquito infectivity of the knockout parasites is also greatly reduced (Furuya et al., 2005).

Recently, we started investigations on the effect of nucleosome distribution and chromotin modification on gene expression and parasite development. Nucleosomes have been isolated from various developing stages and are being sequenced using large-scale sequencing. The purpose of this project is to have a better understanding of gene expression and gene regulation related to parasite development (data unpublished), particularly sexual differentiation.

19.4 Conclusions

In the past few years, major advances have been achieved in malaria genetic and genomics, particularly in high-throughput genotyping. Our main focus in the future will be the characterization and detection of phenotypic differences in malaria parasites, including drug resistance, parasite development and pathogenesis, and associating the differences to variations in the parasite genome. The association will be confirmed using methods of genetics, biochemistry, cell biology, and others.

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Part VII
Immunology: Cellular Immunology

Chapter 20
Modulation of Human Dendritic Cells by Highly Virulent Pathogens

Catharine M. Bosio

20.1 Introduction

Since the recognition of microorganisms as the etiological cause of disease, scientists have studied the intimate interaction between pathogen and host in hopes of developing effective vaccines, diagnostics, and therapeutics. In turn, these studies have greatly enriched our understanding of both the microbial world and our own immune system. Early on, it became apparent that successful infection, replication and dissemination of many of the most virulent pathogens was directly tied to usurpation of host pathways that regulate and temper the immune response or that directly suppress inflammation and antigen presentation in total. Here we will discuss the modulation of host immune responses, with a special emphasis on dendritic cells and *in vivo* models by the highly virulent pathogens, Ebola and Marburg viruses (EBOV and MARV, respectively) and the Gram negative bacterium, *Francisella tularensis*.

20.2 Ebola and Marburg Viruses

MARV and EBOV are both members of the Filoviridae family. MARV was identified following an outbreak in Marburg, Germany in 1967 [1]. Nearly a decade later, following an outbreak in Zaire, EBOV was identified [2]. Since that time, both EBOV and MARV have caused multiple outbreaks, primarily throughout Africa, although isolated incidences have been reported in colonies of non-human primates in Phillipines and in the United States [3, 4]. These outbreaks often occur without warning. Furthermore, the reservoir of MARV and EBOV has not been unequivocally identified. Thus, the study of these lethal viruses, in their natural setting, has been difficult at best. Both viruses are non-segmented, single strand RNA viruses that encode 7–8 genes, respectively [5, 6]. Four of these genes (NP, VP35, VP30, and the RNA-dependent RNA polymerase L) encode the proteins associated with the nucleocapsid [5, 7]. The remaining three genes found in EBOV and MARV include GP, VP40, and VP24 [5]. GP encodes the primary glycoprotein located on the surface of virion particles [8, 9]. VP40 is located beneath the viral membrane and is thought to aid in both maintaining structural stability of the virus as well as mediate assembly and budding from infected host cells [10, 12]. EBOV encode an eighth protein designated sGP [13, 14]. Synthesis of sGP occurs following translation of non-edited mRNA encoding GP. Further, unlike GP, sGP is not membrane bound but is secreted from infected cells [15]. VP35, VP24, and sGP have all been implicated in the modulation of the host immune response and the potential function of each will be discussed below.

20.2.1 *Francisella Tularensis*

Francisella tularensis is a Gram-negative, facultative, intracellular bacterium and the causative agent of tularemia. In 1912, this bacterium was first identified by McCoy and Chapin as being the causative agent of a plague-like illness in ground squirrels in Tulare County, California [16]; However, it was not until 1914 that it was identified as a human pathogen [17]. Since that time, *F. tularensis* has been separated into 4 subspecies. Although at least three of these subspecies have very similar genomes (>90% similar), they vary widely in their ability to infect and kill humans. Two subspecies, *novicida* and *mediasiatica*, are relatively attenuated for humans, with a handful of documented infections only occurring in immunocompromised individuals [18, 19]. The remaining two subspecies, *holartica* and *tularensis*, are well known to cause illness in humans. However, whereas infections with *holartica* rarely cause fatal disease, subspecies *tularensis* can cause severe, lethal disease if left untreated [20, 21]. Within 45 years of the discovery of *F. tularensis,* an attenuated vaccine strain, designated Live Vaccine Strain (LVS), was developed [22]. Unfortunately, LVS provided only moderate protection against median numbers of aerosolized bacteria (i.e., 1,000 bacteria) and failed to impact cutaneous infections among vaccinated laboratory workers [23]. However, LVS became a useful tool to initiate studies

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concerning the immunology of *F. tularensis* since it retained some virulence for mice. It should be noted that virulence in the murine model is highly dependent on the route of infection (e.g., intraperitoneal versus intranansal) [24]. Although this LVS infection model has provided important insight pertaining to the mechanism by which the vaccine engenders protection, it is not clear if data generated from this model will directly correlate with infections mediated by the fully virulent subspecies, *tularensis.*

20.3 Dendritic Cells

As detailed elsewhere [25], there are a myriad of mechanisms in place to provide the host with non-specific defense against invading pathogens. Some of these include physical barriers such as skin and fibrin clots, proteins designed to trap and aid in the phagocytosis of microbes such as pulmonary surfactants, while others include more direct and intimate killing mechanisms such the generation of highly toxic, anti-microbial, oxygen radicals generated in macrophages and neutrophils following engulfment of microorganisms. Macrophages and neutrophils are not the only phagocytic cell type capable of mounting a response following infection. In the early 70's a novel cell type, designated the dendritic cell (DC), was described by Steinman and colleagues [26]. DC are now recognized as an essential cell in the immune system. Taking up residence in nearly every tissue in the body, DC patrol the periphery for antigen. Following uptake of antigen (i.e., microorganism) DC typically become "activated"; changing both the expression of cell surface receptors to enable migration to the draining lymph node and effective antigen presentation to T cells. Activated DC also secrete a wide variety of pro-inflammatory cytokines such as TNF-α, IL-8 and IL-12 to alert the host of the presence of a pathogen (as reviewed, [27, 28]). Given their ability to both alert the immune system to invading pathogens and stimulate antigen specific T cell responses, DC serve to bridge the innate and adaptive arms of the immune response. However, the importance of these cells has made them a natural target for a number of pathogens including, Ebola and Marburg Viruses (EBOV and MARV, respectively) and *Francisella tularensis*, as discussed below.

20.4 Modulation of Innate Immunity

Despite their obvious differences, infections caused by EBOV, MARV, and *F. tularensis* bare striking similarities. Both the Filoviridae and *F. tularensis* cause an acute, typically lethal, disease which , in fatal cases, is marked by septic shock-like symptoms near the end of the disease's course (as reviewed, [29, 30]). In large outbreaks, mortality following EBOV or MARV infection can range between 70%-90% [2], whereas mortality due to *F. tularensis* infections is lower, ranging from 1%–30% [20]. Although the specific dose required for Filovirus infection in humans is not known, it is presumed to be very low, given the ease of transmission to unprotected mucosal surfaces during an ongoing outbreak [31]. Similarly, the reported dose of *F. tularensis* required to cause pulmonary disease in humans is as few as 15 bacteria [32]. The typical mean time to death (MTD) in humans and animal models of all three pathogens averages approximately 5–7 days. However, despite the acute nature of Filoviruses and *F. tularensis* mediated disease, there is strong correlative evidence that, in some individuals, the virus or bacterium can persist for months [33, 34]. Perhaps the most curious similarity between Filovirus and *F. tularensis* mediated disease is that the early stages of infection are marked by an impressive absence of detectable pathology or other indication (e.g., cytokine and chemokine production) of microbial invasion despite exponential replication of the pathogens. This tranquil period is followed by an irreversible, rapid, violent storm of cytokines and systemic tissue damage as the pathogen enters the blood stream exerting a final, fatal "coup de grace."

One unifying theme that may explain the shared ability of both Filoviruses and *F. tularensis* to replicate and to disseminate virtually undetected in the host is their subversion of DC. Both pathogens target DC during the early stage of infection, typically entry, in the host, for rapid, exponential replication. However, as discussed below, the manner in which they accomplish replication without detection by the DC or by other host cells appears to be different.

20.4.1 Ebola and Marburg Viruses

Both viruses can infect and replicate in a variety of cell types, including DC, monocytes, macrophages, and endothelial cells [35, 36, 37, 38]. However, both EBOV and MARV induce secretion of a several chemokines and pro-inflammatory cytokines such as RANTES, MIP-1α, IL-8, IL-6, and TNF-α. Since this profile of cytokines would readily alert the host to infection and is not typically seen until the end stages of disease, it has been suggested that these cell types are not the initial target of EBOV and MARV *in vivo*. Rather, an important *in vivo* study using non-human primates suggested that the first cellular targets of EBOV infection *in vivo* are DC [39]. Further investigation has revealed the importance of DC during the initial stages of EBOV and MARV infections.

In contrast to monocytes, macrophages, and endothelial cells, infection of human DC (hDC) with either EBOV or

MARV fails to induce detectable chemokines or pro-inflammatory cytokines [40, 41]. Further, human DC fail to increase expression of specific cell surface receptors associated with antigen presentation and T cell stimulation, such as HLA-DR, CD86, CD80, and CD40 and poorly induce proliferation of allogeneic T cells in a mixed lymphocyte reaction (MLR) [40, 41]. It is possible that Filoviruses simply enter and replicate hDC undetected, without interfering with the overall responsiveness of these cells. To test this possibility, EBOV and MARV infected hDC were stimulated with an unrelated microbial antigen, lipopolysaccharide (LPS). LPS readily induced production of TNF-α from hDC, regardless of whether EBOV or MARV was present [40]. In contrast, EBOV and MARV infected hDC were completely refractory to production of IFN-α in response to treatment with replication deficient Venezuelan equine encephalitis replicon particles (VRP) [40]. In that report, the active suppression of IFN-α production was directly attributed to the non-structural protein VP35. Previous reports also suggested that VP35 was a potent inhibitor of IFN-α, but this was the first demonstration in terms of an active viral infection [42]. IFN-α is critical for host control of viral infections, therefore the ability of EBOV and MARV to actively suppress IFN-α production among infected cells could be an essential step in the colonization of host, early after infection. Furthermore, this suggested that strategies to promote anti-viral IFN-α production following exposure of the host to EBOV and MARV may be in vain since the vary cells that need to be activated are refractory to IFN-α production. Indeed, treatment of cynomolgus macques with recombinant IFN-α failed to effectively protect animals even when delivered within the first 18 hours of infection [43]. Since the identification of VP35 as an inhibitor of IFN-α production, VP24 has also been shown to interfere with production of IFN-α [44]. However, the mechanism by which VP35 and VP24 suppress IFN-α appear to be different. Whereas VP35 directly prevents activation of IRF-3, a host protein critical for transcription of Type I IFN, VP24 disrupts IFN-α production by blocking accumulation of phosphorylated STAT1 in the nucleus [42, 44, 45]. Together these observations suggests that novel, effective, anti-filovirus treatments will likely require targeting multiple viral proteins for optimal results.

20.4.2 Francisella Tularensis

Modulation of host immune responses by *F. tularensis* was observed in humans during the early 1960s when Greisman et al., observed that humans infected with virulent *F. tularensis* were refractory to endotoxemia [46]. Unfortunately, more than 40 years later, the mechanisms of this tolerance remain elusive. However, in the past few years, renewed interest in *F. tularensis* has provided important clues as to the method by which *F. tularensis* successfully interferes with host immunity to cause lethal disease.

Similar to EBOV and MARV, *F. tularensis* induces very different immune responses, depending on the host cell it is invading. For example, infection of human monocytes with virulent *F. tularensis* elicits a wide array of pro-inflammatory cytokines, including IL-1β and IL-6 [47]. However, as also observed in Filovirus infections, these soluble mediators are all but absent during a large part of the infection and are only routinely observed shortly before the host succumbs to infection [48, 49]. This striking similarity to EBOV and MARV interaction with human DC provoked us to propose that, during the early stages of infection, *F. tularensis* may be modulating responsiveness of DC similar to Filoviruses. In agreement with this hypothesis, infection of human DC with virulent *F. tularensis* failed to elicit secretion of detectable concentrations of pro- and anti-inflammatory cytokines [50]. Further, when cultures containing *F. tularensis* infected DC were exposed to *E. coli* LPS. Both directly infected and uninfected bystander cells were hampered in their ability to produce TNF-α and IL-12 in response to this potent microbial stimulus. These results suggest that interference with hDC function may occur at a more global level than previously appreciated [50]. Furthermore, the global suppression of human DC discussed above is in agreement with *in vivo* studies in which mice infected with a low dose aerosol of virulent *F. tularensis* failed to activate resident macrophages and dendritic cells or recruit neutrophils and monocytes in response to LPS delivered directly in the lung during the first 24 hours after infection [48].

Poor responsiveness to repeated stimulation of cells by microbial antigens is known as microbial tolerance. It is believed that mammalian hosts developed this tolerogenic mechanism as a way to control pro-inflammatory responses while clearing infection. Although a number of microbial products are capable of inducing tolerance, the most well studied molecule is LPS (as reviewed, [51, 52]). *F. tularensis* is a gram-negative pathogen and, thus, possesses LPS. However, *F. tularensis* LPS (FT LPS) is somewhat unique in that 100- to 1000-fold higher concentrations of FT LPS are required to stimulate the same responses observed with *E. coli* LPS [50, 53, 54]. Our laboratory has shown that while high concentrations of purified Schu S4 LPS could suppress human DC responsiveness to *E. coli* LPS, depletion of LPS from *F. tularensis* conditioned medium failed to completely restore responsiveness of human DC to *E. coli* LPS [50]. Rather, treatment of hDC cultures with gentamicin, throughout the culture period with *F. tularensis* to limit extracellular replication of the bacterium, partially restored the ability of hDC to produce TNF-α in response to *E. coli* LPS [50]. Curiously, gentamicin treatment failed to restore the ability of hDC to produce IL-12 in response to *E. coli* LPS.

Furthermore, addition of neutralizing antibodies directed against IL-10 and TGF-β, two host derived cytokines associated with inhibition of hDC function, failed to restore hDC responsiveness to *E. coli* LPS [50]. The role of TGF-β in mediating suppression following *F. tularensis* infection has also been explored. However, similar to *in vitro* results, neutralization of TGF-β *in vivo* failed to completely restore host responsiveness to infection [48]. Together, these observations suggests that suppression of hDC function by virulent *F. tularensis* may occur in by multiple mechanisms. For example, inhibition of TNF-α production may occur when the bacterium interacts with receptors present at the cell surface. In contrast, interference with production of IL-12, specifically IL-12p40 may occur following invasion of the cell and during intracellular replication of *F. tularensis*. Identification of the specific mechanisms of inhibition of hDC and confirmation of these activities *in vivo* will be critical for development of novel prophylaxis, therapeutics, and vaccines directed against tularemia.

20.5 Conclusions

Successful infection and replication of pathogens can be an integral part of their survival. The mammalian immune system has developed to live in harmony with some microorganisms while simultaneously mounting effective defense against more aggressive invaders. DC are an important cell for initiating and mediating the signal for either symbiosis or defense. Thus, when DC are targeted and manipulated by pathogens such as EBOV, MARV, and *F. tularensis,* during early stages of infection, it typically allows an "all access pass" to the host environment. Decoding the specific mechanisms by which these and other pathogens interfere with DC function will be a critical step in designing new therapeutics and vaccines against these highly virulent microbes.

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Chapter 21
Making Friends in Out-of-the- Way Places: How Cells of the Immune System Get Together and How They Conduct Their Business as Revealed by Intravital Imaging

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21.1 Introduction

During embryonic and early post-natal development, many cells migrate over substantial distances, changing their spatial relationship to other cells of the same or distinct differentiation state and engaging in cross-talk that helps establish the tissue and organ structure of the adult. However, once this body plan has been established, such active migration and positional exchange is rare in the undiseased state. Even in epithelial tissues that undergo constant renewal, there is an absence of free cell movement; rather, underlying cells replicate and push the older cells to the surface (skin) or apex (villus) where they are sloughed, having followed a fixed path of movement from birth to death.

A notable exception to this paucity of active cell migration in the adult involves the cells of the innate and adaptive immune systems [1]. These hematopoietic elements move through the body in the blood, some exiting into uninflamed peripheral tissues to take up residence as sentinels and others moving into inflamed tissues to participate in host defense or wound healing. Yet others routinely enter secondary lymphoid tissues such as lymph nodes (LNs), spleen, and Peyer's patches to survey for signs of infection. Within these tissues the T and B lymphocytes distribute into specific subdomains but can rapidly change their locations upon antigen activation and/or inflammatory signals. Dendritic cells (DCs) arrive from the periphery via the lymph and contribute to tolerance and development of protective immunity through interactions with these lymphocytes. Activated lymphocytes leave these lymphoid tissues to enter peripheral sites and act as effectors, penetrating deep into organized tissues or taking up residence again in the bone marrow from which their ancestors arose.

In each of these cases, the immune cells must follow molecular cues that guide their movement into and within a tissue site, whether lymphoid or not. These cells need to know what locations to access or whether, once they have entered, to take up sessile or active migratory residence and how to find other cells when communication is required. How the latter cell pairings are achieved is particularly intriguing when rare antigen-specific lymphocytes or small numbers of antigen-bearing DCs are involved.

Using standard methods of cellular immunology and microscopy, a great deal has been learned about immune cell localization in the healthy individual and the changes in this patterning in the presence of infection, tissue damage, tumor development, or autoimmunity [reviewed in [2]]. However, the underlying highly dynamic nature of these events has been largely invisible until recently. The first inroads into visualizing the actual events involved in immune cell behavior *in situ* came from the application of video-imaging methods to studies of leukocyte adhesion to inflamed vessel walls, studies that helped solidify the rolling, sticking, and transmigration paradigm [3]. More recently, we and others have applied emerging advances in confocal and especially two-photon (2P) imaging to the intravital analysis of a wide variety of immune cells in different tissues and distinct circumstances; we are seeking to gain a better understanding of just how motile the cells really are, what paths they follow as they move, what physical and/or chemical signposts are used to guide this migration, how cells involved in cooperative responses find each other, and where and when effector cells act (reviewed in refs 3–12).

This review summarizes the work done in our laboratory over the past several years and addressed many of the above-mentioned questions. The observations made during these studies have both reinforced existing paradigms and have also suggested new ways of thinking about the dynamic aspects of immunity. We place special emphasis on what we see as a hierarchical organization of lymphoid tissue in support of the cell–cell interactions underlying adaptive immune responses in which gross co-localization in a LN, for example, is translated into locally directed migration that brings cells efficiently into contact within small subdomains of that tissue. Our overview also describes the extension of such intravital imaging to sites outside of organized lymphoid tissue and highlights how related paradigms control immune cell behavior in these locations as well.

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21.2 Explant and Intravital Live Tissue Imaging

The use of improved confocal and especially 2P microscopes, for the dynamic high-resolution imaging of lymphoid and peripheral tissues or organs, has markedly advanced our understanding of the behavior and function of immune cells *in situ*. The details of the methods used for such imaging studies have been extensively reviewed by us and by others [3, 5, 9, 10, 11, 12], so we will limit our discussion to the practical aspects of these techniques.

The choice between using single-photon excitation confocal microscopy and 2P excitation microscopy for explant and intravital applications is generally made based on the depth of imaging requirements. A 2P excitation microscope provides the ability to image much deeper beneath the surface of a given tissue (up to 700 μm in some brain slice preparations and 300 μm in most lymphoid tissues) as compared with a conventional confocal system (maximum depths of 80–100 μm). For organs such as the LN, which have a heterogeneous architecture that varies as one moves away from the tissue surface, 2P excitation represents the only way of making high-resolution observations of many cell types and structures. An additional advantage of 2P excitation is that it tends to induce less phototoxicity in the sample thereby maintaining physiological conditions and allowing for extended imaging sessions (>5 h). Finally, because fluorescence excitation and emission occur only at the focal plane of the objective, the detection optics for a 2P system are greatly simplified compared with a confocal microscope. This fact [specify which fact] has facilitated an increase in the number of home-built 2P microscopes [13] currently being used by many investigators.

However, the advantages gained using 2P excitation-based imaging systems are not without some cost. The axial resolution of a 2P instrument is lower than that of a confocal microscope in part because of the longer wavelength of excitation light used. Infrared (IR) light also produces tissue heating more readily than shorter wavelength illumination (think infrared lamps that heat food or bathrooms); thus, at higher laser power settings, the tissue being imaged can suffer severe heat damage, and it is not difficult to observe charring of the sample surface during a long imaging session when using high input power. Finally, the reliance of the majority of 2P systems on a single IR laser tuned to a specific wavelength can pose problems when imaging multiple different fluorescent probes that may have very different optimal 2P absorption peaks. This [specify what becomes a particular issue] becomes a particular issue when trying to image certain combinations of organic dye-labeled cells (excitation maxima 800 nm) and fluorescent protein-expressing cells (excitation maxima >900 nm). While 2P microscopes consisting of multiple IR laser inputs are becoming more common, the high cost of an individual laser ($150,000) decreases the practicality of these systems.

Regardless of whether confocal or 2P microscopy is used, a fundamental requirement of explant and intravital imaging is that the objective of the microscope must be positioned as close as possible to the surface of the tissue or organ of interest so that one can image to any useful depth, meaning that except for the skin or eyes, one must surgically expose the tissue or organ of interest and make it accessible to the objective. Working out the best surgical approach and the details of how to minimize tissue damage and inflammation while maintaining the physiology of the animal during a prolonged imaging session are among the most difficult aspects of acquiring informative intravital 2P imaging data. Some tissues are better accessed using an upright microscope and other using an inverted instrument; an inverter can be used (at the cost of some loss in signal intensity and flexibility in choice of excitation wavelength) to provide both imaging approaches with a single instrument. Custom-engineered animal holders, special perfusion and heating systems, optical windows, and other inventive methods are required to conduct a broad imaging-based research program involving more than a single target site [14, 15, 16]. Once the surgical issues are resolved, collecting information about a region within a tissue using either a confocal or 2P microscope involves first sweeping the laser beam in the *x*–*y* plane to capture an image at a particular tissue depth. The focus of the microscope is then changed (this is called a step in the *z* dimension) and a new *x*–*y* image collected. This process [specify what process] is repeated until a three-dimensional (3D) region has been imaged. Generation of time-resolved data sets is accomplished by iteratively capturing the same 3D region over time to generate 4D image sets. In making such a collection, one has to balance the competing desire to cover as large a volume as possible with the need to spatially resolve structures (cells, dendrites) and temporally resolve cell interactions. Ideally, we would always capture imaging data sets at the maximum *x*, *y*, *z*, and *t* (time) resolutions achievable by the microscope being used; however, this is impractical for several reasons. Increasing spatial resolution by increasing the number of pixels captured in the *x*–*y* plane and/or the number of *z* planes acquired will drastically increase the amount of time it takes to capture a single 3D stack. The longer the capture time, the greater the exposure of the tissue to potentially damaging laser excitation and the lower the frequency between individual time points. This latter point becomes particularly relevant when tracking cells, given that within a dense field of moving cells the path of a cell over time cannot be accurately followed if the cell moves more than its length or diameter between each volume collection. Thus, the entire series of *x*–*y* images in the *z* dimension must be obtained in less time that it takes the cell to move a short distance. This rate [what rate, please specify] in turn limits how much emitted light can be collected, the laser power

necessary for an adequate signal in that time window, and whether any signal averaging can be performed to limit noise.

By imaging the same volume repeatedly over time, one can merge these time-separated data sets to prepare movies showing the motion of cells and cell processes. The movies available as supplementary data for published papers that utilize these imaging methods are typically prepared by compressing the data in each 3D image stack from a 4D data set into a 2D flat image using the method of maximum intensity projection. Such movies, while easy to view, can be misleading with respect to the *z*-level position of objects of interest, and one must assume that the investigators have analyzed their data in detail, *z*-slice by *z*-slice, to validate the accuracy of semi-automated cell-tracking algorithms and to determine the number and duration of cell contacts (within the axial resolution limits of the data set) in a given 4D data set. Miller *et al.,* [14] have introduced a method for color-coding cells in terms of their *z* position in a volume so that this information is present in the maximal projection movies, but this technique is not practical for displaying multiple cell types with distinct fluorescent labels and when more than a small number of moving cells is present in a field, the constantly changing coloring complicates routine viewing.

These imaging methods can be used to collect data in experimental situations involving just a single color of fluorescence, but this method provides very limited information about the tissue being studied. More typically, two to as many as four or five colors are used simultaneously to allow different cell types and non-hematopoietic components of tissues such as collagen fibers, vascular endothelium, blood flow, or mesenchymal cells to be visualized in the same imaging field [12]. This approach [specify which approach] permits the design of experiments in which wild-type and mutant cells in two different colors can be compared for their behavior within the same region of the same tissue at the same time, limiting the number of unknowns that might influence such behavior when observed separately in different regions of the relevant organ or in the same type of tissue in different animals. It also allows better monitoring of the state of the anesthetized animal and whether inadvertent physiological perturbations induced by the surgery required for the imaging have altered cell behavior. Finally, it also permits better interpretation of the data, because more of the environment in which the cells are moving is visualized, allowing the influence of structural components on migration to be seen directly.

21.3 Lymphocyte Entry, Migration, and Interaction within a LN

Using the methods just described, our laboratory has examined the various steps involved in both the early phases of adaptive immune responses within LNs and the behavior of adaptive effector cells as well as innate immune cells in peripheral tissue sites. We begin this review with a description of the events involved in entry of lymphocytes into organized lymphoid tissues and their migration within those tissues, followed by a discussion of the changes to this behavior that accompany inflammation arising from infection or vaccination.

21.4 LN Entry Via High Endothelial Venules

The anatomic site and molecular basis for naive lymphocyte entry into non-inflamed LNs is well established [1, 17, 20]. Specialized vessels, termed high endothelial venules (HEVs), display the CCR7 ligand CCL21 as well as l-selectin ligands on their luminal membranes. Contact of blood-circulating $CD62L^{+}CCR7^{+}$ T- and B-lymphocytes with the selectin ligand induces rolling. Signaling in these rolling cells induced by CCR7 binding to the CCL21 on the endothelial surface induces firm adhesion (‘sticking’) primarily but not exclusively mediated by the integrin leukocyte function-associated antigen-1 (LFA-1) (CD11a/CD18). These adherent lymphocytes then migrate across the endothelium; although whether they do so by migrating through the endothelial cells themselves or between these cells remains unresolved.

Although this 'rolling, sticking, transmigration' paradigm is well established for lymphocytes and other leukocytes based on a combination of genetic, molecular, and histological studies, and although it [“it” has an unclear reference, specify what] has been visualized using intravital video imaging of various leukocyte populations entering non-lymphoid tissues, it has only recently been directly observed within intact LNs. We have studied the details of lymphocyte entry and migration within the LN environment using a novel method for creating chimeric mice. By transferring syngeneic non-fluorescent bone marrow into lethally irradiated recipients, universally expressing enhanced green fluorescence protein (EGFP) under control of the ubiquitin promoter (Ub-EGFP mice), we have obtained animals with very brightly fluorescent endothelial and other non-hematopoietic stromal cells in LNs whose lymphocyte and myeloid populations are non-fluorescent [21]. After transferring dye-labeled lymphocytes in distinct colors into these chimeric mice, we have been able to image the movements of the lymphocytes with respect to the stromal elements as they enter and move within this organized lymphoid tissue. As expected, naive T cells roll and then adhere firmly to the luminal surface of the green fluorescent cuboidal lining cells of the HEV, following which they penetrate across the endothelial cell layer. Our initial imaging studies were not conducted at sufficiently high resolution to address the question of whether the migration

is trans-endothelial or inter-endothelial, but the technology is capable of providing such an answer. Additional studies are in progress on this important point. The lymphocytes then enter the perivascular channel (PVC) formed by the basal aspect of the endothelial cell basement membrane and the surrounding sheath of fibroblastic reticular cells (FRCs). The T cells exit the PVC through openings between overlapping FRCs, which are organized much like shingles on a roof.

21.5 A Stromal Roadway for T- Lymphocyte Trafficking

Based on the original reports of Miller and colleagues [15, 22, 27] demonstrating the apparently random migration of naive lymphocytes at 7–10 μm/min within the LN parenchyma (represented by black space in the movies produced in these studies), one would have expected newly arrived lymphocytes to move freely and without constraint once clear of the FRC layer around the HEVs. In contrast to this expectation, we noted that as the lymphocytes exited the bloodstream ‘highway’ within HEVs via the FRC-delineated 'off ramps,' they moved directly onto the fiber-like processes of FRCs. These FRCs processes or fibers served as local ‘roads’ on which the T cells migrated within the LN parenchyma with the lymphocytes rarely entering the open space between the intersecting fiber network. Instead, they moved in a polarized fashion along these stromal cell paths and turned at fiber intersections with the same angles as the overlapping FRC strands [21].

The apparent “random walk” behavior noted by others imaging naive T cells, within the LN, thus turned out to be the complex trafficking of the lymphocytes along a defined set of pathways created by the FRC network that was not made fluorescent and hence was not visualized in these prior studies. However, even though our data clearly show that T cells move along specific structural elements in LNs, and are not freely 'swimming' in a roiling cellular 'soup', the FRC network is sufficiently complex, and the turns of T cells sufficiently frequent that the net behavior under steady-state (non-inflamed) conditions is the equivalent of a random walk at the macroscopic level (A. Y. C Huang and H. Qi *et al.*, unpublished observations). Whether there is a subtle directional bias in migration that is missed due to the limited depth and duration of imaging currently possible within the LN remains to be determined. This issue [specify which issue] is an important one to return to as new methods and instrumentation enhance our capacity to see deeper into the LN and track cells for longer periods.

What are FRCs? These cells have been well studied and constitute the primary stromal supporting cell type in the paracortical region of the LN [28, 29, 30], creating a dense 3D network comparable to the intersecting spider webs. They are also present in the white pulp of the spleen, specifically the periarteriolar lymphoid sheath (PALS) or T-cell zone [31]. In the LN, FRCs have been shown to create the conduit system that consists of a sheath of FRCs around fluid-filled reticular fibers containing a central bundle of collagen strands and several extracellular matrix proteins such as laminins and fibronectin [28, 29, 30, 32]. Tracer studies have shown that the conduits provide a distribution system for low molecular weight material (antigens, chemokines, and cytokines) arriving in the subcapsular sinus of the LN via the afferent lymph. DCs are aligned on the FRC conduits [30], and processes from the DCs can penetrate the basement membrane of the sheath to sample antigens and other molecules in the lumen of these structures [32]. Thus, this system provides a means of transmitting information from a peripheral site of infection to the LN via the lymph and within the LN to the DCs.

The sessile network of DCs observed by Lindquist *et al.,* [33] appears to reflect the resident DC population positioned on the FRC network [21]. Activated DCs arriving via the afferent lymph and migrating across the subcapsular sinus into the interfollicular regions of the LN may settle on open spaces on this platform [8] where they are ideally situated to be met by the T cells moving on the FRC network. The resident DCs can themselves be activated by signals received from recognition of pattern-associated molecular patterns (PAMPs) within the conduit flow, as well as by host-generated mediators such as cytokines [e.g., tumor necrosis factor (TNF)] and chemokines.

Given the dense network of FRC-enwrapped conduits, ending on HEVs, it makes sense that there will be a substantial number of activated, antigen-presenting DCs in the region just outside of these lymphocyte entry points. Indeed, Itano *et al.,* [34] have shown that DCs in this location are the first to process and present antigen draining from a skin site, and Bajénoff *et al.,* [35] have demonstrated that T cells preferentially interact with and adhere to antigen-bearing DCs in this location shortly after the T cells arrive in the LN. Together, these data indicate that T cells passing across HEVs enter the T zone on FRC fibers emanating from the sheath around the vessel; as they move along these strands, they rapidly contact DCs that are also attached to the FRC fibers and that have acquired and processed antigen. The antigen-specific T cells stop upon contact with these DCs and undergo activation, which various studies suggest results in the onset of cell division [15, 36] although not necessarily differentiation to effector status [34, 37, 38]. These observations provide a new view of how lymphoid tissues, LNs in particular, are organized anatomically to facilitate interactions between rare antigen-specific lymphocytes and sparse antigen-presenting DCs [21, 39].. Rather than being randomly located throughout the T-cell zone, antigen-bearing

DCs are frequently clustered near the HEV entrances used by naive or central memory lymphocytes. In addition to this concentrating effect, interactions between these cell types are further facilitated by arraying the DCs at strategic sites along defined pathways (the FRC network) that the lymphocytes use for migration within the LN parenchyma. Rather than the conventional view of LNs as sites that enhance adaptive immunity by gross co-localization of antigen and antigen-specific cells within a large organ (the LN), we therefore see that another level of organization exists that further enhances the likelihood of contact between an antigen-specific lymphocyte and its cognate ligand. In addition, this antigen is displayed by a cell whose association with the conduit network permits receipt of inflammatory signals leading to upregulation of the costimulatory molecules and cytokines essential for an effective immune response. As they enter the LN, non-resident DCs already activated in this manner also initially concentrate near HEVs and as they enter the LN and travel through the interfollicular regions rich in HEVs. These migrating presenting cells eventually settle down on the FRC network in the deeper paracortex, where they can interact with T cells that either failed to be captured by a relevant presenting cell soon after HEV entry or with already activated T cells whose further expansion and differentiation into effector or memory cells requires additional antigen-specific stimulation.

What limits T-cell movement to the FRC fiber network and propels the T cells on their way along these paths? Scanning electron microscopic images show microvilli of the T cell contacting FRCs [21], suggesting that integrins known to cluster on such cell processes [very late antigen-4 (VLA-4) in particular] might be involved in T cell–FRC adhesion. However, *in vitro* imaging of perfused thick slices of LNs have failed to show an effect of T-cell pretreatment with antibodies against either VLA-4 or LFA-1 on the pattern or pace of T-cell migration on the FRC fibers (L Y Koo, unpublished observations). Likewise, antibodies to CCL21 alone or in combination with antibodies to CXCL4, which together are the major chemokines recognized by CCR7 and CXCR4 present on naive T cells, failed to markedly reduce migration in this experimental model. However, other studies using LN slices or intact LNs from mice lacking expression of CCL19 and CCL21 (*Plt* mice) have documented a measurable reduction in the migration velocity of T cells under these conditions as compared with the physiological situation [40, 41, 42], indicating that CCR7 ligands contribute to, although fail to fully account for, the stimulus that promotes T-cell mobility. These data suggest caution in interpreting negative results such as those we obtained using the slice method, perhaps because it is difficult to adequately neutralize or block the relevant molecules using this imaging approach and exogenously applied antibodies.

Combined with evidence that T cells treated *ex vivo* with low concentrations of pertussis toxin can access LNs but stop migrating soon after arrival [41] and data showing a role for RGS proteins in regulating T-cell mobility within LNs [43], it seems that T-cell movement along FRC fibers is driven largely, if not exclusively, by signals mediated by Giα-coupled receptors. Given that CCL21 is present at high levels on the surface of FRC fibers [44], it appears likely that lymphocytes migrate within the LN under non-inflammatory conditions primarily as a consequence of chemokinetic stimuli that propel them along the FRCs roadways. In this regard, the observation by Mueller *et al.,* [45] that expression of CCL21 is downregulated by FRCs during the immune response to several pathogens is interesting and suggests that tuning the level of CCL21 in LNs may be an efficient way to regulate the ongoing response by controlling lymphocyte motility on the FRC network. This notion of movement induced by FRC-associated signals is also consistent with our evidence that the T-cell zone of the LN, that is, the region within which T-cell migration is confined under steady state conditions, is precisely delimited by the FRC network, and that the failure of naive T cells to enter B-cell follicles corresponds to the absence of paracortical FRC fibers extending into the follicles [21].

The limitation of naive T-cell movement to the CCL21-decorated FRCs in the paracortex raises several interesting questions, chief among them how activated $CD4^+$ T cells move from the T zone into the B follicles following activation and eventually differentiate into follicular T-helper cells (T_{FH}) localized to germinal centers (GCs) [46,47]. Some insight into this question came from analyzing the behavior of naive B cells within the LN. These lymphocytes enter the parenchyma of the node via HEVs [1, 20], as do T cells, and our data indicate they then move along the same intersecting FRC pathways as do naive T cells [21]. However, in contrast to the T cells, the B cells readily move off the FRC processes as these terminate at the follicle border, with the B lymphocytes now migrating instead along follicular DC (FDC) fibers that course through the B-cell region.

This ability to switch from FRC to FDC stroma is most likely controlled in large measure by CXCR5, a chemokine receptor highly expressed by naive follicular B cells [48]. CXCR5 expression renders B cells responsive to the chemokine CXCL13, which is abundantly produced by and displayed on FDCs in follicles but is absent on the FRC network [49]. Indeed, B cells lacking CXCR5 accumulate at the follicle border but do not enter [48].. Because surface expression of CXCR5 is also a key characteristic of T_{FH} cells [46, 47], CXCR5 is likely to be at least one of the controlling factors that allows activated T cells to migrate on FDC fibers. However, $CD4^+$ T cells lacking CXCR5 can still give rise to a significant, albeit reduced, number of follicle – or GC-localized T_{FH}-like cells [50]. This finding [specify what

finding] suggests factors other than CXCR5 contribute to the follicular homing ability of T cells. We have recently developed model systems and long-term imaging methods for tracking inter-zonal migration of activated T cells to specifically address this issue (H. Qi *et al.*, unpublished observations).

The movement of naive B cells along FDC tracks is also consistent with observations made by other investigators studying cell dynamics in GCs. Several laboratories have reported that GC B-cells make intimate contact with FDCs [51, 52, 53], presumably scavenging for antigen bound to these stromal cells. In contrast to the FRCs, which show little spontaneous movement or deformation due to T-cell migration in the paracortex, FDCs appear to undergo either spontaneous undulations or are readily displaced by the migrating B cells [21].

21.6 Non-Random Migration of T cells in Inflamed LNs

Accepting that LNs have as a major purpose the facilitation of contact between antigen-specific lymphocytes and their cognate antigens, it is useful to consider the quantitative aspects of this activity in the case of T cells and processed antigen presented by specialized cells (DCs). Recent elegant analyses using tetramers to determine the number of naive $CD4^+$ T-cells specific for a particular peptide-MHC combination in a normal, non-transgenic mouse suggest that there are typically 50–200 total antigen-specific T cells in an adult mouse [54]. Assuming a random distribution in the absence of antigen, a given LN would contain only 1–10 such cells at any one time. Although the literature varies in its estimates of the number of antigen-bearing DCs in a draining LN after skin delivery of antigen by natural (infection) or artificial (injection) means [55, 56, 57], it appears that the number can be as few as 10, which is consistent with the ability of 100 systemically injected DCs to prime a mouse [58]. The question thus becomes what strategy would help ensure that the rare specific T cells have a high likelihood of contacting one of the few antigen-bearing DCs present in only a single LN and do so early enough after initial antigen delivery to be useful in fighting an infection [59]. This issue [specify what issue] becomes even more cogent and complex when there is a need for interaction or communication between two different antigen-specific T cells each present at these low frequencies in a naive repertoire, as has been documented in the case of $CD4^+$ T-cell help for $CD8^+$ T-cells [60].

Several groups using 2P imaging have examined the rate at which the T-cell repertoire is scanned by DCs [23, 24]. The numbers obtained in these studies vary over more than an order of magnitude with the highest estimate coming from the work of Miller *et al.,* [24]. These investigators suggest that due to a combination of the 'high-speed' random walk of T cells, and the motion of dendrites extending far from the DC central cell body, a DC contacts 5000 $CD4^+$ T-cells per hour. From this number [specify which number], they calculate that a T cell has a 95% chance of finding at least one of 100 antigen-bearing DCs in a LN within 6 h, which is substantially less than the usual half-life of T-cell LN residence under steady-state conditions and much less than the retention time in an inflamed LN. This analysis [specify which analysis] implies that no additional mechanisms beyond random migration and DC dendrite extension are needed to ensure effective cell–cell interactions during a developing immune response.

What about the three-cell situation? Many studies have shown that in the case of $CD4^+$ T-cell help for $CD8^+$ T-cells, the two lymphocyte types must see their ligands on the same DC, although whether this must be contemporaneous or can be sequential was not determined in these previous studies [reviewed in refs 60,61]. We are now faced with the requirement that two rare T cells find the same DC, not just any of the few antigen-bearing DCs in the LN, if a productive response is to ensue. This requirement (that two rare T cells find the same DC) markedly changes the mathematics; it is unlikely that by random migration alone both the $CD4^+$ and $CD8^+$ T-cells in question will find the same DC in this time frame or even a several fold longer one, in part because once a T cell locates a cell with its antigen, it arrests motion for up to several hours.

Given these considerations, we felt it was unlikely that during an active cell-mediated immune response, all necessary cell interactions would occur efficiently based on random walk behavior. We were further encouraged to think otherwise, because it is well documented that activated DCs and T cells produce copious amounts of chemokines [62], and it seemed unlikely that such mediator secretion would have no effect in the LN environment, although this was an open question because most of the chemokines produced under such circumstances were not believed to have cognate receptors expressed on non-antigen-activated T cells [63].

To examine this [specify which issue] issue, we undertook a series of experiments examining cell dynamics in LNs under conditions in which $CD4^+$ help for $CD8^+$ T-cell memory generation could be readily demonstrated [64]. The model system involved the co-transfer of two sets of DCs, one set pulsed with a peptide (OVA323) recognized by the OT-II T-cell receptor [65] and the other set mocked pulsed, with each set of DCs labeled using a distinct fluorescent dye. After subcutaneous injection and time for migration to the draining LN, the mice were given two populations of naive T cells labeled with two other dyes, OT-II $CD4^+$ T-cells and polyclonal or monoclonal $CD8^+$ T-cells. Both of the latter gave the same results, because cognate antigen for the monoclonal $CD8^+$ T-cells was not introduced into the system, to

prevent rapid adhesion of these lymphocytes to DCs as they entered the LN.

Using this four-color system, we collected 2P imaging data and compared the frequency with which $CD8^+$ T-cells contacted the mock-pulsed versus the OVA323 (specific peptide)-pulsed DCs in the same imaging field in the same LN (a parameter we termed the 'hit rate'). Because there was some variation in the number of the two DC types in an imaging field and in the simplest case the frequency of contact with T cells would be a 'second-order reaction,' we corrected the absolute contact number measured from the imaging files by the relative frequency of DCs in the volume [64]. This experimental design provides optimal controls for the comparison of $CD8^+$ T-cell–DC interactions by normalizing the region of LN imaged, the state of the experimental preparation during the imaging session, the density, number, and location of the injected DCs, and so forth, as compared with the collection of data in separate animals given only one or the other DC preparation [12].

The results of the study were striking. $CD8^+$ T-cells interacted with the DCs that presented antigen to (and could be seen to have interacted with or be interacting with) the OT-II $CD4^+$ T-cells at four to five times the rate as they interacted with the control DCs unable to activate the antigen-specific $CD4^+$ T-cells, and this distinction could be seen even when considering DCs that were only a cell diameter (50 μ) apart in the same region of a LN. These data could not be explained by a random walk model and clearly implied the existence of some mechanism of local guidance of $CD8^+$ T-cell movement toward the DCs able to engage $CD4^+$ T-cells in a specific manner.

The obvious candidates for mediating such guidance were chemokines, but attempts to inhibit the preferential interactions with antibodies to chemokines known to interact with the CCR7 and CXCR4 receptors expressed on naive T cells were unsuccessful. While this result could be a technical failure rather than a true indication of no role for these chemokines (see the above discussion of the *in vitro* slice data using blocking antibodies to evaluate the role of CCL21 in basal migration), we considered the possibility that some of the so-called inflammatory chemokines produced by activated DCs might be involved and attempted to block the specific interactions with antibodies to several such mediators. These studies revealed a dramatic effect of blocking either CCL3 [macrophage inflammatory protein-1α (MIP-1α)] or CCL4 (MIP-1β); administration of either antibody *in vivo* reduced the hit rate for $CD8^+$ T-cells with the OVA323-pulsed DCs to the level seen with the mock-pulsed DCs [64].

These observations led to a new question: How could CCL3 or CCL4 have this effect on naive $CD8^+$ T cells, recalling that we did not include specific antigen for the $CD8^+$ T-cells in these experiments? The cognate receptor for these chemokines, CCR5, was presumed to be expressed on T cells only after antigen activation [63]. However, we found that injection of an alum-CpG adjuvant mixture or injection of PAMP-stimulated DCs without any specific antigen induced a variable but substantial fraction of the naive $CD8^+$ T-cell population to show surface CCR5 expression within a short time after LN entry [64]. Furthermore, CCR5-deficient $CD8^+$ T-cells showed the same hit rate with the OVA323-pulsed DCs as with the control DCs, rather than the four to fivefold elevated rate of interaction with the OVA323-pulsed DCs that was seen using wildtype $CD8^+$ T-cells in the presence of OT-II cells.

While the imaging data were striking and showed unequivocally that CCL3/4 played a major role in regulating the rate of $CD8^+$ T-cell contact with DCs presenting antigen to $CD4^+$ T-cells, these studies on their own did not establish the physiological importance of this effect. To examine this issue, we used a model for $CD4^+$ help-dependent $CD8^+$ T-cell memory generation, asking if inhibition of the CCL3/4-dependent recruitment of $CD8^+$ T-cells to sites of $CD4^+$ T-cell–DC interaction in the first hours to days after vaccination would affect the contribution of $CD4^+$ T-cell help to $CD8^+$ T-cell memory measured more than a month later. Strikingly, treatment of vaccinated animals with anti-CCL3/4 antibodies had little effect on the acute $CD8^+$ T-cell response, but it entirely eliminated the ability of the $CD4^+$ T-cells to provide help for $CD8^+$ memory T-cell survival and function [64, 66].

Our experiments have provided an entirely new perspective on how cell interactions involving multiple cell types occur within secondary lymphoid tissues and on the critical role of actively guided associations in development of effective adaptive immunity. For at least some of the interactions, specific guidance cues in the form of locally generated chemokines play a key role in promoting what would otherwise be statistically rare associations under random walk conditions. We do not have evidence that the DC–$CD4^+$ T-cell interaction is guided by chemokines, and it could occur as has been proposed [24, 67, 68], based on random walk kinetics, although one might imagine that chemokine guidance could enhance these interactions as well. In the case of $CD8^+$ T cells, PAMPs and/or inflammatory mediators draining into the LN from the site of inoculation lead to rapid upregulation of surface CCR5 levels; experiments are in progress to identify the specific mediators involved in this process and to determine the mechanism of the increased chemokine receptor expression (enhanced transcription, translation, or transport from stores within the T cells).

When considered together with the data on FRC-guidance of naive T cells along paths that enhance the likelihood of interaction with fiber–co-localized DCs [21], we begin to get a picture of the LN as a highly organized organ, with multiple levels of control of cell trafficking that each contributes to enabling rare cells in the lymphocyte pool to rapidly and efficiently locate antigen-bearing DCs as well as other rare

lymphocytes with which they need to cooperate for productive adaptive immune responses. The concept that seemed to emerge from the earliest 2P studies of the LN as a bag in which the relevant marbles (cells) eventually find each other as they were tossed around randomly (migrated without direction) has now been replaced by one in which there is a hierarchical set of features that promote useful interactions in a non-random manner [39] (Fig. 21.1). First, a LN draining a site of infection or vaccination serves as the locus of accumulation of antigen and of antigen-bearing DCs arriving in the afferent lymph. It also is the site of enhanced entry of lymphocytes from the naive circulating pool, brought to that LN in larger numbers by increased local arterial blood flow [69] and facilitated in their entrance by increased levels of CCL21 and intercellular adhesion molecule-1 (ICAM-1) on the HEVs. Once in the LN parenchyma, the T cells travel on discrete pathways decorated with resident and migrating antigen-bearing DCs, both of which are enriched immediately around the HEVs [21, 32, 35]. This co-localization to FRCs further decreases the search space involved in making a productive interaction between the T cells and DCs. In addition, at least after $CD4^+$ T-cells find an antigen-bearing DC, local chemokine cues change the chemokinetic and chemotactic behavior of $CD8^+$ T-cells, which have acquired new chemokine sensitivities, possibly by exposure to mediators delivered via conduits that terminate on the HEVs [64]. The secretion of chemokines such as CCL3 and CCL4 by PAMP-activated DCs, perhaps further enhanced by signals from antigen-engaged $CD4^+$ T-cells, provides cues that bias the turning choices of $CD8^+$ T cells on the FRC network [21, 64, 70], leading these cells more directly and efficiently to the relevant FRC-associated DCs.

Some concern has been raised that the directed attraction to a DC of all or most $CD8^+$ T-cells in an inflamed node through unspecific upregulation of CCR5 expression would lead to competition for access to that DC or $CD4^+$ T-cell–DC pair. We have measured the duration of contact between $CD8^+$ T-cells interacting with $CD4^+$ T-cell engaged DCs in the LN and found at best a marginal increase in the dwell time (duration of contact) as compared with the situation in which no $CD4^+$ T-cell activation is occurring [64]. Thus, the flux of $CD8^+$ T-cells in the 'receiving line' is not grossly altered by the chemokine attraction, and the attracted cells do not congregate around the activated DCs, perhaps because they are stimulated to acquire enhanced mobility by the high density of chemokine in this region. This [specify what would cause them to move off] would cause them to move off along efferent FRC fibers, and partial desensitization of CCR5, by transient residence in a high density of this chemokine, would limit the likelihood that the lymphocytes would turn back.

This hierarchical system is akin to human movement in a large complex environment, for example, an airport. Highways lead to access roads that deposit an individual (=lymphocyte) in the departure area; corridors funnel travelers to the security areas, and signs direct the passengers to the specific gate where they can enter (interact with) their specific plane (=DC). Clearly, just depositing an individual in an airport and allowing that person to wander randomly in the hope that he/she will arrive in time at the proper plane is an inefficient strategy and it seems that the same is true for the cellular components of the adaptive immune system.

21.7 Changes in T-Lymphocyte Migratory Behavior upon Contact with Antigen-Bearing DCs

We have summarized above the role of the stromal cell network and chemokines in facilitating interaction between T cells and antigen-bearing DCs. What happens when the two meet? This was the initial question we and others posed as the field developed the tools for imaging the dynamics of immune cells in complex tissues such as LNs. The first report on this topic was from this laboratory. Using the adoptive transfer of antigen-pulsed dye-labeled DCs given subcutaneously so they could migrate by the natural route into a draining LN, followed by intravenous injection of TCR transgenic $CD4^+$ T-cells that would also access the LN by a physiological route, we conducted imaging of explanted LNs using

Fig. 21.1 (continued) Lymphocytes adhere to this network and migrate along its component fibers (**e**) with a speed that is regulated by the homeostatic chemokines SLC (CCL21) and possibly ELC (CCL19), which densely cover its surface (**d**). This network also provides an anchoring substratum for sessile DCs than continuously sample the content of the conduit system (**f**). By creating roads on which T cells roam and to which DCs adhere, the FRC network presumably enhances the probability of the critical T/DC encounters required to initiate an adaptive immune response. This is a second level of concentration/co-localization. Upon infection, antigen-loaded DCs interact for several hours with antigen-specific CD41 T cells, and these interacting cells release CCL3 and CCL4, two chemokinesthat are likely to adhere to the numerous glycosaminoglycans decorating the adjacent FRCs. This creates chemokine pathways that help guide nearby CD81 T cells (expressing CCR5 due to inflammatory signals in the LN) to the relevant DCs and CD41 T cells for delivery of help for effective memory cell generation (**g**). This provides a third level of concentration/co-localization. Thus, sequential guidance cues involving chemical and structural elements help the immune system produce effective adaptive responses in an efficient manner despite the rare nature of antigen, antigen-presenting cells, and antigen-specific T cells early after an infection. B, B follicle; T: T-cell zone; S.C.S: subcapsular sinus; SLC: secondary lymphoid chemokine (CCL21); ERTR-7 recognizes an unknown extracellular matrix protein produced by FRCs, while desmin stains the FRCs themselves

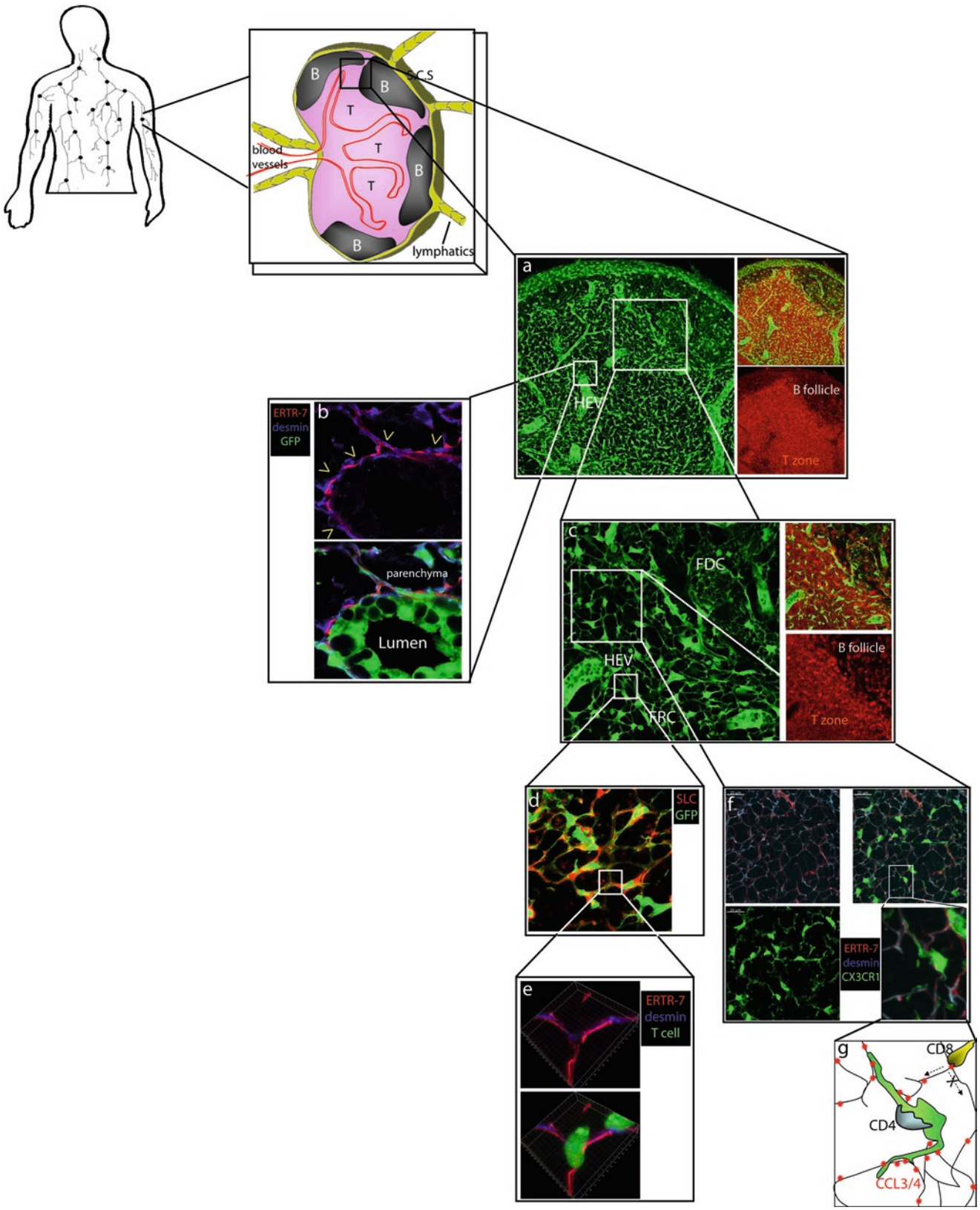

Fig. 21.1 Microanatomy orchestrates leukocyte entry, migration, and positioning in LNs – a 'fractal' view of LN function. LNs are the site of accumulation of antigen, antigen-presenting cells, and lymphocytes. This is the first level of concentration/co-localization involving the key elements underlying adaptive immune responses. Within the LN is an intricate meshwork of various stromal cell types that are easily visualized in ubiquitin promoter–GFP transgenic animals that have been irradiated and reconstituted with wildtype bone marrow (**a**). Blood circulating lymphocytes enter the LNs using HEVs, specialized paracortical blood vessels on which lymphocytes roll, then adhere and transmigrate to the adjacent parenchyma. This last step is regulated by a rim of FRCs [yellow arrowheads in (**b**)] that create a cellular gauntlet around HEVs. Lymphocytes access the parenchyma by squeezing between the processes of two adjacent FRCs. In the parenchyma, FRCs create a dense 3D network (**c**).

confocal instruments [36]. This limited [specify what limited] our examination to the more superficial region of the LN and did not permit us to visualize the events occurring at the time of T-cell passage across the HEVs. However, these limitations have been corrected in later studies using intravital 2P methods, with similar results to those originally obtained using explants (A. Y. C. Huang, unpublished observations). In both cases, with PAMP-activated DCs pulsed with moderate to high levels of specific peptide antigen, we observed the rapid establishment of stable complexes of T cells and DCs. Although we have not returned to a systematic study of the very earliest time points after T-cell entry into the LN, both our original study looking at time points as early as 4–6 h after T-cell transfer and more recent analyses using 2P intravital imaging at shorter time intervals, showed that most T cells stop migrating upon touching an antigen-bearing, activated DC under these particular conditions. The total duration of the ensuing interaction is not well determined. Our initial study used intermittent imaging, and if we are correct that the same T cell remained associated throughout the imaging session as appears to be the case, then many interactions lasted as long as 8 h [36]. More recent data sets using high rate 2P imaging methods have been limited to data collections of <2 h. During this period, few if any T cells detach [15, 23, 71], so the length of the association would appear to be many hours at a minimum (in agreement with our original report).

This prolonged association terminates at approximately 30–36 h, in conjunction with the onset of cell division among the activated T cells. Again, it is not technically possible to observe a T–DC pair from its time of initial formation to this late time point, so one cannot be certain that the T cells do not detach after a several hours and rapidly re-associate with a different DC for several more hours, repeating this process until division ensues, but there are no data directly supporting such an intermittent interaction model. As we improve our capacity to make direct observations in living animals for longer and longer periods [we have recently been able to image at high collection rates for as long 8–10 h (J. G. Egen and H. Qi, unpublished observations)], this question will be addressed directly.

Putting together the results obtained in our initial study, we proposed two phases of T cell–DC interaction in the LN in an immunogenic setting [36, 72]. The first was a prolonged period of tight and apparently stable antigen-driven interaction beginning shortly after if not immediately upon initial T–DC contact within the LN and lasting until the time of first division. The second phase began at that point, when we could observe the T cells migrating rapidly and making only transient contacts with antigen-bearing DCs. The initial contact clearly served to allow the cell to initiate clonal expansion, but when the $CD4^+$ T cells acquire the capacity for effector cytokine production or cues for memory cell formation remains unknown.

Shortly after this first report, Mempel *et al.,* [15] published an analysis of $CD8^+$ T-cell–DC interaction using intravital rather than explant methods. They also introduced the use of anti-CD62L antibody treatment to synchronize the population of T cells being studied by allowing only a limited time for LN entry before the antibody treatment blocked any additional cells from entering. In this model, an additional phase of T-cell behavior was noted, one that lasted for the first several hours after LN entry and consisted of transient rather than stable T–DC contacts, followed by the same two behavioral phases as initially reported for $CD4^+$ T-cells by our laboratory. Recently, this same laboratory has reported that the duration of the early phase (Phase 1 in their terminology) depends on the strength of binding between the T-cell receptor and the peptide-major histocompatibility complex ligand used and the density of this ligand on the DCs [73]. With high densities of a strongly binding ligand, Phase 1 is extremely brief, resembling the results noted above in our hands. One model proposed as an explanation for these data is that a T cell needs to accumulate T-cell receptor signals to a threshold level to engage in a stable contact with a DC. This could occur immediately with high densities of good ligands on each DC, or require sequential interactions with poorer quality ligands at lower density to ratchet up the signal level in the T cell until it passes the threshold level and a stable interaction ensued [15, 73]..

An alternative possibility is that only DCs with sufficient stimulatory capacity can form stable conjugates; antigen-presenting cells meeting this requirement would be rare when poor ligands or low concentrations of peptide are used [74]. The lower frequency of adequate DCs in this condition means that it will take more time for a T cell to find such a DC, as it migrates within the LN. Other contacts will be non-productive and appear as the transient interactions seen in such circumstances. However, in this model, there is no accumulation or build-up of signals or receptor sensitivity among the T cells to explain the eventual appearance of stable T–DC pairs. This issue [specify what issue] can be addressed by recovering T cells during the early phase in which stable contacts are rare and conducting dose response analyses *in vitro* to examine if the cells have acquired heightened sensitivity or not. If not, then the lag time to finding a 'good' DC may be the right model.

21.8 Cell-aided Antigen Delivery to B cells

The scenario just laid out makes sense for T cells that require direct physical interaction with specialized presenting cells that have acquired, processed, and displayed on their surface complexes of peptides and major histocompatibility complex molecules for T-cell receptor recognition. In contrast, the

immunoglobulin receptors of B cells bind antigens directly, often in their native form. Furthermore, there is good evidence that passive diffusion may bring low molecular weight soluble antigens directly into primary follicles for naive B cells to recognize [75]. However, particulate antigen of large size (e.g., viruses and bacteria) may not readily gain free access to follicular area. For example, conduits do not seem able to pass large molecules across to the LN parenchyma – the limit is in the 40–70 kDa range [28, 76], less than the size of many important bacterial or viral antigens and certainly less than that of particulate antigens such as these pathogens themselves. A cell-aided process for carrying and displaying such antigens to B cells may thus be important to ensure access of these lymphocytes to the widest possible range of antigen sources. Indeed, antigen associated with DCs can induce antibody responses [77, 78, 79, 80, 81], even though very few DCs can be found in primary follicles. Furthermore, DCs are able to preserve a substantial amount of acquired antigen in intact form over an extended time frame [82], long enough for DCs to traffic into the LN and physically interact with and deliver antigen to B cells. For these reasons, we took advantage of live imaging analysis to examine a possible role for DC-associated antigen in B cell antigen recognition within LNs [83].

As a model system, we choose the small protein antigen hen egg lysozyme (HEL), which has been intensively studied with respect to B-cell activation and tolerance [84]. HEL is very cationic and binds strongly to negatively charged surface proteins on cells that then undergo endocytosis, thus simulating binding and uptake of microbial antigens by scavenger receptors on various myeloid cell types. In addition, this protein has been shown to recycle from inside DCs to the plasma membrane in intact form [85].. Finally, a useful B-cell receptor (BCR) transgenic model [MD4 [86]] existed for testing the effects of DC-associated HEL on B cells *in situ*.

In vitro experiments showed that DCs pulsed with HEL at 37 °C retained about 80% of the total pool of HEL at intracellular sites; further, removal, using pronase of all the surface HEL on such antigen-exposed cells, failed to inhibit the ability of these DCs to trigger BCR signals in MD4 HEL-specific transgenic B cells, as assessed by Ca^{2+} elevation and an increase in major histocompatibility complex class II and CD80/86 expression [83]. We then used such antigen-pulsed DCs labeled with fluorescent dye to assess whether, and if so, where, and with what effect, these antigen-bearing DCs interacted with antigen-specific B cells in the LN. After subcutaneous administration of control or antigen-pulsed DCs, alternatively labeled MD4 transgenic B cells or polyclonal B cells were administered intravenously to an anesthetized mouse, and imaging begun immediately. This treatment allowed us to visualize the entry of B cells into the LN through the HEVs and to be certain that we were observing the behavior of antigen-naive B cells before their entry into the follicle [83].

Using this [specify which approach] approach, we found that antigen-specific but not antigen-unspecific B cells transiently arrested their migration in the T zone between the HEVs and the B follicle when they contacted antigen-pulsed but not control DCs. While the increased duration of interaction as compared to unspecific controls was only 5–10 min per instance of cellular contact, much less than the hours of stable contact measured between specific T cells and antigen-bearing DCs, specific B cells typically reiterated such interactions with many antigen-carrying DCs in the vicinity of HEVs. Using B cells pre-labeled with the indicator dye fluo-4, we also documented rises in intracellular Ca^{2+} as a result of these antigen-specific interactions between B cells and DCs. The Ca^{2+} elevations were sustained for >10 min, consistent with antigen receptor-triggered Ca^{2+} signaling. In addition, such calcium responses were not observed when the DCs lacked antigen or polyclonal B cells were used, indicating that the response was the result of antigen-specific interactions between B cells and DCs. Additionally, the B cells that engaged DCs carrying specific antigen began to round up in concert with the rise in intracellular calcium, similar to what has been reported for T cells undergoing antigen-induced 'stopping' behavior *in vitro* [87]. Because the loss of cell polarity following T-cell antigen receptor signaling is associated with cessation of active migration, we further analyzed the larger scale migration of B cells interacting with DCs *in vivo*. Indeed, antigen-specific B cells were retarded in net migration upon interaction with DCs carrying nominal antigen. In concert with their increased period of physical contact with antigen-carrying DCs and this reduced migration, antigen-specific B cells were found to acquire HEL antigen from the DCs and to remain at the T–B border for an extended period of time without entering the B follicle.

These data provide the most direct evidence to date that B cells can detect antigen on DCs in LNs. They also indicate that B cells in transit through the peri-HEV region can be a potentially relevant population in the generation of humoral immune responses [83]. More recently, several reports have indicated that particulate antigen and immune complexes brought from subcutaneous sites by afferent lymph flow can be made available to B cells migrating in the follicle close to the subcapsular sinus wall, possibly via macrophages residing at this location [88, 89]. Together with our study, these results suggest a common anatomical feature of pathways involved in cell-aided antigen delivery to B cells: they take place outside or at the edge of follicles and do not involve the bulk of B cells within the follicle proper. It is thus tempting to speculate that DCs and macrophages transport antigen from peripheral tissues and/or lymph-draining sinuses and conduits to B cells in transit to and through the follicular

edge. These cell-aided processes may become increasingly important, as diffusible antigenic components dwindle over time following inoculation with a non-replicating vaccine. From this [specify which perspective] perspective, it would be very interesting to test whether in LNs B cells spend more time transiting outside of follicle and visiting the follicular edge than residing deep in the follicle center. If they do, this would be consistent with the idea that these lymphocytes are programmed to search intensively for cell-associated antigen on macrophages near the subcapsular sinus and on DCs in the interfollicular and T/B border regions and that such antigens are of substantial importance in the generation of humoral immune responses.

A unique aspect of DC-mediated antigen presentation to B cells is its potential to rapidly co-localize antigen-specific T and B cells together on the same antigen-carrying DCs. In fact, the location at which we observed the majority of antigen-specific B cell–DC interactions is the same as the site in which cognate T–B interactions have been shown to first occur (interfollicular area and T/B border) [90]. Our ongoing work is therefore examining whether there is a critical role for DCs in the initial establishment of cognate T–B interactions, continuing our research focus on understanding how rare cells find each other within the LN environment.

21.9 Imaging Studies of Events Outside Organized Lymphoid Tissues

To date, most studies of the immune system that have employed intravital 2P imaging have utilized the LN model, although some laboratories have used this method to analyze B cells [91] and $CD8^+$ T-cells [92] in bone marrow and a confocal approach has been utilized to examine NKT cells in the liver [16]. Very recently, reports have appeared analyzing effector T-cell behavior in tumor beds [93,94] and in the central nervous system [95]. We have employed the 2P imaging method in several model systems to examine how activated T cells move and to begin to monitor their effector functions in diverse tissue sites, as well as to investigate the interaction of pathogens with the innate and adaptive defense systems of the host.

21.10 Antigen- Sampling by Gut- Associated DCs

The equilibrium that exists between host and commensal bacteria in the gut is not independent of the immune system. Rather, both host and microorganism have evolved to allow the existence of protective immunity without immunopathology and without full clearance of the bacteria [96, 97] B cells play a key role in this homeostatic relationship, with secretory IgA in concert with the mucous layer and anti-bacterial proteins having a major role in maintaining an effective barrier against transepithelial penetration of the bacteria [98, 99].

How do B cells involved in producing this IgA see their antigen, and what is the pathway leading to antigen stimulation of the T cells involved in the production of some but not all of this IgA response? The conventional answer would be movement of bacteria across the epithelium via specialized M cells, binding of the transported antigen to BCR on B cells in the follicular region of Peyer's patches, uptake of the material by Peyer's patch-associated DCs in the subepithelial dome region, and activation of T cells in the perifollicular region of the Peyer's patch that would then cooperate with the antigen-activated B cells [100]. There is also transport of antigen via afferent lymphatics into the mesenteric LNs, where all the events described above for induction of adaptive response could occur [101]. Peyer's patches are relatively rare in the small intestine; however, whereas numerous DCs reside on the basolateral side of the villus epithelial monolayer throughout the gastrointestinal tract. Based on this distribution of DCs, another route of antigen acquisition has been described that may also contribute to both equilibrium with commensals and the promotion of active immune responses to pathogens present in the gut lumen.

Rescigno *et al.,* [102] first reported in an *in vitro* model with human epithelial cells and DCs that the DCs could extend processes across a tight epithelial junction and sample antigens on the luminal surface of the epithelial layer. Histological studies also suggested that such extension could occur *in vivo*, a conclusion that was amplified by high-resolution static imaging studies showing many such DC extensions in the small bowel and indicating a role for CX3CR1 in the extension process in response to microbial signals [103, 104]. In collaboration with Rescigno, we have examined this phenomenon in greater detail, developing tools for visualization of the DC extension process in real time in intact or explanted gut segments, and exploring the signaling cascade involved in promoting DC sampling.

Using either mice with GFP expressed in DCs under control of the CD11c promoter [105] or expressed as a covalent fusion with the β-chain of the major histocompatibility complex class II A locus [106], we have been able to detect the active protrusion of parts of DCs across the epithelial layer of the pericaecal region of the small bowel [107]. A systematic study of the frequency of these extensions, which often terminate in a spherical protuberance we term a 'balloon body (BB),' (Fig. 21.2) showed that in the steady state in C57Bl/6 mice such BBs were most frequent in the proximal ileum and rare in the terminal ileum. These structures also showed a decrease in number from the tips of villi toward the base. To examine if this expression distribution was constitutive or dictated by the gut flora, we treated mice with broad-spectrum

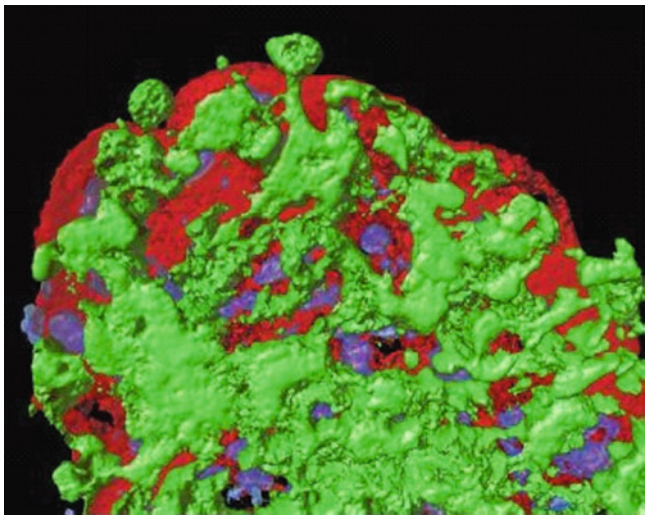

Fig. 21.2 BBs projecting in the intestinal lumen. MHC CII-GFP mice, whose DCs appear green, were deprived of food for 4 h before *Salmonella typhimurium* oral administration. An everted explant of the terminal ileum was prepared and the epithelium was stained using SNARF-1 (red) and Hoechst 33342 fluorescent nucleic acid stain (blue). The explant was then analyzed by confocal microscopy. The image was obtained using a × 40 objective and displayed using IMARIS. *z* slice =9.50 μm

antibiotics; intriguingly, the frequency of extension in the proximal ileum decreased upon such treatment. In agreement with the implication that the extensions might be prompted by stimuli from the gut flora, the addition of non-invasive *Salmonella* organisms to the gut promoted increases in BBs in the terminal ileum where these structures were typically rare in the steady state.

These data suggested that bacteria or bacterial products actively induced BBs, which in turn implicated pattern recognition receptors (PRRs) in this process. To examine the possible role of one subset of such PPRs, the Toll-like receptors (TLRs) in such signaling, we treated animals with purified TLR ligands such as lipopolysaccharide (LPS), peptidoglycan (PG), flagellin, poly I:C, or CpGs, These experiments revealed that some but not other TLR stimuli were active in this regard. The TLR5 ligand flagellin in particular was ineffective, and this finding correlated with reports that this TLR is not expressed to a substantial extent on the apical surface of intestinal epithelial cells [108,109]. To determine if the relevant TLRs were those expressed by the epithelial cells, as this latter correlation suggested, by the DCs themselves, or by both, we prepared bone marrow chimeras using as bone marrow donors the CD11c-GFP or major histocompatibility complex class II-GFP reporter strains and as recipients, TLR2, TLR4, or myeloid differentiation factor 88 (MyD88) gene-deficient mice. These chimeras thus lacked TLR or TLR adapter expression by non-hematopoietic cells, including epithelial cells, but were intact for TLR signaling among the GFP^+ DCs we tracked for BB formation.

Treatment of the TLR2 knockout (KO) chimeras with PG failed to yield an increase in terminal ileum BBs, whereas LPS, a TLR 4 ligand, still did so. The opposite was seen with TLR4 KO chimeras, where LPS failed to work but PG was still active. The MyD88 KO chimeras had almost no BBs in the absence of stimulation, and these animals could not be induced to increase this low number by application of either purified TLR ligands or *Salmonella*. Taken together, these data clearly identified a necessary role for effective TLR-mediated signaling by non-hematopoietic elements in the induction of a BB response, irrespective of the presence of fully functional TLR signaling systems in the DCs themselves [107]. While not showing it explicitly, these data were most consistent with the epithelial cells being the key elements involved in sensing the microbial stimuli and then signaling in an as yet unknown manner to the underlying DCs in the *lamina propria* to extend processes across the epithelial layer into the gut lumen.

Using dye-labeled bacteria, we could show that the BBs interacted with luminal organisms and could bring these into the DCs in the *lamina propria*. The work of others has shown that DCs with acquired organisms can be found in mesenteric LNs [103, 104],, although neither we nor they have shown by direct imaging that these are the same DCs that acquire the bacteria by transepithelial extension. However, it is obviously tempting to speculate that the DC-associated antigen acquired in this manner plays a crucial role in inducing and sustaining the appropriate responses to commensal organisms and to invading pathogenic species.

Numerous questions remain to be answered about this antigen capture system. What is (are) the phenotype(s) of the DCs involved in the sampling process and how is their state of activation/differentiation regulated to allow the proper class of immune response to commensal versus pathogenic material? What signals from the TLR-activated epithelial cells result in BB extension and how do BBs pass between epithelial cells without disrupting the barrier function of the epithelial layer, as we [107] and others [102] have found? Where do the antigen-laden DCs go and with what cells do they interact? Are the regional differences in extension frequency a consequence of local anti-microbial factors that dictate the relative bacterial burden in that region of the gut? What accounts for the differences between laboratories with respect to the distribution of BB extension in the small bowel and of the role of the fractalkine system in the extension phenomenon?

21.11 Dynamics in Mycobacterial Liver Granulomas

Resistance to mycobacterial infection involves the coordinated action of myeloid cells and lymphocytes, especially $CD4^+$ T-cells. These cell types together form a prototypical

structure called a granuloma that constrains these bacteria, limiting their replication and spread. Such granulomas form in various tissue and organ settings, with the lungs the predominant site in the case of *M. tuberculosis* acquired by the aerosol route and the spleen and liver the primary target organs for blood-borne infection. Together with A. Sher and A. Rothfuchs, we have used systemic infection of mice with the attenuated vaccine organism bacille Calmette-Guérin (BCG) as a model for analyzing cellular dynamics and interactions during the process of granuloma formation in the liver, an organ that is amenable to intravital microscopic observations (ref. 16, J. G. Egan *et al.*, in press).

In these studies, we have taken advantage of a series of useful reagents, in particular DsRed-expressing BCG bacteria [110] that permit their direct visualization during intravital imaging and a variety of mice expressing fluorescent proteins in different myeloid lineage cell populations. By utilizing these tools in combination with fluorescent blood tracers that label the liver sinusoid network, we have been able to visualize the initial interactions between host immune cells and bacteria following infection, providing insight into the early events involved in granuloma initiation.

While dynamic events such as host-pathogen interactions can be visualized directly using intravital methods, the weeks-long process of granuloma formation is well beyond the scope of this imaging technique. However, by combining more traditional methods such as sequential static high-resolution multiplex immunofluorescent imaging with dynamic intravital snapshots at specific time points, we have begun characterizing the migratory behavior of specific myeloid populations during granuloma development. These studies are yielding intriguing data showing an important relationship between initially infected tissue-resident macrophages in the liver and the subsequent aggregation of myeloid cells that constitute the major cell type in the maturing lesion.

One of our primary interests in visualizing cell behavior in a variety of lymphoid and non-lymphoid tissues is to look for conserved or homologous mechanisms that regulate cellular function at various stages during an immune response. Thus, many of the same questions that have been and are being asked in our studies of the LN are also applicable to our studies of non-lymphoid sites. Using the mycobacterial granuloma system, we are beginning to address such topics as the role of antigen-specific T-cell-presenting cell interactions in regulating T-cell motility and the importance of chemokines and adhesion molecules in maintaining T-cell locomotion and defining migrational boundaries. These studies should reveal some of the specific strategies used by the immune system to facilitate critical processes such as cell migration and cell–cell communication, ultimately leading to a better understanding of how an inflammatory response is regulated.

21.12 Neutrophil Migration in the Skin

The host defense cell type typically most sensitive to disturbance of tissue integrity is the neutrophil. Indeed, when developing the liver imaging methods used for the analysis of BCG infection, we found that minor tissue injury induced during the surgical procedure could promote neutrophil infiltration within 1 h, a response that we discovered was associated with death of some of the hepatocytes in the damaged region (J. G. Egen, unpublished observations). This rapid and dramatic response of the neutrophil population was even more marked in the skin after tissue damage and/or infection. Using gene-targeted animals containing EGFP under control of the lysozyme promoter (Lys-GFP mice) [111], we have recorded massive influx of these innate cells into the parenchyma of the skin from blood vessels following injection of an inflammatory stimulus. These neutrophils clearly show directed migration toward the site of injection, implicating chemical guidance cues in this process. Our current efforts are aimed at elucidating the molecular players directly involved in initiating this chemotactic response, as well as understanding the role of inflammatory mediators and/or pathogen-derived factors in regulating neutrophil migration.

A particular technique that has aided in these neutrophil studies is the ability to adoptively transfer dye-labeled populations of these cells into normal or mutant hosts. Because of the very high density of emigrating cells in the intact Lys-EGFP reporter mouse model, such tranfers reduce the complexity of analysis. More importantly, however, they allow us to use genetically modified versus wildtype neutrophils in the same imaging field to compare their behavior. This approach has the same benefits described earlier for the analysis of $CD8^+$ T-cell recruitment to DCs that do or do not interact with antigen-specific $CD4^+$ T cells with respect to a relatively homogenous imaging field environment and a single state of animal physiology.

21.13 Visualization of Osteoclasts

Another tissue that is amenable to 2P imaging is the bone marrow. von Andrian and colleagues [92] initially reported this method, introducing the use of the mouse skull as a suitable site for such imaging because the bones are only approximately 100-μm-thick and the underlying marrow cavity thus is within the range of 2P imaging, though with less resolution and depth because of the scattering properties of the hard tissue. We have adopted this approach for an analysis of the factors that regulate the attachment and detachment of osteoclast precursors to the bone surface.

Bone is constantly remodeled by a balanced action of bone-forming osteoblasts and bone-destroying osteoclasts. The latter cells arise from monocytoid precursors under the control of various cytokines and signals, with receptor activating NF-κB-ligand (RANK-L) being especially important in this regard. In various disease states, osteoclast formation and activity is elevated and imbalances the activity of osteoblasts, leading to bone destruction. Understanding what controls the attachment of osteoclast precursors to the bone surface where they can undergo fusion to form mature multinucleate osteoclasts is a question of both basic interest and medical importance.

To develop the tools for directly measuring osteoclast precursor attachment to and release from the bone surface and to potentially visualize the process of cell fusion, we have examined whether osteoclast/monocyte-lineage cells, marked by expression of GFP under control of the colony-stimulating factor receptor (CSF-R), can be imaged in the marrow cavity and what their behavior is with respect to migration as well as entry and exit from the marrow space. Preliminary studies indicate that these fluorescent cells can be tracked by 2P imaging in the skull bone marrow and that under steady-state conditions, they exhibit limited if any movement during a typical 30 min imaging session. We have developed methods for simultaneously visualizing the blood flow in the marrow sinusoids and for detecting enzymes characteristic of mature osteoclasts.

Ongoing studies are now examining the influence of various chemoattractants on positioning and movement of monocytes within the marrow space and on the bone surface, as well as the effect of prolonged RANK-L administration on osteoclast development in the latter location. With further development, this model should prove valuable in generating a dynamic picture of how osteoclast formation and activity is regulated during health and disease.

21.14 Concluding Remarks

We opened this review by emphasizing that cell mobility in the adult was a defining characteristic of the immune system and pointing to the recent revolution in technology that now permits us to examine this crucial dynamic aspect of immunity directly within intact tissues. As the specific results we have described here from our studies reveal, there already have been many surprises about how fast, in what manner, under guidance of what signals, and where within organized tissues various lymphocytes and myeloid cells traffic as they conduct their activities. The role of chemokines as critical players in organizing interactions among distinct immune cell subsets has been made clear, and the question of which of these mediators is acting in what circumstance to promote effective positioning of various cell types is now a subject of intensified study. The critical importance of non-hematopoietic stromal elements in providing not only a supporting structure to lymphoid tissues but also in guiding the movements of lymphocytes and perhaps myeloid cells has been uncovered, leading to further questions about the molecular basis for the interactions between these stromal components and the immune cell populations with which they associate. Changes in the duration and nature of T-cell interactions with antigen-bearing DCs over time during a developing response, the discovery of a multiplicity of mechanisms for B-cell acquisition of antigen, and an early sense of the complexity of cell movements and interactions in non-lymphoid tissues during effector responses are all now topics for further investigation using a combination of classical immunological tools and these new imaging strategies.

The development and application of dynamic imaging tools has synergized with a large existing body of knowledge and methods for immune system exploration to provide a much more informed picture of how host defenses operate. With further technical developments that will permit molecular aspects of signaling cascades and the positioning of surface proteins to be tracked along with gross cell dynamics, with the development of new reporter cells and animals that can reveal when and where effector genes are active, and with an enhanced capacity to image many more colors at one time, to image deeper into tissues, and to image for longer times, there is little doubt that this burgeoning field of study can look forward to a long period of growth and critical contributions to our understanding of the immune system.

In summary, a central characteristic of the immune system is the constantly changing location of most of its constituent cells. Lymphoid and myeloid cells circulate in the blood, and subsets of these cells enter, move, and interact within, then leave organized lymphoid tissues. When inflammation is present, various hematopoietic cells also exit the vasculature and migrate within non-lymphoid tissues, where they carry out effector functions that support host defense or result in autoimmune pathology. Effective innate and adaptive immune responses involve not only the action of these individual cells but also productive communication among them, often requiring direct membrane contact between rare antigen-specific or antigen-bearing cells. Here, we describe our ongoing studies using two-photon intravital microscopy to probe the *in situ* behavior of the cells of the immune system and their interactions with non-hematopoietic stromal elements. We emphasize the importance of non-random cell migration within lymphoid tissues and detail newly established mechanisms of traffic control that operate at multiple organizational scales to facilitate critical cell contacts. We also describe how the methods we have developed for imaging within lymphoid sites are being applied to other tissues and organs, revealing dynamic details of host-pathogen interactions previously inaccessible to direct observation.

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Chapter 22
Role of Regulatory/Suppressor T Cells in Immune Responses

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22.1 Introduction

Although the concept of suppression mediated by T lymphocytes was originally proposed more than 30 years ago, studies over the past 15 years in animal models of autoimmunity have rekindled interest in the existence of a unique lineage of thymic-derived T lymphocytes that specifically suppress immune responses. This population of naturally occurring suppressor T cells can be identified by its co-expression of the CD4 and CD25 antigens, as well as by the presence of the transcription factor, Foxp3. $CD4^+CD25^+Foxp3^+$ T cells (~10% of $CD4^+$ T cells) are now commonly referred to as T regulatory cells (Treg). Our group was among the first to demonstrate that Treg cells were potent suppressors of the activation of both CD4 and CD8 lymphocytes *in vitro*, in response both to polyclonal stimuli and specific antigens by an as-yet unknown cell contact-dependent mechanism. A population with identical phenotypic and functional properties has also been identified in man. While the initial studies focused on the effects of these cells in suppressing responses to autoantigens, Treg have now been shown to play a central role in controlling the immune responses to alloantigens, tumor-associated antigens, allergens, and, most importantly, pathogen-derived antigens (Fig. 22.1). Although considerable progress has been made in this field, a number of central issues remain to be addressed, including (i) the molecular basis for cell-contact mediated suppression; (ii) the relationship (if any) between the *in vitro* studies and the mechanisms whereby Treg suppress *in vivo*, and (iii) how the function of these Treg can be manipulated *in vivo* so as either to diminish or enhance their function.

22.2 *In vitro* Studies of the Suppressor Function of $CD4^+CD25^+Foxp3^+$ Treg

It has proven difficult to determine the mechanism of action, antigen specificity, or cellular targets of Treg. To analyze the mechanism of action of these cells, we have established an *in vitro* model system that mimics the function of these cells *in vivo* [1]. In this model system, Treg are co-cultured with conventional T (Tcon) cells in the presence of a source of antigen-presenting cells (APC) and the suppressive effects of the Treg cells on the proliferation of the Tcon cells assessed. This co-culture system has been widely adopted by all workers in this field and has also proven to be useful to analyze the suppressive functions of the Treg cells in cultures of human lymphocytes. We believe that these types of *in vitro* studies have contributed greatly to our understanding of the potential inhibitory effects of the Treg cells *in vivo*, but are well aware that the important differences may exist between the *in vivo* and *in vitro* studies. Treg cells are non-responsive to stimulation via the T cell receptor (TCR) secondary to their inability to produce IL-2. They also fail to proliferate in the presence of high concentrations of exogenous IL-2, even though they express all three components of the IL-2 receptor complex. Suppression *in vitro* is mediated by a cytokine-independent, cell-contact dependent mechanism that requires activation of the Treg via the TCR. Although Treg will not respond to stimulation via the TCR or IL-2 alone, they readily expand in the presence of both of these stimuli. When Tregs are stimulated with anti-CD3 and IL-2 for seven days [2], the stimulated and expanded cells remained non-responsive to TCR stimulation and exhibited enhanced (three-to four-fold) suppressive capacity. The pre-activated cells were powerful suppressors of the antigen-specific responses of Tcon cells that expressed any transgenic TCR. There were no apparent MHC restrictions on this *in vitro* suppressor activity. If similar non-specific suppressor activity can be generated, following stimulation of Treg *in vivo*, it is likely to be subject to immunoregulatory pressures as these cells, if present in the circulation, would manifest potent general immunosuppressive activity.

22.2.1 *Cellular Targets of Treg-Mediated Suppression*

The cellular target for the suppressive activity of the Treg remains controversial. We initially suggested that the APC was the cellular target as Treg only suppressed APC-dependent

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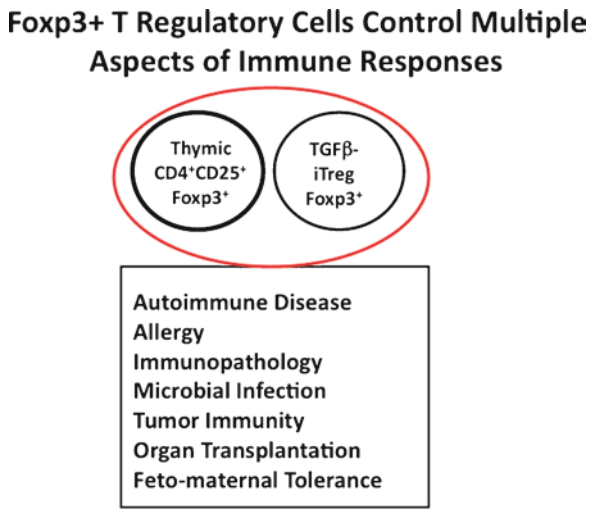

responses and suppression could be overcome by addition of IL-2. One possible interpretation of these results was that the suppressor population was inhibiting the generation or delivery of co-stimulatory signals required for IL-2 production. The potent suppression observed at low ratios of responders to suppressors (4:1) was also consistent with the APC as the primary target. It thus remains possible that the suppressor population in some fashion deactivated the APC. Further insight into the mechanism of action and cellular target of the Treg was derived from an analysis of their suppressive effects on $CD8^+$ responder cells [3]. Most of the effects of the $CD4^+$Treg on $CD8^+$ responders were similar to those seen with $CD4^+$ responders, but several important differences were noted. Firstly, in addition to T-cell proliferation, the capacity of fresh $CD8^+$ T cells to manifest effector functions such as the production of interferon-γ (IFN-γ) were also suppressed; secondly, $CD4^+$Treg inhibited the activation of $CD4^+$ responders by primarily blocking IL-2 production, while they regulated $CD8^+$ T-cell responses both by blocking IL-2 production, as well as by lowering responsiveness to exogenous IL-2 and thereby potentially disrupting CD4 help for $CD8^+$ T cells; finally, $CD4^+$Treg inhibited the activation of $CD8^+$ T cells by acting directly on responder T cells, in the absence of APC. Soluble tetramer stimulation of $CD8^+$ T cells from TCR transgenic (Tg) mice resulted in both proliferation and IFN-γ production, in the absence of APC and APC-derived co-stimulatory signals. Pre-activated Treg induced marked suppression of both of these tetramer-induced responses. The results from this two-cell system conclusively demonstrate that $CD4^+$Treg can mediate suppression via a T-T cell interaction and that the APC is not required for the delivery of the suppressive signal to the responding $CD8^+$ T cells. However, this result does not exclude the possibility that $CD4^+$Treg might also exert inhibitory effects on APC or use the APC surface as a platform on which the suppressor cells physically interact with $CD4^+$ or $CD8^+$ effectors *in vivo*.

22.2.2 Role of cytokines in Treg-mediated suppression

Our initial studies conclusively demonstrated that Treg-mediated suppression was not mediated by IL-4 or IL-10, as Treg from IL-4 deficient ($^{-/-}$) and IL-10 $^{-/-}$ mice were as effective as Treg from wild-type (WT) mice in mediating suppression of T-cell activation *in vitro*, and autoimmune gastritis (AIG) *in vivo*. Studies using neutralizing monoclonal antibodies (mAbs) to transform growth factor-β (TGF-β) also failed to indicate a role for TGF-β in Treg-mediated suppression *in vitro*. However, TGF-β plays an important role in maintaining immune homeostasis, in general, and in regulating T cells, in particular. TGF-$\beta^{-/-}$ mice manifest a severe, spontaneous autoimmune syndrome and die at three weeks of age. The importance of T cells in the genesis of this syndrome is supported by the observation that T-cell specific disruption of TGF-β signaling leads to a similar, although milder, syndrome. These *in vivo* models raise the possibility that TGF-β may play a critical role in immune suppression mediated by Treg. It has been proposed that Treg express cell-surface latent TGF-β and mediate suppression via a cell-contact dependent presentation of latent TGF-β to a receptor on the target Tcon cells. This conclusion was based on the ability to detect the expression of cell surface TGF-β by flow cytometry and to abrogate suppression completely with high concentrations of anti-TGF-β. However, this study did not conclusively prove that TGF-β acted directly on the responder T cells. We [4] took advantage of a number of different genetic models to test directly whether Treg-mediated suppression was dependent on the ability of Treg to produce TGF-β or on the ability of target T cells to respond to TGF-β. We demonstrated that neutralization of TGF-β with high concentrations of anti-TGF-β mAbs or soluble TGF-βRII-Fc did not reverse *in vitro* suppression mediated by resting or activated Treg. Responder Tcon from Smad3 $^{-/-}$ or dominant-negative TGFβRII Tg mice, who are both unresponsive to TGF-β-induced growth arrest, were as susceptible to Treg-mediated suppression as T cells from WT mice. Furthermore, Treg from neonatal TGF-$\beta^{-/-}$ mice were as suppressive as Treg from WT mice. Neutralization of TGF-β *in vivo* also did not abrogate suppression of autoimmune gastritis (AIG). Taken together, our studies do not support the view that secreted or cell-surface associated TGF-β contributes significantly to the *in vitro* suppressive functions of Treg. Although Treg from Smad3$^{-/-}$ mice appeared to function as efficiently as Treg from WT mice, it remains possible that

TGF-β could play a role in the induction of Treg suppressor activity. Such a stimulatory effect on Treg suppressor activity would function in a Smad3-independent fashion. TGF-β may also play a critical role in the induction and maintenance of suppressor function in Treg (see below).

22.2.3 Co-Stimulatory Requirements for Activation of Treg

While it is widely accepted that activation of naïve Tcon requires two signals: one through the TCR by interaction with MHC/peptide and a second through co-stimulation delivered by APC, the nature of the co-stimulatory signals required for activation of Treg suppressor effector function are unknown. Treg are the only cells in the naïve animal that express detectable levels of CTLA-4 in the absence of activation. Indeed, it has been proposed that CTLA-4 plays a critical role in the inhibition mediated by Treg cells as addition of anti-CTLA-4 or its Fab fragment completely abrogated suppression in co-cultures of Treg and Tcon. In addition, AIG could be induced in normal animals with anti-CTLA-4 and that Treg-mediated inhibition of inflammatory bowel disease (IBD) induced by transfer of $CD45RB^{hi}$ T cells to SCID recipients could be reversed by anti-CTLA-4. Thus, the possibility has been raised that engagement of CTLA-4 on Treg by its target ligands plays a critical role in the activation of the suppressive function of the Treg. We have used a two-step culture system to examine the contribution of both cytokines and the CD28/CTLA-4 co-stimulatory pathway in the activation of Treg [5]. Although Treg proliferate when triggered via their TCR in the presence of IL-2, about 10-fold higher concentrations of IL-2 are required to generate a level of proliferation equivalent to that seen with Tcon. The concentration of IL-2 required for induction of suppressor effector function was two-to three-fold higher than that needed for the generation of a maximum proliferative response. This result suggests that IL-2 may also function as a differentiation factor for the induction of T suppressor function *in vitro*. IL-4 and, to a much lesser extent IL-7, were capable of inducing proliferation of Treg in the presence of a TCR signal. The suppressor effector function generated in the presence of IL-4 was equivalent to that induced in the presence of IL-2, while only modest suppressor activity was seen when cells were expanded in the presence of IL-7. IL-6, -7, -9, -10 and TGF-β were incapable of inducing proliferation or suppressor effector functions in these assays. The generation of suppressor activity *in vitro* did not require CD28-derived co-stimulatory signals as blocking co-stimulation with anti-CD80/CD86, CTLA-4 Ig, or the use of APC from $CD80/CD86^{-/-}$ mice had no effect. Addition of Fab fragments of anti-CTLA-4 reversed suppression in co-cultures, but the same Fab fragments also enhanced the proliferative responses of Tcon in the absence of Treg. Thus, in the co-culture, it is likely that the anti-CTLA-4 blocks the interaction of CD80/CD86 with CTLA-4 at the level of the responder Tcon cells, thereby raising their threshold for CD25-mediated suppression. As Treg can readily suppress the activation of $CD8^+$ T cells, and activation of $CD8^+$ T cells is much less dependent on CD28-derived co-stimulatory signals, we assayed the ability of Treg cells to suppress the responses of $CD8^+$ T cells under conditions of co-stimulatory blockade. Marked inhibition of the responses of the $CD8^+$ T cells was observed. We concluded that CTLA-4 is not required for Treg function, nor is the activation of suppressor effector function *in vitro* dependent on co-stimulation mediated by CD28/CTLA-4 interactions with CD80/CD86. We cannot exclude that some of the effects of anti-CTLA-4 were actually mediated by interaction with CTLA-4 on the surface of the Treg. However, it is not clear that such effects are secondary to blockade of the physiologic interaction of CTLA-4 with its ligands on APC, rather than secondary mAb-induced disruption of the interaction of CTLA-4 with the CD3 complex during formation of the immunological synapse in Treg.

22.2.4 Role of IL-2 in Treg-Mediated Suppression

The IL-2/IL-2R pathway is important in the development and expansion of Treg *in vivo*, as IL-2, IL-2Rα, and $IL\text{-}2R\beta^{-/-}$ mice all die early in life of severe lymphoproliferation and autoimmune disease and $IL\text{-}2^{-/-}$ and $IL\text{-}2R\beta^{-/-}$ mice have few or no Treg cells. Furthermore, $Stat5^{-/-}$ mice have very few Treg, while mice transgenic for the active form of Stat5 possess a greater frequency of these cells, thus confirming the requirement for IL-2 signaling in Treg homeostasis. The addition of IL-2 or CD28-mediated co-stimulation are thought to break the anergic state of the Treg. One of the major problems with interpretation of this data is that T-cell proliferation has been used as the major readout of suppression and both Treg and Tcon proliferate under these conditions. We have used a quantitative assay of suppression of IL-2 transcription to re-evaluate the role of IL-2 in suppressor function of Treg [6]. Transcription of IL-2 mRNA remains fully suppressed in the presence of high concentrations of exogenous IL-2 and in the presence of proliferation of both the Treg and Tcon. In contrast, addition of anti-CD28 to the suppression assay resulted in abrogation of suppression, as transcription of IL-2 mRNA was partially restored. Thus, abrogation of the anergic state of the Treg does not break suppression. More importantly, the addition of anti-IL-2 completely abrogated the suppressive effects of Treg on IL-2 mRNA transcription. This demonstrates that not only is

IL-2 required for the generation and peripheral maintenance of Treg *in vivo*, but that IL-2 is also required for the acquisition of suppressor function *in vitro*. It might be regarded as paradoxical that IL-2 is required for subsequent suppression of its own production. However, the initial activation of effector Tcon, with resultant production of IL-2, may be needed for regulation of the function of the Treg, for two distinct reasons. Firstly, it may facilitate the non-specific expansion of the Treg *in situ* at the site of the response; secondly, the requirement for initial activation of effector cells prior to induction of suppressor function imposes a time constraint on when suppression can become manifest. Indeed, it would be highly desirable in the immune response to infectious agents that a certain level of effector function be established prior to the induction of suppressor function, which subsequently would lead to contraction of the response and prevention of an over-exuberant inflammatory response.

22.3 Gene Expression Analysis of Foxp3$^+$ Treg

We have used DNA micro-array technology to compare the patterns of gene expression in CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells [7]. Our goals in the application of this technology were three-fold: (i) the identification of genes differentially expressed by resting CD4$^+$CD25$^+$ T cells, whose products might be used to develop more specific reagents for use in functional studies; (ii) analysis of molecules induced at different time points after T-cell activation to search for cell surface or secreted molecules that may be involved in the effector phase of suppression, and (iii) determination of genes expressed in the resting or activated state of the CD4$^+$CD25$^+$ T cells, that relate to their anergic phenotype. The two populations of T cells were found to differ in only a small number of the >11,000 genes and ESTs tested; over >1,000 genes/ESTs were found to be similarly modulated in the two populations, in response to anti-CD3. Only a small number of genes (29) were differentially expressed between resting CD25$^-$ and CD25$^+$ T cells, and a larger number (77) were differentially expressed following activation. Nine of these genes were shared between the resting and activated state, making the total number of genes differentially expressed 97. The patterns of genes expressed in the CD25$^+$ cells were not consistent with the hypothesis that CD25$^+$ cells simply represent a population of previously activated cells, as many of the observed differences were closely correlated with the distinct functional properties of this subpopulation.

Several genes (CTLA-4, Ly6, OX40, 4–1BB, CD103, and GITR) were identified that distinguished resting CD25$^+$ T cells from resting CD25$^-$ T cells; however, CTLA-4, Ly-6, OX40, and GITR were also induced on the CD25$^-$ subset following activation and, thus, resemble CD25 in their expression profile. CD103 was only expressed in a minor (20–30%) subpopulation of CD25$^+$ T cells, and the level of expression was not modulated by T-cell activation. The CD25$^+$CD103$^+$ subset manifested somewhat greater (~three- to five-fold) capacity to suppress T-cell activation *in vitro*, when compared to the CD25$^+$CD103$^-$ subset. This subpopulation has subsequently been shown by others to have unique homing properties *in vivo*. One subset of genes (CIS, SOCS-1, and SOCS-2) that was more highly expressed in the CD25$^+$ subset belonged to the suppressor of cytokine signaling (SOCS) family. As IL-2 is required for the survival/maintenance of the CD25$^+$ population, the SOCS proteins may be induced in response to this cytokine, or other cytokines needed for homeostatic control of these cells. The capacity of the CD25$^+$ T cells to upregulate these inhibitory molecules may diminish their suppressive function during protective immune responses.

22.3.1 Role of GITR/GITR-L Interactions in Modulating Suppression

As one of the major goals of the micro-array studies was to define molecules involved in the suppressor activity of the CD4$^+$CD25$^+$ T cells, we tested neutralizing antibodies to both cytokines and cell-surface molecules, that were either selectively expressed on resting CD25$^+$ T cells or induced after activation, for the their capacity to reverse CD25-mediated suppression. A polyclonal antiserum to the glucocorticoid induced TNF-like receptor (GITR) reversed suppression suggesting that this molecule played an important role in the suppression process [7]. The GITR (TNFRSF18) is a 228 amino acid type I transmembrane protein that resembles other members (4-1BB and CD27) of the TNFRSF in its intracellular domain. It was unlikely that the GITR was involved in suppressor effector function of the CD25$^+$ subset as two populations that expressed GITR, resting CD25$^+$ T cells and activated CD25$^-$ T cells do not manifest suppressor activity. Our initial studies strongly supported the view that the anti-GITR was acting on the CD25$^+$, rather than the CD25$^-$ subset; as culture of the CD25$^+$—but not CD25$^-$—population with anti-GITR and IL-2, in the absence of a TCR stimulus, induced a vigorous proliferative response. This study supported the view that anti-GITR functions as an agonist for the GITR in this assay. We proposed that engagement of the GITR by this agonistic antibody or, potentially, by its physiologic ligand, results in a signal that not only prevents suppressor function from being manifest, but also reverses the non-responsive state of these cells to exogenous IL-2.

We have identified the mouse ortholog of GITR-L (TNFSF18) generated an antagonistic antibody against it[against what?], and determined its tissue distribution [8]. the GITR-L is expressed on the cell surface of APC with the highest level of expression seen on B-1 lymphocytes, intermediate levels on conventional B-2 B lymphocytes and macrophages, and $B220^+$ DC, with lower levels on the $B220^-$ DC subset. The GITR-L is unique among members of the TNFSF, in that it is expressed on resting APC and its expression is downregulated by triggering the B-cell receptor, CD40, or different Toll-like receptors. Other members of the TNFSF (4-1BB-L, OX40-L, LIGHT, CD70, CD30-L) are not detectable on resting APC and their expression is upregulated by activation of the APC via Toll-like receptor stimulation. We have used the anti-GITR and anti-GITR-L antibodies together with $GITR^{-/-}$ mice to re-examine the cellular target of anti-GITR mediated reversal of the suppressive effects of the $CD25^+$ T cells. We found that both the anti-GITR and GITR-L transfected cells enhanced the activation of $CD25^-$ T cells, in the absence of $CD25^+$ T cells. In addition, the anti-GITR-L partially inhibited the activation of $CD25^-$ T cells, in the absence of $CD25^+$ T cells. Most importantly, the addition of anti-GITR to co-cultures of $CD25^+$ and $CD25^-$ T cells from WT and $GITR^{-/-}$ mice demonstrated that the target for antibody-mediated reversal of suppression was the $CD25^-$ T cells This result was further supported by experiments using rat $CD25^-$ responder and mouse $CD25^+$ suppressor T cells in co-cultures, which conclusively demonstrated that GITR ligation on the $CD25^+$ T cell subset does not abrogate their suppressive function.

Lymph node cells from $GITR^{-/-}$ mice were found to be profoundly hypo-responsive to TCR stimulation; however, responsiveness could be completely normalized by removal of the endogenous population of $CD4^+CD25^+$ T cells. This result suggests that, in the absence of GITR/GITR-L interactions, suppression is dominant. Indeed, the suppressive effects of the $CD25^+$ T cells, in the absence of GITR engagement, were mediated by inhibition of both IL-2 production and expression of CD25 by the $CD4^+CD25^-$ responder population. The co-stimulatory signals delivered by GITR and CD28 appeared to be distinct, but interrelated. In the absence of regulatory cells, the responses of $CD4^+$ and $CD8^+$ cells from $GITR^{-/-}$ mice were equivalent to those of WT mice. In contrast, $CD4^+$ and $CD8^+$ T cells from $CD28^{-/-}$ were non-responsive under these culture conditions. Conversely, in the presence of exogenous IL-2, the responses of $CD4^+$ and $CD8^+$ T cells from the $GITR^{-/-}$ mice were not restored, while partial restoration of the responses of cells from the $CD28^{-/-}$ mice was observed. CD28 derived co-stimulatory signals are required for upregulation of GITR expression on $CD4^+CD25^-$ T cells, as anti-CD80/CD86 mAbs markedly inhibited GITR upregulation. Collectively, these results favor the view that engagement of GITR on both $CD4^+CD25^-$ and $CD8^+$ T effector cells by GITR-L early during the course of an immune response serves primarily to render the effector population resistant to the suppressive effects of the $CD25^+$ T cells. During the course of the response, inflammatory signals ultimately result in down-regulation of GITR-L expression, which would increase the susceptibility of the effector cells to CD25-mediated suppression. An additional important function of the CD28 signaling pathway during T-cell activation is to license T-cell resistance to CD25-mediated suppression by enhancing the expression of the GITR.

22.3.2 Analysis of Subsets of $Foxp3^+$ Treg

We have performed a detailed comparison of the development and phenotype of $Foxp3^+$ T cells, in relation to the expression of conventional MHC molecules, and correlated the phenotypic properties of $Foxp3^+$ T cells developing in major histocompatibility complex (MHC)-deficient mice with their expression of cell-surface antigens and function, both *in vivo* and *in vitro* [9]. A major population of thymic-derived effector $Foxp3^+CD8^+CD103^+$ T cells that are MHC class I restricted was identified in mice lacking MHC class II antigens, and a population of thymic-independent $CD4^+Foxp3^+CD103^+$ T cells was seen in mice that are lacking all conventional MHC antigens. Most effector $CD4^+Foxp3^+CD103^+$ T cells in wild-type mice are thymic-derived, although some are likely to be thymic-independent. Expression of MHC class II exclusively on thymic cortical epithelium allowed the selection of naïve $CD4^+Foxp3^+CD103^-$ thymocytes. However, the development of the effector $CD4^+Foxp3^+CD103^+$ subset requires MHC class II expression in the thymic medulla. All the effector populations can constitutively suppress T-cell activation *in vitro*, and can rapidly localize to inflamed lymphoid organs or tissues. We reason that these effector $Foxp3^+$ T cells may play a specialized antigen non-specific role during the early phases of an immune response, perhaps by acting during the priming phase as an initial means to modulate the adaptive immune response.

22.3.3 TGFβ-induced $Foxp3^+$ Treg

We have fully characterized the cytokine and costimulatory molecule requirements for the TGF-β-mediated induction and maintenance of Foxp3 by $CD4^+Foxp3^-$ T cells [10]. Induced Treg (iTreg) were anergic and fully capable of suppressing the proliferation of naïve T cells when restimulated *in vitro*. We found an absolute requirement for IL-2 in concert with TGF-β for induction of $Foxp3^+$ iTreg. Thus far, we have not identified another cytokine that could substitute for

IL-2, including other cytokines that use the γc-chain as part of their receptor complexes. Once induced Foxp3 expression was stable for five-10 days *in vivo* and *in vitro*, in the absence of IL-2. The role of CD28 in the induction of iTreg appears to be solely related to its capacity to enhance the endogenous production of IL-2. It is still unknown what percentage of circulating Treg is derived from the thymus and what percentage is generated in the periphery by this TGF-β-dependent induction pathway. Although IL-2 was required for the induction of iTreg, the generation of IL-17 producing T cells in the presence of IL-6 and TGF-β was markedly inhibited by the presence of IL-2 [11].

Some studies have suggested that iTreg lack regulatory function, even though they express Foxp3 and that they rapidly lose Foxp3 expression following transfer *in vivo*. As adoptive transfer of Treg into neonatal Scurfy mice that are completely deficient in Treg, prevents the development of disease, we tested whether iTreg would also be effective in rescuing Scurfy mice. We found that polyclonal iTreg generated from peripheral $CD4^+Foxp3^-$ T cells could completely suppress all the pathologic manifestations of the severe autoimmune disease that develops in Scurfy mice, in both lymphoid sites and in tissues [12]. Weekly injections of iTreg prolonged the life of the scurfy mice for more than 35 days. Thus, the TCR repertoire of the iTreg generated from normal adult mice appears to be sufficient to prevent autoimmune disease in the scurfy mouse. The iTreg maintained high levels of Foxp3 expression in the inflammatory environment, whereas the same cells when transferred to normal mice lost expression of Foxp3, suggesting that certain factors such as proinflammatory cytokines in the sick Scurfy mouse might act as survival or expansion signals for the iTreg. Taken together, these results demonstrate the potency and stability of the iTreg in curing the most severe autoimmune disease.

22.3.4 *Foxp3⁺ Treg Mediate Infectious Tolerance in a TGFβ-Dependent Manner*

The demonstration that TGF-β is a potent inducer of Foxp3 expression and Treg function *in vitro* and *in vivo*, together with studies demonstrating that Treg production of TGF-β is required for protection against certain autoimmune diseases raised the possibility that one potential mechanism of action of Treg is to induce Treg *de novo* from naïve T cell precursors in a TGF-β-dependent manner, thus facilitating a type of infectious tolerance [13]. Previous studies have demonstrated that the TGF-β propeptide, latency associated peptide (LAP), and thus, presumably, TGF-β are expressed on the surface of Treg. Cell surface LAP was only expressed by activated, but not resting, $Foxp3^+$ T cells and not by activated $Foxp3^-$ T cells. Since activated Treg express functional LAP/TGF-β complexes on their cell surface, we determined whether Treg would be able to confer infectious tolerance by inducing Foxp3 expression in $CD4^+Foxp3^-$ T cells. Co-culture of pre-activated $Foxp3^+$ Treg, but not pre-activated $CD4^+Foxp3^-$, T cells with naïve $CD4^+Foxp3^-$ T cells resulted in the induction of Foxp3 expression in 10–30% of the naïve T cells. The addition of LAP completely inhibited the induction of Foxp3, demonstrating that Treg-dependent induction of Foxp3 is TGF-β-dependent. Pre-activated Treg from TGF-$\beta^{-/-}$ mice or from mice with T-selective defect in processing TGF-β (furin$^{-/-}$ mice) were incapable of inducing Foxp3 in naïve responders, thus demonstrating that the Treg were the source of the TGF-β. The $CD4^+Foxp3^+$ T cells converted from $Foxp3^-$ cells were suppressive *in vitro* and could inhibit the development of colitis when co-transferred with $CD4^+Foxp3^-$ T cells into RAG$^{-/-}$ mice. Co-transfer into normal mice of pre-activated, antigen-specific Treg with naïve antigen-specific responder T cells also resulted in induction of Foxp3 in the responders, after immunization of the recipients with antigen. Treg-mediated Foxp3 induction appears to be tightly regulated and dependent on T-cell activation. We propose that that *in vivo* simultaneous stimulation of both the Treg and the antigen-specific T cells likely occurs on the platform of the DC in the form of a three-cell interaction. As a single DC can present more than one organ-derived antigen, it is possible that the Treg-TGF-β pathway of Foxp3 induction can result in expansion of Treg of broad antigen specificities and thus serve as an important adjuvant in the use of Treg for cellular biotherapy.

22.4 Use of Foxp3⁺ Treg for the Treatment of Autoimmune Disease

22.4.1 *In vivo Studies of Treg in AIG*

We originally considered the use of the day three thymectomy (d3Tx) model for induction of autoimmune immune disease in susceptible strains of mice for analysis of Treg function *in vivo*. However, disease induction in this model varies greatly as to time of onset and the percentage of mice that ultimately develop the autoimmunity. AIG is one of the few spontaneous models of organ-specific autoimmune diseases in which the effector-cell target antigen, the proton pump of the gastric parietal cell (H/K ATPase), has been defined. We have generated two strains of TCR Tg mice that we believe are ideal for further *in vivo* studies of Treg function. We originally isolated several clones from a mouse that

developed AIG, following d3Tx [14]. One clone, designated A23, was specific for the gastric parietal cell antigen, H/K ATPase α-chain$_{630–641.}$ The T-cell receptor α and β genes from this clone were used to generate A23 Tg mice [15]. All A23 Tg animals spontaneously developed severe AIG, and evidence of disease was detected as early as day 10 of life. Gastritis could be transferred to immunoincompetent mice with as few as 10^3 Tg thymocytes, but 10^7 cells would only induce mild disease in WT recipients, presumably due to the presence of endogenous Treg. The A23 T-cell clone was of the Th1 type and produced large amounts of IFN-γ. Similarly, the disease that developed in the A23 Tg mice was characterized by a significant production of Th1 cytokines in the draining lymph node (LN) and in the gastric mucosa.

We have developed a second line of TCR Tg mice, whose TCR recognizes a different epitope, H/K ATPase α-chain$_{889–900}$ [16]. This TCR was derived from the A51 clone that expressed a Th2 phenotype and produced IL-4, -5 and –10 upon antigen-specific stimulation. Upon transfer to *nu/nu* recipients, this clone would induce a predominantly eosinophilic gastritis with production of Th2 cytokines. We generated this TCR Tg line to test whether the phenotype of disease is related to the particular TCR expressed by the pathogenic cell. Surprisingly, constitutive expression of the A51 TCR in the Tg mice resulted in AIG of an indolent phenotype, with late onset of disease, presence of large numbers of eosinophils in the gastric mucosa, and elaboration of Th2 cytokines. Thus, the A51 Tg mice represent a unique model for the study of autoimmune disease and illustrate that the *in vivo* response to a self-antigen can be Th2 in phenotype, yet also result in pathological autoimmunity. This model also suggests that there is a narrow window of T-cell activation by self-antigens dictated by the TCR itself, in which autoimmune reactivity may coexist with a distinct cytokine phenotype.

One difficulty in the interpretation of many of the early studies on the function of Treg *in vivo*, including the d3Tx model, is that almost all involve induction of a generalized state of partial lymphocyte depletion or transfer of cells to a lymphopenic host, in addition to depletion of Treg. Indeed, it has been proposed that the lymphopenic state itself may promote the activation of autoreactive effector cells and that reconstitution of a depleted animal with either Treg or Tcon would prevent disease development. A much more direct test of the role of Treg in regulating the development of autoimmunity *in vivo* would be their selective removal from the intact animal. To define a role directly for Treg in the control of organ-specific autoImmunity in the absence of lymphopenia-induced proliferation, we selectively depleted them by treatment with anti-CD25 mAb [17]. Depleted animals only rarely developed AIG, the most common manifestation of a deficiency of Treg in BALB/c mice. The failure to observe autoimmune disease following depletion of Treg was not secondary to inadequate depletion or emergence of new thymic emigrants because transfer of T cells from Treg-depleted animals to *nu/nu* recipients for up to six weeks following depletion, resulted in the induction of autoimmune disease in 100% of recipients. This disparity between the ability to transfer disease to a lymphopenic animal, while lymphocyte-sufficient, Treg-depleted donors did not develop autoimmune disease forced us to re-examine the contribution of lymphopenia for the induction of autoimmune disease. We failed to observe any suppressive effects of co-transfer of Treg on the lymphopenia-induced proliferation of Tcon for 21–28 days after transfer. In contrast, co-transfer of Treg exerted a profound suppressive effect on the accumulation of Tcon when the animals were analyzed two to six months after transfer. However, most recipients of Tcon will develop manifestations of autoimmune disease during this period of time and it is likely that they have undergone autoantigen-specific stimulation, in addition to lymphopenia-induced proliferation. It is the former component of the response that is suppressed by co-transfer of Treg. Collectively, our data are most consistent with a model in which Treg play no role in the control of lymphopenia-induced proliferation, but that the proliferative response functions as a second signal that is required for the differentiation of autoreactive effector cells. A number of other stimuli can provide this second signal for activation of autoreactive effector cells, including high levels of autoantigen expression, as immunization of Treg-depleted mice with the H/K ATPase in incomplete adjuvant—which never induces disease in normal mice—resulted in the induction of disease in all Treg-depleted animals.

22.4.2 Role of DC in the Presentation of Autoantigens

We have also analyzed the role of tissue DC in the presentation of natural autoantigens [18]. Immunofluorescence microscopy showed physical association of DC with parietal cells in the gastric mucosa. The H/K ATPase protein could be localized within the vesicular compartments of a few DC in the draining gastric LN, and these antigen-containing DC increased in number during autoimmune disease. Gastric DC could activate the TXA-23 T-cell clone to produce IFN-γ *in vitro*, in the absence of added antigen, demonstrating that these DC can process the endocytosed antigen. Such a process took place *in vivo* as T cells from the TXA23 Tg animals proliferated extensively in the gastric, but not peripheral, lymph nodes three days after transfer to *nu/nu* recipients. These findings indicate that the H/K ATPase antigen is constitutively processed and presented by gastric DC *in vivo* in nonimmunogenic conditions.

22.4.3 Prevention of AIG with Polyclonal Treg

Most of our studies have utilized effector cells from the TCR transgenic mouse, TxA23. Low numbers of $CD4^+$ SP thymocytes from TxA23 mice can transfer AIG to *nu/nu* recipients. The cell-transfer model into *nu/nu* recipients allows us to examine the effects of Treg on preventing the induction of AIG, including the effects of Treg on antigen-specific priming, differentiation, and migration into the organ targeted for autoimmune destruction. We have used this experimental model in which a small number of self-reactive T cells, both naïve and fully differentiated, responding to a well-defined, naturally expressed and presented autoantigen can be examined for their location, number, and function during the course of an autoimmune response in the presence or absence of polyclonal Treg or antigen-specific iTreg.

Co-transfer of polyclonal Treg from normal BALB/c mice with a small number of autoantigen-specific TCR Tg T cells was quite effective in inhibiting the development of AIG in *nu/nu* recipients, as demonstrated by suppression of parietal cell destruction and lack of autoantibody production [19]. Importantly, Treg failed to inhibit the migration of the autoreactive T cells to the gastric LN or into the target organ and did not inhibit the antigen-driven expansion of autoreactive T cells in the gastric LN. The primary effect of the Treg appeared to be inhibition of the differentiation of the autoantigen-specific T cells to Th1 effector cells in the gastric LN, as reflected by a decrease in antigen-stimulated IFN-γ production and reduction in T-bet expression. Suppression of the antigen-specific induction of IFN-γ production could be observed as early as seven days after transfer of the polyclonal Treg. It seems highly unlikely that this suppression is secondary to the activation and expansion of the limited number of gastric antigen-specific Treg present within the polyclonal population. An alternative possibility is that Treg are able to control various responses because they are continuously recognizing and being partially activated by ubiquitous self-peptides, and simply need a second signal such as IL-2 provided by the responding effector T cells to activate their suppressive activity.

22.4.4 Prevention of AIG with Antigen-Specific ITreg

Several studies have suggested that antigen-specific Treg from TCR Tg mice are more efficient than a polyclonal population of Treg at preventing autoimmunity. Naïve H/K ATPase-specific $CD4^+Foxp3^-$ T cells from the thymus of TxA23 mice can be easily expanded and induced to express Foxp3 when activated in the presence of TGF-β. Antigen-specific iTregs were more efficient at preventing autoimmunity than polyclonal Tregs used in our previous studies. The iTregs were long lived *in vivo*, maintained Foxp3 expression, and protected mice from disease. The mechanisms of action of the antigen-specific iTregs were quite distinct from the polyclonal Tregs [20]. The major effect of the iTreg was to inhibit priming of autoreactive T cells within the gastric LN. A suppression of proliferation characterized by a 40-fold reduction in the numbers of autoreactive effectors was observed within the first five days after priming. We hypothesized that the failure of effector-cell expansion was a secondary to inefficient priming, due to an effect of the iTreg on antigen-presenting DC. We therefore isolated DC from the gastric lymph node two days after transfer and measured their ability to present endogenous H/K ATPase. DC that had been exposed to iTregs *in vivo* had a reduced capacity to present endogenous antigen, compared with those from non-injected mice, as well as mice injected only with effector T cells. The concept that Treg can mediate their suppressive effects *in vivo* by affecting antigen-presenting DC is supported by recent studies demonstrating that antigen-specific Tregs interacted with antigen presenting DC, and that the presence of the Treg led to fewer long-lasting interactions between effector T cells and DC. Our results suggest an alternative model in which the Treg exert their negative effects by decreasing the stimulatory capacity of DC, rather than by competing with the effectors for antigen, or by acting directly on the effector cells to prevent interaction with DC.

22.4.5 Tregs Inhibit Disease Induction by Differentiated Th1 and Th2 Cells

Most studies of Treg function in models of organ-specific autoimmune diseases have focused on prevention of disease induced by naïve T cells, rather than on treatment of ongoing disease or prevention of disease induced by fully differentiated effector T cells. We have compared the capacity of fully differentiated Th1, Th2 and Th17 cells, derived from the TxA23 mouse, to induce AIG upon transfer to *nu/nu* recipients and determined the susceptibility of these fully differentiated effector T cell populations to control by transfer of polyclonal Treg [21]. We could readily induce differentiation of thymocytes from the TxA23 mouse to Th1, Th2, and Th17 cell lines. After transfer, all three effector T-cell populations preferentially accumulated in the gastric LN and the gastric mucosa, and elicited destructive AIG with distinctive pathologic profiles. Th17-mediated disease seemed to be the most aggressive type and was accompanied by the highest number of infiltrating polymorphonuclear leukocytes, including a

very high percentage of eosinophils. Polyclonal Treg could suppress the capacity of Th1 cells, moderately suppress Th2 cells, but had little effect on Th17-induced AIG. In contrast to our studies with naïve T cells, significant inhibition of effector cell expansion was seen when polyclonal Treg were co-transferred with Th1 and Th2 effectors, and the inhibition of expansion was an excellent correlate of protection from destructive AIG. One reason for this result is that the effector populations expressed only low levels of CD62L and could not enter the gastric lymph node, while the Treg express high levels of CD62L and could easily be detected in the gastric LN immediately after transfer. A small percentage of the polyclonal Treg population expressing receptors for antigens derived from the target organ may have been retained in the node, expanded, and then exerted inhibitory effects on DC, resulting in inhibition of the expansion of the susceptible effector populations upon their arrival in the node. T effector cells isolated from protected animals were not anergic and were fully competent to produce effector cytokines *ex vivo*. These findings are most consistent with a model where Tregs *in vivo* are not shutting off effector cytokine production by T cells directly, but are regulating their expansion by acting on DC.

22.5 Characterization of Human Tregs

Expression of the CD25 antigen correlates with Treg function and Foxp3 expression in the mouse. The availability of a number of mAbs to human Foxp3 allowed us to perform a detailed analysis of the correlation between CD25 expression and Foxp3 expression. In contrast to our experience with mouse T cells, we found that only those $CD4^+$ T cells that express CD25 at high levels are uniformly $Foxp3^+$. A population of $CD4^+$ T cells expressing intermediate levels of CD25 also contained some $Foxp3^+$ T cells, but attempts to include these $CD25^{int}$ cells in our preparations always resulted in significant contamination with $Foxp3^-$ T cells. We purify human $Foxp3^+$ T cells by first isolating all $CD4^+CD25^+$ T cells with magnetic beads and then use preparative cell sorting to obtain highly enriched (95% $Foxp3^+$ T cells) Tregs. A number of assays have been used in the literature for measurement of the function of human Tregs *in vitro*. Some are modeled after the assays originally developed by us in the mouse and utilize responder cells, APC and soluble anti-CD3, while others prefer to use anti-CD3/CD28 beads to activate the responders, in the absence of APC. As Treg suppression of mouse T-cell activation induced by solid phase stimuli is remarkably inefficient, we routinely now use a culture system in which human $CD4^+CD25^-$ T cells are activated with soluble OKT3 in the presence of T-depleted PBMC.

22.5.1 Role of TGF-β in the Induction of Foxp3 Expression in Naïve Human T Cells

While it is clear the Foxp3 is the definitive marker for mouse Treg cells, several studies in man raised the possibility that Foxp3 can also be induced upon activation of human non-Treg cells. In some studies, these cells expressed a Treg phenotype, whereas in other studies the induction of Foxp3 did not correlate with a Treg phenotype. As our studies in the mouse clearly documented that stimulation of naïve T cells in the presence of TGF-β and IL-2 resulted in induction of Foxp3 expression and a complete Treg phenotype, we analyzed the effects of TGF-β on the induction of Foxp3 on human T cells [22]. When $Foxp3^-$ T cells were stimulated for five days with anti-CD3/CD28 and IL-2 in 5% autologous serum, ~30% of the cells expressed Foxp3, while ~80% expressed Foxp3 when TGF-βwas added to the cultures. When the cells were stimulated in the presence of anti-TGF-β less than 4% expressed Foxp3. Thus, human $CD4^+Foxp3^-$ T cell resemble mouse cells, in that Foxp3 expression can be induced by TGF-β. However, the TGF-β-induced cells lacked all the functional characteristics of real Treg, as they proliferated when stimulated via their TCR, failed to exert suppressor activity when co-cultured with freshly isolated $CD4^+CD25^-$ T cells, and produced IL-2 and IFN-γ upon re-stimulation.

22.5.2 Human Treg Suppress Mouse Responder Cells

We have demonstrated that human $Foxp3^+$ T cells, activated with plate-bound anti-hCD3, are almost as potent inhibitors of the proliferation of mouse $CD4^+Foxp3^-$ responders (stimulated with soluble anti-mouse CD3 and mouse DC), as mouse $CD4^+Foxp3^+$ Treg. This result strongly suggests that at least *in vitro*, a major component of the suppressive function of $Foxp3^+$ Treg is preserved across the species [23]. One advantage of this model is that allows us to attempt to block the suppressive capacity of the human T cells with mAbs to human cell-surface antigens that will not interfere with the activation of the mouse responder cells. We have tested a panel of several mAbs and noted that suppression by the human Tregs is completely reversed by anti-hCD11a (LFA-1), but not by anti-human CD54 (ICAM-1). We conclude from this that hCD11a on the human Tregs interacts with mouse CD54 on either the mouse responder T cell or DC, rather than with human CD54 on the human Treg. Human Tregs are fully competent suppressors of mouse $CD4^+Foxp3^-$ T cells from $CD54^{-/-}$ mice in the presence of wild-type mouse DC, indicating that one target of the human Tregs is the mouse

DC, rather that the mouse responder T cell. Thus, the suppressive effects of human Treg on mouse responder T cells closely mimics the potential function of Tregs cells *in vivo*, where our recent studies have focused on the DC as the target (see above).

22.5.3 Expansion of human Tregs for cellular immunotherapy

The major obstacle for the use of human Tregs in cell-based therapy is the difficulty of obtaining a highly pure population after *ex vivo* expansion. A $CD4^+FOXP3^+$ population of > 90% purity can be isolated by Fluorescence-Activated Cell Sorting (FACS) of the top 2–4% of $CD4^+$ T cells with high CD25 expression ($CD25^{hi}$) from peripheral blood, but frequently the percentage of $FOXP3^+$ T cells decreases to 75% after one week and to 50% after two weeks of expansion by stimulation with anti-CD3/CD28 and IL-2. A more complex problem is the validity of FOXP3 as a bona fide marker of human Tregs. We have demonstrated that expression of FOXP3 can be induced by TCR stimulation of human $CD4^+CD25^-FOXP3^-$ T cells in the presence of TGF-β, but the induced cells lack all the functional properties of Tregs. As TGF-β is present in the serum used for cultures, a similar induction of FOXP3 expression in contaminating $FOXP3^-$ T cells may occur during expansion cultures of partially purified Tregs. While the expanded population might appear to be highly enriched in $FOXP3^+$ cells, many of these cells may be induced $FOXP3^+$ cells that lack Treg functions.

We have identified three unique cell-surface markers, latency associated peptide (LAP) and IL-1 receptor type I (CD121a) and II (CD121b) that are selectively expressed on activated Tregs, but not on activated $CD4^+FOXP3^-$ or induced $FOXP3^+$ cells [24]. We have used these cell surface markers to design a protocol that allows for purification of $FOXP3^+$ Tregs from *ex vivo* expansion cultures, starting with leukapheresis preparations and using only magnetic bead targeting reagents. The final Treg product is composed of > 90% $FOXP3^+$ cells and is highly anergic and suppressive *in vitro*. This method provides an important advance for the preparation of Tregs for cell-based immunotherapy to treat or prevent autoimmunity and transplant-related complications. Moreover, given the limitation of human blood samples, particularly in the pediatric population, and the importance of obtaining a highly purified Treg population for functional and genomic analyses, we demonstrate that with this technique it is possible to expand and purify Tregs from 5–10 ml blood volume to achieve high numbers and purity for further studies.

22.6 Conclusions

There is little doubt that the study of suppressor/regulatory T cells is presently one of the most active areas of research in contemporary immunology. It is likely that a complete elucidation of all aspects of their action will be available within the next five-10 years. One hopes that these basic discoveries will lead to the development of small molecules and biologics that will allow us to manipulate the function of these cells *in vivo* and move from the bench to the bedside. Transient depletion or inactivation of Treg function should then be a powerful adjunct to the augmentation of immune responses to tumor vaccines or weak vaccines to infectious agents. Transient augmentation of the numbers or function of Tregs is a somewhat greater challenge, but hopefully should become part of the therapeutic armamentarium for treatment of autoimmune and inflammatory diseases.

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Chapter 23
Proliferation versus Contraction of Immune Cells, the Non-Apoptotic Role of Caspase 8 In Immune Homeostasis

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23.1 Introduction

Upon antigen stimulation, immune-responsive cells, particularly antigen-specific T-cells, are activated and begin to proliferate, which is a requirement for the immune system to counteract effectively pathogen invasion and other pathogenic disturbances. For a long period of time, the major interests of immunological researchers have been focused on how to promote immune responses against given antigens; specifically, to make effective vaccines. Research has now shown that in the immune system, all mature T cells are positively selected through recognition of self-antigens in the context of MHC (major histocompatibility complex). Thus, unrestricted expansion of any specific population of T cells may not always be a benefit for a host; rather, autoimmunity and other toxic reactions can occur in certain circumstances [1]. Therefore, it has been gradually appreciated that in a healthy and balanced immune system, the proliferation and contraction of immune cells during an immune response are equally important. Following the proliferation and accomplishment of effector functions against antigen-induced disturbances, the expanded T-cells need to be removed, bringing the immune system to a new homeostatic status. Early studies on T-cell apoptosis have proposed a hypothesis that programmed death (PCD) of activated T cells serves as a suicidal retrenchment mechanism which counterposes the proliferation of T cells in a clonally specific manner [2, 3]. Since then, substantial evidence has uncovered malfunctions of lymphocyte apoptosis, one of the major mechanisms for PCD, that directly impair immune contraction, causing overpopulation of T cells and other immune cells. These have led to the discoveries of genetic defects of CD95 and CD95 ligand accountable for the lymphoproliferative (*lpr*) disorder and the general lymphoproliferative disease (*gld*) in mice [4, 5]; and the autoimmune lymphoproliferative syndrome (ALPS) in humans [6–8, 41].

The signaling pathways that underlie the apoptosis of lymphocytes have been extensively explored, with significant progress being achieved during last 20 years [1, 9]. One noteworthy discovery, revealed by scientists at the National Institutes of Allergy and Infectious Diseases (NIAID), was the observation that a group of patients who had autoimmune disorders presented very similar clinical manifestations to the CD95 mutant *lpr* mice. Further investigations on this group of patients have led to the discovery of causative heterozygous mutations on CD95/Fas gene [6]. Additional studies have shown that the mutant CD95 receptor proteins not only fail to signal CD95-induced apoptosis in activated T cells upon TCR- or CD95 stimulation, but these mutant CD95 can also dominantly interfere with normal CD95 signaling. As a consequence, activated T cells sustain and accumulate in these patients. ALPS has been clinically characterized by chronic, benign splenomegaly and generalized lymphadenopathy, together with laboratory tests showing hypergammaglobulinemia, and autoantibody formation, such as anti-nuclear antibody (ANA) elevation. ALPS patients associated with CD95 gene mutations are designated as ALPS type-Ia [1]. There are also several ALPS cases in our cohort study that have been identified with CD95 ligand (CD95L) gene mutations. Along with other's reports [10, 11], it appears that mutant CD95L proteins, parallel to CD95 mutants, are unable to initiate apoptosis signals through CD95. ALPS patients linked with CD95L gene mutations are categorized as ALPS type-Ib [12]. Currently, the clinical diagnostic criteria and the molecular classification of ALPS are largely reliant on clinical autoimmune lymphoproliferative manifestations that associate with identified causative gene mutations, as shown in Tables 23.1 and 23.2, respectively. Obviously, ALPS classification is subject to future change with new discoveries of genetic mutations associated with the disease.

23.2 Aspects of Type I ALPS

The majority of ALPS cases (50~70%) belong to the type-Ia, which is characteristically associated with the co-expression of mutant and wild-type CD95 protein in the same cells. The mutant CD95 protein dominantly interferes with normal CD95 signals and causes apoptosis defects in T cells and other

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Table 23.1 Diagnostic criteria for Autoimmune Lymphoproliferative Syndrome (ALPS)

Diagnostic features	Description
Required	Chronic nonmalignant lymphadenopathy and/or splenomegaly
	Peripheral over population of $CD4^-$ $CD8^-$ TCRα/β T lymphocytes (DNT cells)
	Defect in TCR- and/or CD95-induced apoptosis
Supportive	Family history of ALPS
	Characteristic histopathology
	Autoimmune disease
	Germ-line mutations in CD95, CD95 ligand, or caspase-10 genes
	DNT cell somatic mutation in CD95 gene

Table 23.2 Molecular classification of ALPS and related disorders

Classification (Type)	Gene mutation
Ia	CD95 (TNFRSF6/Fas/Apo1)
Ib	CD95L (TNFSF6/FasL)
Ic or Im	CD95 somatic mutation (in DNT cells)
IIa	Caspase-10
CEDS*	Caspase-8 (* Caspase-8 Deficiency State)
III	Molecularly undefined
IV	NRAS (defect in IL-2 withdrawal apoptosis)

immune cells. Although the penetrance of these symptoms seems to be incomplete for the inherited CD95 mutations in our family studies, CD95 mutation is clearly a critical risk factor accountable for a predisposition to ALPS [1, 13, 14].

The studies of human ALPS cases and the molecular mechanisms of the disease have greatly reinforced the original hypothesis that programmed demise of activated T cells during an immune response is critical for immune homeostasis [6, 7]. A healthy immune system can efficiently recognize, react and control pathogenic and self-antigenic disturbances, as well as sense and restrain lymphocyte expansion to avoid overreaction that might lead to autoimmune damage.

Predictably, functional mutations of molecules involved in the CD95 signal pathway might result in an apoptosis defect in lymphocytes. The identification of caspase-8 and -10 mutations in ALPS has actually brought up some unexpected, but interesting controversies regarding apoptosis signaling pathways [15–18]. Caspase-8 and -10, belonging to the cysteinyl-aspartate protease family, were identified through their physical connections with a Fas (CD95)-associated death domain (FADD) protein. A cluster of genes encodes the caspase-8 and -10 proteins in humans, along with another protein called cFLIP (cellular FLICE inhibitory protein) that is highly homologous to caspase-8 and -10, but without a functional enzyme domain. Caspase-8 and -10 are considered the most proximal caspases that deliver death receptor (DR)-induced apoptosis signals [19–21]. Structurally, pro-caspase-8 and -10 each contain two N-terminal death-effector domains (DED), as well as large and small subunits of the enzymatic domain. To illustrate this concept of death signaling with CD95, upon DR occupancy, these proenzymes are immediately recruited onto membrane CD95 through homophilic interactions with FADD, forming the death-inducing signal complex (DISC) [22]. Our lab and others have shown that both caspase-8 and -10 are quickly auto-activated during DISC formation, and in turn, the highly activated caspase-8 and -10 can trigger a cascade activation of downstream caspases [23–25]. Multiple studies have shown that cellular aggregation of caspase-8 seems to be sufficient for initiating its auto-cleavage and auto-activation. Therefore, it can be concluded that fully activated caspase-8 delivers apoptosis signals through its proteolytic activity, setting off a cascade activation of other cellular caspases that leads to irreversible cellular execution of apoptosis [9, 26].

23.3 Characterization of Type II ALPS

The success in identification of CD95 and CD95L mutations in ALPS patients has greatly stimulated our interest in capturing new genetic mutations that directly affect the DR signal pathways. We identified several cases of heterozygous mutations of caspase-10 gene in ALPS type-II patients [15, 16]. From *in vitro* experimental data, it appears that heterozygous loss-of-function mutations in caspase-10 gene causes a predisposition to ALPS type-II disease. Supporting this idea, recent studies from another group also found that inactive mutations of caspase-10 were associated with incidence of non-Hodgkin lymphomas and some gastric cancers [27, 28]. These results suggest that mutant caspase-10 proteins can disrupt CD95-induced DISC signaling in peripheral T cells and mononuclear dendritic precursor cells, causing a defect in CD95-induced cell death. It appears that in one aspect, overpopulation of immune cells can cause autoimmunity, whereas on the other hand, a sustained survival advantage of lymphocytes can result in elevated tumor incidence.

Undoubtedly, the *in vitro* data clearly support the hypothesis that normal caspase-8 and -10 are critical for TCR- and CD95-induced apoptosis. To examine the caspase-10 gene mutations that had been identified in some ALPS patients, we used a caspase-8 deficient Jurkat I9.2 line, a human lymphoma line that has no caspase-8 but only residual endogenous

caspase-10 expression and is resistant to CD95-induced apoptosis. It has been shown that the restoration of CD95 PCD in this cell line is dependent on exogenous expression of caspase-8 and/or -10 [29]. We found the mutant caspase-10 genes encoding for loss-of-function proteins, failed to restore CD95-induced apoptosis. In addition, the mutant caspase-10 protein appears to interfere with the functions of wild-type caspase-8 and -10 proteins [24]. Together with the patients' clinical manifestations, these findings indicate that loss-of-function mutations of caspase-10 molecule can jeopardize T-cell apoptosis, and thereby hinder homeostatic control of the proliferating lymphocytes during immune responses, while contributing to autoimmune disorders. Nevertheless, conventional targeted disruption of caspase-8 gene in mice was originally discovered to be embryonic-lethal, due to major developmental defects at day E11.5 [30], which had once essentially halted the *in vivo* assessment of this hypothesis.

Interestingly, unlike humans and other mammals, mouse strains do not have caspase-10 locus, based on the completed maps of human and mouse genomes. Caspase-8 has therefore been considered as a non-redundant apical caspase that mediates DR-induced apoptosis signals in mouse. By contrast, in humans and other animals, there is a caspase-10 gene that is highly homologous to caspase-8. The discrepancy between mouse and human proposes the existence of a possible redundancy and raises uncertainties regarding the biological functions of caspase-8 in human.

23.4 Identification and Characterization of Caspase-8-Deficiency (CED) Patients

Following evaluation of various patients with ALPS-like clinical features, we have found that some patients do not carry gene mutations in CD95, CD95 ligand, FADD, or caspase-10. These patients have been temporarily classified as ALPS type III. After performing functional and genetic screens on these patients, we identified kin of children having an inherited caspase-8 deficiency (CED) [17]. Two siblings, a 12-year old proband female and an 11-year old male, shared ALPS-like clinical manifestations, including lymphadenopathy, splenomegaly, eczema and asthma. Additionally, the peripheral blood lymphocytes from these two patients were defective in CD95-induced apoptosis. Unexpectedly however, straying from typical ALPS cases that manifest with autoimmune symptoms [6, 31], the two affected individuals appeared to have herpes virus infections, recurrent sinopulmonary and gastrointestinal infections, as well as poor response to immunization; also, laboratory tests showed they lacked DNT cells and autoantibodies that are characteristic in Type-I ALPS, thus indicating a coexistence of lymphoproliferation and immunodeficiency. Nevertheless, the affected siblings were developmentally normal. Studies of their mother and an unaffected sibling revealed no ALPS-related manifestations, except their periphery blood lymphocytes (PBLs) showed partial resistance toward CD95-induced apoptosis [17].

Biochemical analysis of the DISC composition showed that the two affected individuals had normal CD95 and FADD expression, as well as CD95-induced FADD recruitment. However, their PBLs seemed to have a poor expression of caspase-8 and in addition, a weak recruitment of caspase-8 to the DISC. CD95 stimulation failed to activate the residual caspase-8 and downstream caspases, indicating that an existing small amount of caspase-8 was not functional in their PBLs. DNA sequencing analysis identified a caspase-8 gene mutation that was homozygous in both of the affected siblings, but was heterozygous in all of the other core family members. The mutation causes a substitution of the amino acid arginine with tryptophan (R248W) in the large subunit of the enzymatic domain. This mutation was not found in more than 100 normal and other patient-DNA samples, suggesting it is very unlikely that the R248W mutation was a polymorphism. Further studies demonstrate that R248W mutation causes caspase-8 to lose its enzymatic function, making it incapable of delivering apoptosis signals through CD95 [17].

23.5 Inflammatory Role of Caspase-8 in Immune Cells

It was well anticipated that the loss-of-function mutation of caspase-8 would cause a defect in CD95-induced apoptosis. Yet surprisingly, in addition to an apoptosis defect, the two homozygous CED patients but not other heterozygous family members, were clearly immuno-deficient, indicating that caspase-8 may play a role in the protective immune system. Further studies have demonstrated that T lymphocytes isolated from the patients displayed a dramatic reduction in NF-κB induction, cytokine production, and up-regulation of activation-markers upon TCR stimulation. Furthermore, B cells and NK cells seemed also to have defects in responding to their corresponding stimuli. On the other hand, by increasing wild-type caspase-8 protein expression in the primary T cells derived from the patients, we were able to partially correct the defect in T-cell activation. Lending further support to our hypothesis, recent conditional caspase-8 knockout mice revealed that caspase-8-null mouse T cells have deficiencies in activation and proliferation. In addition, caspase-8 in hematopoietic cells also has a fundamental role in lymphocyte and organ development, besides its regulatory role in apoptosis [32, 33]. Taken together, these data strongly indicate that caspase-8 has dual roles in proliferation and programmed death of immune cells [34].

In contrast to ALPS type Ia, where a CD95 mutation in patients leads to an apoptosis defect that predominantly associates with lymphoproliferative autoimmune disorders, CED seems to present a contradictory scenario regarding apoptosis defect combined with immunodeficiency. Along with others, we have found by *in vitro* experiments that inhibiting caspase activity can suppress normal T-cell activation [17, 18, 35]. To address the molecular mechanisms that underlie the inflammatory role of caspase-8 in T-cell activation, we first examined the transcription factor NF-κB activation in T cells exposed to caspase inhibitors. Upon TCR stimulation, the cytoplasmic Inhibitor of κB (IκB) protein degrades rapidly, which causes NF-κB nuclear translocation and transcriptional activation. We found that normal primary T cells treated with zVAD-fmk, a pan-caspase inhibitor peptide, showed significant blockade of NF-κB nuclear translocation [18]. Further experiments using a more specific caspase-8 inhibitor, zIETD-fmk, or a caspase-8 deficient T-cell lymphoma line (I9.2) verify that lack of caspase-8, but not other caspases in T cells, accounts for the obstruction of TCR-induced NF-κB activation. In addition, B cells and NK cells derived from a CED patient are unable to initiate NF-κB nuclear translocation through stimulation of CD40, FcγRIII, or NK-cell activation receptor 2B4. Interestingly, TNF-α or CD95-induced NF-κB activation appears to be normal when caspase-8 is defective. These observations demonstrate that caspase-8 is selectively required for antigen-receptor induced NF-κB activation in T, B, and NK cells.

23.6 Molecular Functions of Caspase-8 in TCR-Induced NF-κB Activation

One obvious question that arose is how caspase-8 can interact with the TCR signaling pathways. It has been known that protein kinase Cθ is phosphorylated during early events of TCR stimulation, by which it initiates recruitment of CARMA1-Bcl10-MALT1 (CBM) signaling complex. Also, the TCR-induced recruitment of CBM complex appears to link with immediate activation of the Inhibitor of kappa-B kinase (IKK) complex [36]. However, it was unclear how NF-κB activation signals were passed through the two signaling complexes that are seemingly segregated [37]. We have observed normal PKCθ activation, but significantly decreased IKK phosphorylation and IκBα degradation in caspase-8 deficient conditions, indicating caspase-8 may act at the nexus of PKCθ and IKK phosphorylation [18].

The physical involvement of caspase-8 in T-cell activation signaling pathways emerges from a kinetic analysis of the components associated with TCR-activation complexes. We have found that TCR stimulation induces sequential recruitment of caspase-8 and IKKα,β into the CBM complex, which is followed by IKK phosphorylation and IκBa degradation. Lacking caspase-8 largely abrogates IKK activation through CBM complex, whereas over expression of constitutively active IKK can restore NF-κB activation in caspase-8-deficient Jurkat I9.2 lines. This evidence, together with the data showing that PKCθ activation is normal in caspase-8 deficient cells, suggests that caspase-8 plays a role in connecting PKCθ and IKK activation [18].

Another interesting question arises as to whether the protease activity of caspase-8 is important for NF-κB activation. The observation that caspase inhibitors block TCR-induced NF-κB activation appears to support this argument [17]. However, we found that neither the prodomain nor the active enzyme domain of caspase-8 was able to restore TCR-induced NF-κB activation in caspase-8 deficient T cells. On the contrary, the full-length wild-type caspase-8 can fully restore the NF-κB signaling events through TCR stimulation [18]. Although the direct substrate of caspase-8 remains unidentified in the TCR signal complex, we conclude that physical presence and minimum enzymatic activation of caspase-8 are critical for delivering TCR-induced NF-κB activation signals (illustrated in Fig. 23.1).

It is counter-intuitive that caspase-8, originally identified as an initiator caspase that mediates DR-induced apoptosis signals, is essential for TCR-induced activation signals. Considering the immune system as a self-maintained homeostatic environment, the double-sided roles of caspase-8 can provide the system with tight regulation of over proliferation versus programmed death of the immune-reactive cells. This idea should not be surprising because caspase-8 is not the only protein that has opposing roles in signaling T-cell survival and death. FADD, an essential adaptor protein for DRs to recruit caspase-8/-10, is involved in signaling lymphocyte survival, in addition to its indispensable role in CD95-induced apoptosis [38]. There is also evidence implying that CD95L and CD95 can play roles in initiating apoptosis, as well as providing a costimulatory signal for T-cell activation [39–41]. In a further extension of this concept, it is evident that TCR stimulation can induce T-cell activation in naïve cells, but also cause apoptosis when the T cells are already activated, suggesting that the same ensemble of signaling proteins can achieve different biological functions in the context of different available microenvironments [1]. These facts also argue that during evolution, proteins can find more than one locus to contribute to their function, even with a conflicting role. This engenders a more complex system, with even greater regulation of the immune system.

It is currently unclear if the autocleaved and fully activated capase-8 enzyme has the same role as the minimally activated full-length caspase-8 in TCR-induced NF-κB activation. Fully activated and cleaved caspase-8 may dissociate from the CBM complex, serving as a dampening signal precluding immune hyperactivity. Also, the fully activated caspase can efficiently trigger the full cascade of caspase activation, causing intracellular damage that prevents cellular proliferation and leads to apoptosis.

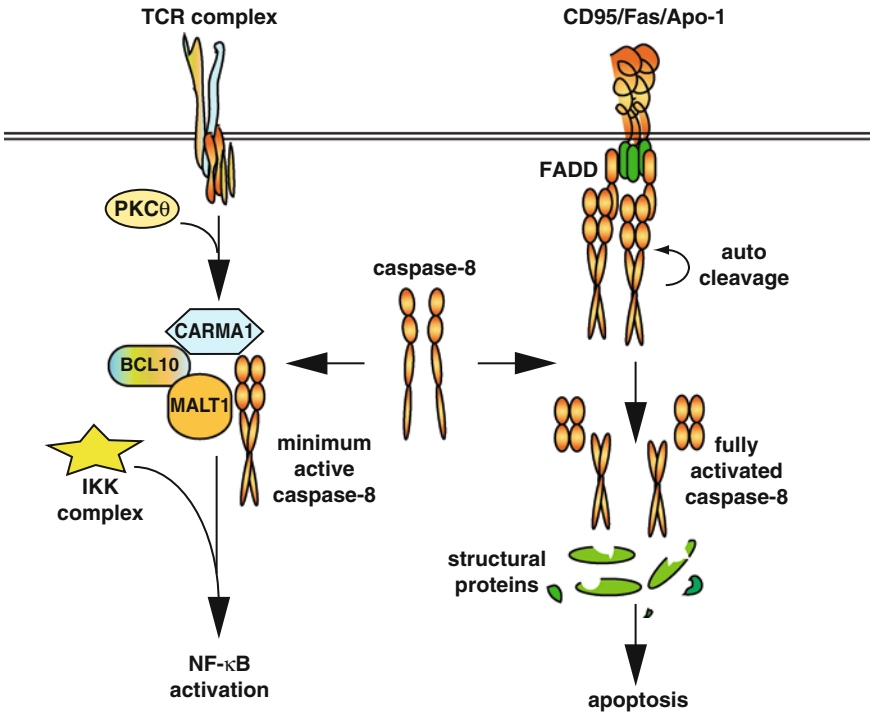

Fig. 23. 1 Caspase-*8 participates in both pro-inflammatory and apoptotic signaling in T lymphocytes.* The full-length, partially active caspase-8 is physically associated with the CARMA1-BCL10-MALT1 (CARD-containing membrane-associated protein 1, - B-cell lymphoma 10, - mucosa-associated lymphoid tissue protein 1, CBM) complex. Minimum active caspase-8 is critical for the transduction of inflammatory signals from TCR complex toward activation of the inhibitor of κ B kinase (IKK) and thus for NF-κB activation. During CD95 signaling, procaspase-8 is recruited to the membrane CD95 through the adaptor Fas-associated death domain (FADD). Aggregation of caspase-8 triggers self-cleavage and auto-activation, which generates fully activated caspase-8 and leads to apoptosis execution

23.7 Conclusion Marks

A systemic exploration of the molecular mechanism that underlies ALPS has led us to conclude that proliferation and contraction of immune cells are equally important for maintaining a healthy immune system. Caspase-8, along with other proteins involved in programmed lymphocyte death, are playing important roles in receptor-mediated activation and the proliferation of immunoreactive cells. Dysregulation of this homeostasis can lead to either immunodeficiency and/or autoimmunity. Our observations also provide a basis for the improvement of clinical diagnosis and vaccination strategies, as well as the potential treatment of autoimmune disorders.

The cysteine-aspartate-protease (Caspase) 8 has been identified originally as an apical caspase that mediates death-receptor (DR) induced apoptosis. An unexpected pro-inflammatory role of caspase-8 in immune cells has emerged from the identification of a homozygous loss-of-function mutation of a caspase-8 gene in a kin with two children, who manifested defects in DR-induced apoptosis combined unexpectedly with immunodeficiency. Further studies have revealed that a full-length pro-caspase-8 with minimum enzymatic activity appears to be required for antigen-receptor induced NF-κB activation in immune cells, especially in T lymphocytes. Thus, caspase-8 plays dual roles in regulating immune responses, promoting inflammatory signals for immune activation and mediating lymphocyte apoptosis during immune responses.

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Chapter 24
Features of Plasma Cell-Related Neoplasms in Mice

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Abbreviations

AID	activation-induced cytidine deaminase
BCR	B cell receptor
BM	bone marrow
Chr	chromosome
CpG	cytosine phosphate guanosine
CSR	class switch recombination
DC	dendritic cell
FDC	follicular DC
FOL	follicular
F_{TH}	follicular T helper cell
GC	germinal center
KI	knockin
KO	knockout
MM	multiple myeloma
MZ	marginal zone
PCT	plasmacytoma
PL	plasmacytoid lymphoma
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
TD	T cell dependent
TG	transgene
TI	T cell independent.

24.1 Introduction

Plasma cells are terminally differentiated cells of the B-cell lineage. They can develop from any of several mature B-cell subsets, including germinal center (GC), memory, marginal zone (MZ), and B1 cells. Plasma-cell tumors of mice, termed plasmacytomas (PCT), are increasingly recognized as sharing many features with the major type of plasma-cell tumors in humans, termed multiple myeloma (MM), an almost uniformly lethal disease. Consequently, an increasing number of mouse models of plasma-cell neoplasia are being developed to dissect the mechanisms underlying the initiation, progression, and maintenance of the transformed phenotype with the long-term goal of improving diagnosis and therapy. Studies of plasma-cell neoplasms in mice and humans have been remarkably informative for identifying genes and signaling pathways critical to normal B-cell differentiation, function, and survival, as well as neoplastic transformation (Fig. 24.1). Examinations of mouse plasma-cell neoplasms initiated in the National Cancer Institute over 50 years ago have been joined synergistically with studies in the National Institute of Allergy and Infectious Diseases, together with their collaborators, of normal and transformed plasma cells from retrovirus-infected and autoimmune mice. Together, they have painted an increasingly rich picture of the later stages of normal B-cell differentiation and the changes that redirect these cells to neoplasia. Other recent publications provide reviews of other plasma-cell tumor models, as well as other aspects of the studies described here [1,2].

24.2 Origins of Normal Plasma Cells

Responses to viral, bacterial, and other pathogens are initiated within minutes of challenge by cellular components of the innate immune response that include dendritic cells (DC), macrophages, and granulocytes [3,4]. Interferons and inflammatory cytokines produced by these cells and intracellular resistance mechanisms provide an immediate protective response that gives way to adaptive, antigen-specific immune responses by T cells and follicular (FOL) B cells that take a week or more to develop. The time frame between the immediacy of the innate response and the delayed acquired response is filled in by a "primed acquired" response manifested by the ability of antigen-specific MZ and B1 cells to develop into highly secretory plasma cells within hours [5], as well as the activities of γ/δ and NKT cells [6].

The MZ and B1 B cell subsets in mice are mostly localized to unique anatomic compartments: B1 cells to the peritoneal and pleural cavities [7] and MZ B cells to the splenic MZ [8]. The MZ comprises MZ B cells and specialized populations

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of MZ macrophages and DC. It is geographically located in the area of the spleen where arterioles empty into sinuses, generating a slow rate of blood flow that allows efficient trapping of blood-borne antigen-antibody complexes and circulating bacteria or viruses. MZ B cells derive from bone marrow (BM) progenitors and are generally sessile; however, exposure to antigen results in a rapid migration through the follicle to form extrafollicular foci of plasma cells [9,10]. There they secrete large amounts of mostly IgM before undergoing apoptosis within seven to eight days.

There are two subsets of peritoneal B1 cells that uniformly express CD11b but are distinguished by their expression of CD5: B1a cells are $CD5^{+}$, while B1b cells are $CD5^{-}$. These phenotypic differences are associated with unique responses to particular pathogens. $CD11b^{-}$ B1a-like cells are also found in small numbers in spleen in association with follicular DC (FDC) [11]. Unlike MZ or FOL B cells, B1 cells develop mostly from fetal liver precursors [7,12].

Analyses of the Ig repertoires of both MZ and B1 B cells have provided evidence that they are highly selected and that the compartments are enriched for cells broadly reactive to self and microbial antigens [13,14]. Both B-cell subsets secrete primarily IgM antibodies, derived preferentially from germline rearrangements of the B cell receptor (BCR) or from minimally mutated sequences. Both are capable of undergoing class-switch recombination (CSR), with switched B1 cells generating most of the secreted IgA in the gut [15], while splenic MZ B cells produce systemic IgA [16]. It is important to note that class-switched responses can develop in response to specific viral infections and other stimuli in the absence of help from T cells (T-independent (TI) antigens) [17,18].

Like MZ B cells, FOL B cells are BM derived; however, in contrast with MZ and B1 cells that appear to be highly selected, FOL B cells appear to be less enriched for particular specificities. In addition, they are distinguished from MZ and B1 cells by their residence in follicles that lie inside the

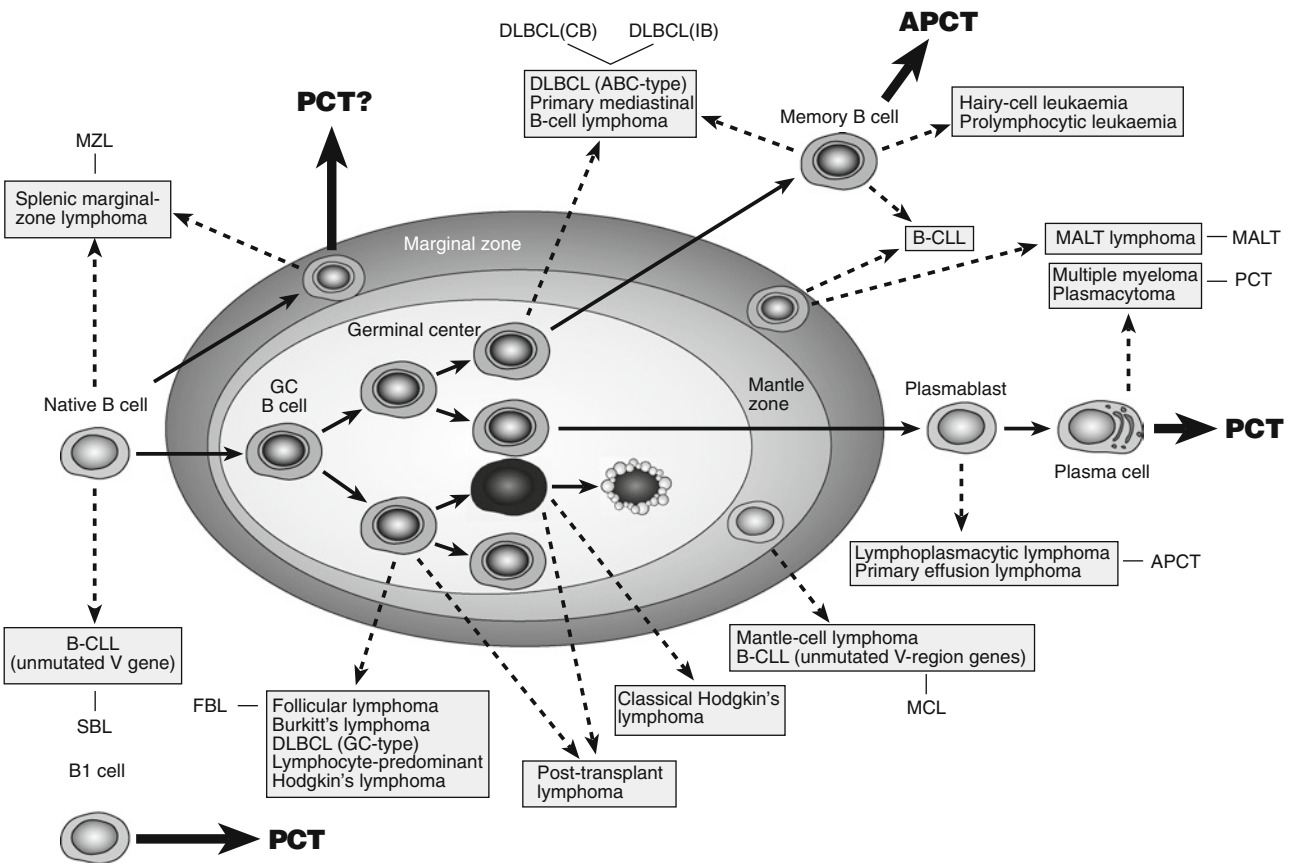

Fig. 24.1 Origins of human and mouse B cell lineage neoplasms. Solid thin arrows indicate normal patterns of B cell differentiation. Dashed thin arrows show the relations of normal cells at particular stages of this pathway as precursors to specific human neoplasms shown in the shaded boxes. Short thin lines connect the human tumor types to the most similar subset of mouse B cell-lineage tumor. Heavy solid arrows indicate the relations between a normal differentiated cell type and the mouse plasma-cell neoplasms described in the text. PCT is used to indicate plasmacytic plasmacytomas and APCT to indicate anaplastic and plasmablastic plasmacytomas

splenic MZ, or in lymph nodes and their requirement for T-cell help to develop into plasma cells in T-dependent (TD) responses. After interacting with T cells and antigen, FOL B cells migrate into FDC-rich areas of the follicle where they form GC [19]. In humans, but less so in mice, GC exhibit so-called dark and light zones, with the former comprising primarily large, rapidly proliferating centroblasts and the latter small, non-cycling centrocytes. In response to follicular T helper cells (F_{TH}) that secrete IL-21 and express CD40L [20,21], IL-21R$^+$ CD40$^+$ GC B cells express high levels of the enzyme activation-induced cytidine deaminase (AID) [22]. AID is responsible for mutating Ig variable region sequences to generate antibodies with increased affinity for inducing antigen, a process termed somatic hypermutation (SHM). AID is also responsible for inducing CSR to produce antibodies with different functional capabilities, such as complement fixation (IgGs), secretion (IgA), or binding to mast cells (IgE). Humans or mice deficient in AID are unable to generate V region mutated high-affinity or class-switched antibodies [23]. B cells that have been processed through GC can progress immediately to become plasma cells or they can enter the pool of long-lived, recirculating memory B cells. When re-exposed to cognate antigen, memory cells can rapidly differentiate into plasma cells. Plasma cells generated in response to TD antigens can reside in the spleen as a short-lived population or migrate to the BM, where they become long-lived plasma cells.

In summary, plasma cells generated from physiologic responses to TI antigens derive mostly from MZ or B1 cells, while those generated in response to TD antigens derive from post-GC plasma cells or memory B cells. Understanding how each of these pathways is triggered and can be manipulated will be critical to developing finely tuned responses to antigenic components of different vaccines.

24.3 Plasma Cell Development in Autoimmunity

Autoimmune disorders can be subclassified into those that are organ specific, such as type I diabetes mellitus or Hashimoto's thyroiditis, and those that are generalized, such as systemic lupus erythematosus (SLE) or Sjögren's syndrome (SS). Organ-specific T cells usually mediate the destructive aspects of the former diseases while the pathology in the systemic disorders, with exceptions, is caused by deposition of complement-coated immune complexes of antibody with DNA, RNA, or other self antigens in glomeruli, blood vessels, skin, and other organs.

In the systemic diseases, antibodies reactive with self antigens often derive from plasma cells with precursors that are AID experienced, as they exhibit SHM and are class switched. This is consistent with the presence of ectopic GC in salivary glands of patients with SS, thyroids of patients with Hashimoto's thyroiditis, and synovial tissues of patients with rheumatoid arthritis. In mouse models of SLE, these plasma cells appear to derive from chronically activated cells that accumulate in extrafollicular foci. These foci are distinct from those produced by activated MZ B cells, as their development is dependent on a unique F_{TH}-like T cell subset. Notably, systemic disease is greatly attenuated in mice deficient in AID.

24.3.1 Relation of An Inflammatory Environment to Plasma Cell Malignancies In Mice

Plasma cell development in normal responses to TD antigens is now known to progress from the GC reaction to cells defined as immunoblasts, followed by a transition to plasmablasts and then to fully mature plasma cells. The essential features of mature plasma cells—condensed chromatin with a 'clock face' appearance of an eccentric nucleus, a perinuclear pale area now recognized as the Golgi, and a spherical cytoplasm—were described by Marshalko [24]. The disease associated with the malignant counterpart of mature plasma cells is now termed MM. Of interest, the mature-appearing cells in cases of MM are described today as Marshalko cells.

Dunn first described spontaneous ileocecal neoplasms of mature plasma cells in mice in 1954. This observation was followed rapidly by demonstrations that PCT were readily induced in BALB/c mice treated intraperitoneally with agents, such as the mineral oil pristane, that induce a chronic inflammatory response with PCT developing between 150 and 300 days as reviewed in Potter [25]. In mice treated with pristane, oil granulomas develop rapidly on peritoneal surfaces. The granulomas comprise activated inflammatory cells, including macrophages full of poorly metabolized mineral oil, granulocytes, and eosinophils. Early on after treatment with pristane, they also exhibited collections of what appear to be normal B cells surrounded by increasing numbers of plasma cells. With time, the plasma cell populations in the granulomas take on the appearance of transformed plasma cells and are shed into ascitic fluid that accumulates in the peritoneum [1].

The importance of inflammation-associated oil granulomas to PCT induction was illustrated by three observations. First, treatment of mice with cortisol inhibited granuloma formation and PCT development. Second, susceptibility to PCT induction by pristane is markedly strain dependent, with BALB/c and NZB mice being highly susceptible to induction, whereas DBA/2, C57BL/6, and many other strains are almost totally resistant [26–28]. Although both

BALB/c and NZB mice were sensitive to PCT induction by pristane, they differed in the proportions of mice that developed PCT, the latency for disease development, and the distributions of switched Ig classes produced; NZB mice developed fewer tumors with longer latencies and had substantially increased proportions of PCT that secreted IgG subclasses, as compared to the dominance of IgA among BALB/c PCT [28, 29].

While all the resistant strains also developed granulomas in response to pristane, studies of these mice showed that the number of preneoplastic foci that developed in response to pristane was less than in susceptible BALB/c mice. A third critical determinant of BALB/c PCT induction was subsequently found to be production of IL-6 by the granuloma tissue [30]. Indeed, mice deficient in IL-6 by virtue of a gene knockout (KO) were totally resistant to PCT induction [31], whereas mice constitutively producing IL-6 systemically from a transgene (TG) generated tissue-based PCT in the absence of pristane [32]. Finally, pristane-primed mice chronically treated with the nonsteroidal antiinflammatory drug indomethacin were completely resistant to PCT induction, by virtue of complete blockage of IL-6 production by peritoneal macrophages [33,34].

24.4 Genetic and Molecular Determinants of PCT Induction by Pristane

The genetic basis for strain-dependent differences in susceptibility to PCT induction by pristane was studied systematically in mice derived from crosses between BALB/c and the resistant strain DBA/2. These studies showed that the predisposition of BALB/c mice to PCT induction is multigenic [35] and associated with loci mapping to chromosome (Chr) 1 and two regions on Chr 4, designated *Pctr1* and *Pctr2* [35–37]. The BALB allele of *Pctr1* is an "efficiency" allele of the p16 (INK4a) gene, which has a compound defect in both coding and promoter region sequences compared with DBA [38–40] that and leads to reduced phosphorylation of the Rb tumor suppressor. *Frap/mTOR* has been identified as a candidate gene for *Pctr2* by positional cloning, and the BALB allele is again less efficient in phosphorylating the p53 tumor suppressor [41]. The *Pctr2* locus has been shown to delay onset of plasmacytomagenesis [37]. Both p16 (*Pctr1*) and mTOR (*Pctr2*) have been shown to have tumor suppressor activity in NIH3T3 cell assays [39,41]. The PCT-susceptible NZB strain carries the same high-efficiency allele of p16 as the DBA strain; however, its mTOR allele is the less efficient BALB version of the gene [38,41]. In the same backcross that led to identification of the *Pctr1, 2* loci, another gene on Chr 1, referred to as a modifier of PCT resistance/susceptibility, *Pctm*, was identified [35]. This modifier was the only susceptibility locus identified in the backcross that was linked to heterozygosity (C/D alleles), as opposed to homozygosity of BALB alleles, suggesting that DBA mice carry a PCT susceptibility gene that displays the susceptible phenotype when the locus is heterozygous with BALB.

A fundamental understanding of genomic changes associated with plasmacytomagenesis, however, awaited the advent of molecular genetics. The 1970s, '80s and '90s were marked sequentially by: (i) the advent of molecular characterizations of oncogenes captured by avian or murine acutely transforming retroviruses and the ability to adapt them for infections of mice; (ii) the identification of tumor-specific translocations and the characterizations of genes affected by the translocations; and (iii) the ability to manipulate mice genetically, through the introduction of TG and by germline modifications to generate gene KO and knockins (KI).

All three elements have contributed to the understanding that specific, AID-dependent chromosomal translocations resulting in deregulated expression of the *Myc* proto-oncogene are the initiating events in PCT development [reviewed in Potter [1]]. First, studies from the laboratories of Klein and Potter revealed that PCT were regularly associated with the presence of one of two chromosomal translocations, T(12;15) and T(6;15) [42,43]. Because Chr 12 and Chr 6 were known to carry the IgH and Ig kappa loci, respectively, that undergo rearrangement during normal plasma-cell development, these findings suggested that aberrant rearrangements of Ig genes might result in their association with a "supergene" on Chr 15 [43]. This hypothesis was rapidly validated by the demonstration that in a panel of established PCT cell lines, the proto-oncogene *Myc*, normally located on Chr 15, was translocated and recombined to the IgH locus Cα gene [44]. This finding marked a pivotal turning point in the understanding of B-cell neoplasia in mice and humans, as *Myc* had previously been identified as the transforming sequence captured by the MC29 family of oncogenic avian retroviruses [45], and in chickens infected with avian leucosis viruses, bursal B-cell lymphomas were almost always associated with somatically acquired proviral insertions just upstream of the cellular *Myc* gene [46]. A flurry of studies in humans soon described the association of aberrant Ig translocations juxtaposed to *Myc* and a series of other proto-oncogenes now designated *Bcl1*, *Bcl2*, etc. [47].

Second, the critical importance of *Ig/Myc* translocations to B-cell transformation was clarified by studies of mice bearing an Eμ-*Myc* TG that developed a high incidence of pre-B and immature B-cell lymphomas [48]. The influence of Ig regulatory sequences adjacent to *Myc* for features of mature B-cell and plasma-cell transformation has been clarified by studies of KI mice, with *Myc* inserted into the IgH locus to replicate Myc translocations that are found in human Burkitt lymphomas or mouse PCT [49,50].

Finally, the mechanisms responsible for generation of DS DNA breaks in the Ig and *Myc* loci that precede development of the translocations characteristic of PCT and other B cell-lineage neoplasms have become increasingly well understood. Molecular processes that normally remodel Ig genes include VDJ recombination, SHM, and CSR. *IgH/BCL2* and *IgH/BCL1* translocations found in human follicular lymphomas and mantle cell lymphomas, respectively, have breakpoints immediately adjacent to IgH J-region sequences, with fine features that reflect the activity of RAG during the stages of B-cell development in the BM [51,52]. Very recently, the Lieber group has suggested that these breaks are uniquely targeted to cytosine phosphate guanosine (CpG) clusters in the major breakpoint regions, with the RAG complex acting on AID-deaminated methyl-CpGs in these clusters [53].

Although the involvement of AID in the *BCL1* and *BCL2* translocations remains to be cleanly established, AID is critical to normal processes of SHM and CSR and to translocations associated with both types of genomic alteration. Involvement of SHM in translocations is inferred from the examination of breakpoints within or adjacent to rearranged V genes that suggest their generation as byproducts of SHM. These include *IgH/MYC* translocations in endemic and sporadic Burkitt lymphomas and *IgH/BCL6* translocations in diffuse large B-cell lymphoma [52].

Normal CSR involves generation of AID-dependent DS DNA breaks in both the switch μ (sμ) and a switch region associated with one of the downstream CH genes. Studies of mice homozygous for a null mutation of *Aicda* showed them to be highly resistant to PCT induction by pristane [54]. The PCT that did develop were atypical, appearing with long latencies in the absence of ascites and with translocations involving unusual juxtapositions of Ig and *Myc* family members. Mice heterozygous for a null allele of *Aicda* exhibited haploinsufficiency for both isotype switching and plasmacytoma development [55]. Remarkably, the Nussenzweig laboratory has recently reported that the DS DNA breaks in *Myc* that lead to translocations are also dependent on AID [56].

MYC is recognized as a critical regulator of cell growth that encodes a DNA-binding transcription factor that can activate or repress RNA transcription. MYC regulates expression of an unusually large number of target genes involved in the control of critical cell functions including growth, cell cycle progression, differentiation, and apoptosis in almost all cells. It has been described as having a global transcriptional role in human B cells [57–59]. Nonetheless, Ig/*MYC* translocations can be found in normal B cells, indicating the requirement of secondary genetic events for transformation and development of PCT. IL-6 produced by stromal cells clearly plays a major role, but other B-cell-intrinsic mechanisms are less well understood.

Recently, it was suggested that signaling through the NOTCH pathway may be an important component of PCT progression or maintenance [60]. These studies showed that genes involved in NOTCH signaling were expressed at increased levels in primary IL-6 TG PCT. In addition, inhibition of signaling in established cell lines was associated with reduced expression of NOTCH targets, impaired proliferation, and increased apoptosis. These observations have important implications, in that they suggest that Myc and NOTCH may synergize in PCT development. Support for this concept comes from studies of human T-cell acute lymphoblastic leukemia in which NOTCH and MYC were shown to regulate directly interconnected transcriptional programs with common targets that affect growth of the primary tumors [61], as well as studies of NOTCH signaling in MM [62], in which almost all cases overexpress MYC.

Other recent work suggests that aberrant expression of the protein elongation factor EEF1A2 may contribute to induction or progression of PCT and possibly a subset of MM (Z. Li, manuscript submitted). EEF1A2 is overexpressed in breast, ovarian, and other solid tumors, where it has been suggested to function as a proto–oncogene [63]. Increased expression in PCT was not associated with mutations or copy number changes, but resulted in delayed G1-S progression and decreased signaling through the JAK/STAT and AKT/PI3K pathways. (Z. Li, manuscript submitted). Further studies of primary PCT and MM will be required to evaluate effects on initiation, progression, or possibly maintenance of these neoplasms.

24.5 MYC-Independent Plasma-Cell Neoplasms in Mice

A number of reports have shown that patients with autoimmune diseases including SLE, SS, and highly active rheumatoid arthritis are at increased risk for development of B-cell lineage neoplasms. The mechanisms involved in tumor development under these circumstances are not well understood, although chronic stimulation by autoantigens may be contributory. Studies of mice bearing mutations of *Fas* or *Fasl* that develop severe autoimmunity showed that they develop plasma-cell-related neoplasms around a year of age [64]. Most of the tumors exhibited CSR to IgG subclasses or IgA, but some were IgM positive. Although the tumors were difficult to identify in primary tissues because of the characteristic dominance of $CD4^{-}CD8^{-}$ T-cells in these mice, they had many plasma cell features on transplantation, but primary tumors or cell lines derived from them had no *Ig/Myc* translocations.

Subsequent studies showed that the tumor, termed plasmacytoid lymphoma (PL), derived from antigen-experienced autoreactive cells that secreted antinuclear or rheumatoid factor antibodies and exhibited skewed IgV gene repertoires [65]. Gene expression profiles of PL showed that they were

readily distinguished from all other B-cell-lineage tumors examined and suggested that they represented a less mature stage of plasma-cell differentiation than pristane-induced PCT and another plasma cell-rich neoplasm, the lymphomas of SJL mice.

Another population of Myc-independent PCT was identified during studies of hematopoietic neoplasms that develop in NFS.V$^+$ mice congenic for ecotropic MuLV induction loci from the high virus strains AKR and C58 [66]. These strains produce high levels of ecotropic MuLV throughout their lives and develop a wide spectrum of B-cell-lineage lymphomas, including small lymphocytic; marginal zone; follicular; diffuse large B-cell lymphoma, and high–grade diffuse blastic types. During the course of these studies, we recognized that the small proportion of these tumors (~1.8%) diagnosed as PCT were less mature than the PCT of pristane-treated or IL-6 TG mice [67]. Instead, they appear to include among them a spectrum with features ranging from those of immunoblastic lymphomas to immature plasmablastic lymphomas, with some sharing a few features with fully mature Marshalko-type/plasmacytic PCT characteristic of pristane-induced PCT. PCT with this spectrum of features were recognized among those that developed in Eμ-v-abl TG mice [68,69]. A number of additional cases were soon recognized among PCT occurring in iMyc$^{E\mu}$knockin mice [50].

Histologic features allowed these diverse PCTs to be graded into three classes: the least mature as anaplastic (APCT) and those of greater maturity (but not fully mature) as plasmablastic, both from NFS.V$^+$ and iMyc$^{E\mu}$knockin mice; the most mature plasmacytic cases were those characteristic of pristane-induced PCT. Molecular studies of the anaplastic and plasmablastic cases showed that they were clonal but lacked Ig/*Myc* translocations [67]. In addition, oligonucleotide microarray gene expression profiling showed them to be less mature than conventional PCT or SJL tumors and more like PL or immunoblastic tumors.

Another Myc-independent model of plasma-cell neoplasia was described by Radl and collaborators [70,71]. They identified a series of plasma-cell tumors that developed in old C57BL/KaLwRij mice that, in contrast to other systems, homed to the BM, causing osteolytic lesions such as those seen in MM.

24.5.1 Considerations Regarding the Role of the BCR, Antigenic Stimulation, and Cellular Origins of Plasma-Cell Neoplasms In Mice

BCR dependency is a requirement for normal B-cell survival and selection from the pairing of surrogate light chains with μH and the later vetting of immature B cells in fetal tissues or adult BM; selection into FOL, MZ, or B1 subsets; and survival and exit from the GC reaction to form memory or plasma cells. Similar selection pressures also seem to occur in B-cell malignancies, as almost all B-cell lymphomas of mice and humans still express a BCR, even if it is only at low levels. The fact that some B-cell tumors, such as follicular or primary central nervous system lymphomas, exhibit ongoing V-region mutations during the process of clonal expansion is in keeping with this view [72–74] and indicates that they are still exposed to AID and other influences characteristic of residence in the GC.

Because most plasma-cell-related neoplasms do not express surface Ig, evidence of their prior BCR-dependent selection processes would have to be mined from studies of their rearranged Ig genes and secreted immunoglobulins, or developed from model systems that allow the influence of antigen selection to be read out in PCT. The following observations relate to these issues, keeping in mind that plasma-cell neoplasms that develop under different settings may have distinctly different drives, including antigen-independent and innate immune mechanisms [17,18]. Some of the strongest evidence for a contribution of antigenic drive in PCT development comes from studies showing that germ-free [75] and specific pathogen-free BALB/c mice [76] are highly resistant to PCT induction by pristane. In addition, studies of lambda light chain amino acid sequences indicated that antigen selection was operating to induce amino acid changes in specificity-determining regions [77].

Equally strong evidence for antigen-driven BCR activation in mouse plasma-cell tumors comes from the PL of autoimmune *Fas* and *Fasl* mutant mice. The tumors secrete antibodies directed to autoantigens for significantly mutated Ig V regions that derive from skewed V_H and V_K repertoires found in other autoimmune mice [78,79]. This, combined with the observation that almost all the tumors have undergone CSR, would indicate that the tumors were GC derived, but arrested in their progression to terminal plasma cell differentiation. In this regard, it is noteworthy that T-cell-deficient nude mice treated with pristane developed B-cell lymphomas but no PCT, whereas nude mice reconstituted with T cells before tumor induction developed PCT [80]. These data could be interpreted to indicate that T-cell-dependent GC development was a prerequisite for PCT development.

Binding studies of pristane-induced PCTs have revealed binding activities for a variety of antigens including DNP; phosphocholine; phosphatidylcholine; galactan; glucans, and flagellin, among others [reviewed in Potter [81]]. A more recent study identified a series of PCT with binding specificities against cytoskeletal proteins and DNA, with many being polyreactive [82]. Sequencing of the IgH V regions from these tumors revealed SHM activity with high replacement to silent ratios in four tumors and another with a single

mutation, while light-chain sequences provided evidence for intraclonal heterogeneity. The identification of tumors with somatic mutations, intraclonal heterogeneity, and high replacement to silent ratios is strongly suggestive of continuing antigenic selection.

In another study to be described more extensively below (K. Williams, et al, manuscript in preparation), PCT were induced by pristane treatment of wild-type BALB/c mice or BALB/c mice bearing a M167μκ (V1μ plus Vκ24) TG that is specific for phosphatidylcholine [83]. As part of this study, IgH and Igk V-region sequences were determined for 24 PCT from TG mice, 10 from wild-type mice, and 9 from CB.20 mice, BALB/c mice congenic for the C57BL/6 IgH locus. The results showed that around 80% of the tumors in each of the three groups exhibited SHM, with mutations in IgH found in all the tumors with mutated genes but one, while 30% to 45% of the Vκ sequences remained germline. Most of the mutations resulted in amino acid substitutions with clustering in the complementarity-determining regions. The high replacement to silent ratios and clusterings of mutations in the complementarity-determining regions are strongly suggestive of antigen-driven selection.

24.6 Cells of Origin for Pristane-Induced PCT and APCT

Analysis of PCT induction in M167μκ TG mice was undertaken as part of an effort to understand the cell of origin of pristane-induced PCT. The study was based on the understanding that normal MZ and B1a cells are enriched for cells with specificities for bacterial and self antigens, when compared with normal follicular B2 cells and that different Ig TG were associated with expanded MZ or B1 cell populations. Mice bearing the M167μκ TG have a MZ B-cell population that is increased around threefold over that of non-TG mice [84], and mice bearing a VH12μ TG have a greatly increased population of B1a cells [85]. To determine whether PCT induction might be influenced by BCR-determined expansions of these B-cell subsets, TG mice of both types and wild-type controls were treated with pristane and followed for PCT development. PCT developed in 65% of M167μκ TG mice, in contrast to an incidence in non-TG mice of 35% (K. Williams, et al., manuscript in preparation). Parallel studies showed that the incidence of PCT in VH12μ TG mice and their controls was almost identical at 27%. These data imply that MZ B cells are more susceptible to pristane-induced transformation than B1 cells and might be the cell of origin for pristane-induced PCT of BALB/c mice.

The possibility that B1 cells might be the preferred targets for transformation was suggested by induction studies using mice mutant for tyrosine kinase, BTK. These mice lack B1 cells and were found to be markedly resistant to PCT development [86]. The fact that BTK is involved downstream from SYK in BCR signaling regardless of B-cell subset, however, indicates that the mutant mice may have no normal B cells, making the results difficult to interpret.

Additional studies were designed to determine whether a cell of origin could also be determined for APCT. Previous studies of gene expression profiles suggested that they might reflect a stage of plasma cell development intermediate between post-GC immunoblasts and plasmablasts [67], but possible origins from B1, MZ, or memory B cells had not been excluded. To examine these possibilities, genes differentially expressed between PCT and APCT were compared with expression profiles generated from normal naïve, GC, and memory B cells, as well as plasma cells [87]. Remarkably, these comparisons showed that APCT were more related to memory cells than to plasma cells (C.-F. Qi, et al, manuscript in preparation). These suggested parallels between APCT and normal memory cells were strongly supported by quantitative real-time RT-PCT analyses of transcripts from PCT and APCT, as well as by immunohistochemical studies and western blot analyses of proteins expressed by these two tumor types.

24.7 Conclusions

The studies described here demonstrated that plasma-cell neoplasms of mice are considerably more varied than was appreciated when pristane-induced PCT were the only tumors available. Analyses of the pristane-induced tumors were remarkably informative for defining the role of AID-dependent *Myc*-activating chromosomal translocations in transformation; contributions of NOTCH signaling; the requirements for IL-6 and EEF1A2 to tumor progression and maintenance; the contributions of antigen specificity to positive selection, and the probable identification of MZ B cells as the cell of origin or the cell type most susceptible to pristane-induced PCT. Studies of spontaneous PL that develop in autoimmune mice and the APCT of NFS.V$^+$ mice showed that subsets of plasma-cell tumors can develop independent of *Myc* activation, while providing firm evidence for the contribution of antigenic selection in PL and origins of APCT from memory B cells. These models, with their proven similarities to human MM, will provide important preclinical systems for evaluating the contributions of novel signaling pathways to plasma-cell transformation, as well as for generating new understandings of normal plasma-cell differentiation and function.

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Chapter 25
A Role of IRF8 in Transcriptional Control of B-Cell Development

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Abbreviations

BCR	B cell receptor
BM	bone marrow
CLP	common lymphoic progenitor
MP	myeloid progenitor
CSR	class switch recombination
DC	dendritic cell
FO	follicular
GC	germinal center
HSC	hematopoietic stem cell
IL	interleukin
IRF	interferon regulatory factor
LMPP	lymphoid-primed MPP
MPP	multipotent progenitor
MZ	marginal zone
PC	plasma cell
SHM	somatic hypermutation
SLC	surrogate L chain
T1	transitional 1 (B cells).

25.1 Introduction

B-cell development follows ordered cellular stages, beginning with hematopoietic stem cells (HSCs) in the fetal liver before birth, and in the bone marrow (BM) after birth (Fig. 25.1a). HSCs give rise to multipotent progenitors (MPPs), which subsequently differentiate into lymphoid-primed multipotent progenitors (LMPPs) and myeloid progenitors (MPs). MPs give rise to granulocytes, macrophages, dendritic cells (DCs), megakaryocytes, and erythrocytes. The differentiative potential of LMPPs is restricted to myeloid and lymphoid progeny cells. A subset of LMPPs expresses interleukin (IL)-7 receptor (R) and differentiates into common lymphoid progenitors (CLPs). CLPs give rise to $B220^+$ pre-pro-B cells, the earliest known stage of the B-cell lineage. These pre-pro-B cells, along with other cell types, were originally defined as Fraction A cells by Hardy and colleagues [1]. Pre-pro-B cells have dual potential to become B and T cells [2]. Full commitment to the B-cell lineage is marked by expression of CD19 and loss of T-cell lineage developmental potential, a process controlled by multiple transcriptional factors such as PU.1, IKAROS, EBF, E2A, PAX5, and ID2 [3–5].

$CD19^+$ B-cell precursors, or pro-B cells, undergo D_H-J_H and V_H-D_HJ_H gene rearrangements to generate IgM heavy (μH) chain proteins. μH chains associate with surrogate L chains λ5 and $V_{preB,}$ as well as the signaling module Igα-β, to form pre-B cell receptors (pre-BCRs). These cells become pre-B cells. Deposition of pre-BCRs on the cell surface triggers a signaling cascade, leading to several rounds of proliferation before they exit the cell cycle. In small pre-B cells, the expression of RAG1 and RAG2 genes is upregulated and L-chain gene rearrangements are initiated. Successful production of H and L chains leads to expression of BCRs on the cell surface. These IgM^+ cells are immature B cells. A small fraction of these immature B cells may undergo *de novo* differentiation into mature B2 cells [6,7].

The majority of BM immature B cells migrate to the spleen, where they continue to mature through several transitional stages before they are selected into one of three mature B-cell pools: the marginal zone (MZ), follicular (FO) B2 and B1a cells (Fig. 25.1b). While all three subsets are relatively long lived, they differ significantly in frequency, phenotype, location, and function. Although MZ B cells are low in numbers (3% to 5% of total splenic B cells), they respond rapidly to blood-borne pathogens and differentiate into antibody-secreting plasma cells (PCs) within three days. This makes them an important component of a bridge between innate immunity and fully developed T cell- and FO B2 cell-mediated acquired immunity. In contrast, FO B2 cells, which represent 65% to 80% of total B cells, participate in a late response involving engagement with T cells and DCs in germinal centers (GCs). Antigen-driven selection, somatic hypermutation (SHM), and class switch recombination (CSR) during GC reactions lead to generation of high-affinity antibody-producing PCs and memory B cells.

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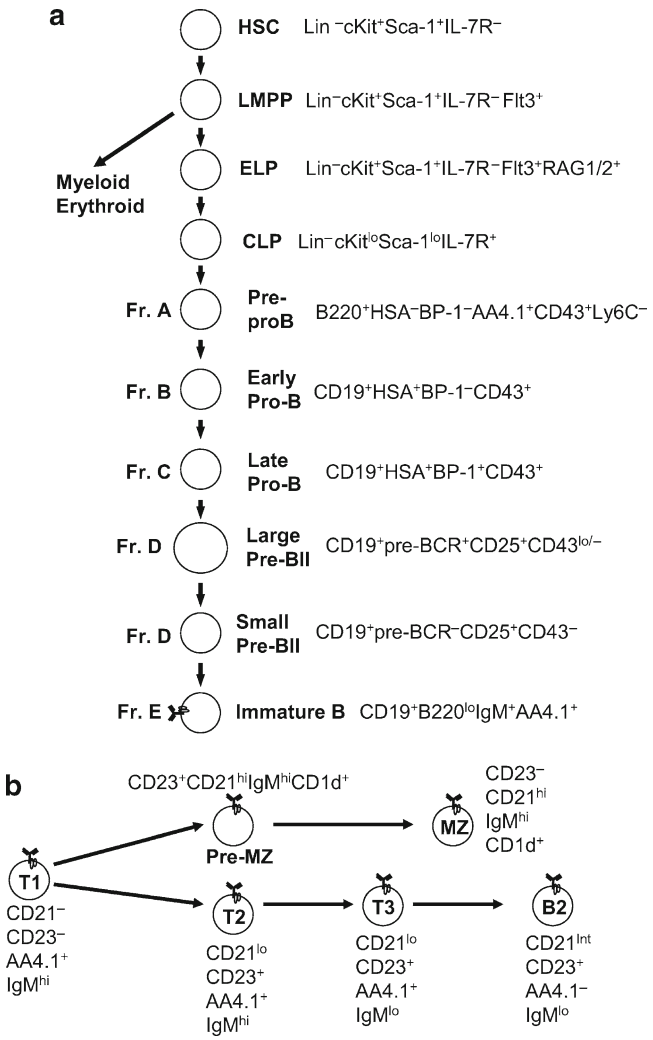

Fig. 25.1 Differentiation of hematopoietic stem cells and their progeny in the bone marrow (**a**) and spleen (**b**). CLP, common lymphoid progenitor; ELP, early lymphoid progenitor; Fr. A to E, B lymphocytes of Hardy fractions A–E; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor

About 5% of splenic B cells are CD5⁺ B1a cells. The majority of B1a cells reside in pleural and peritoneal cavities. Besides B1a, a subset of CD11b⁺ and CD5⁻ B-cells, termed B1b, is also localized in the peritoneum. While the origin of B1a and B1b cells is controversial in literature, both B1a and B1b cells are critical players in innate immunity against microbial infections [8–10]. Compared with FO B2 cells, B1 cells are prone to switch to the IgA class [11] and thus are valuable for mucosal immunity against commensal and pathogenic microbial organisms.

Regulation of developmental transitions from one stage to the next is largely mediated by a certain group of transcription factors. For example, PU.1, EBF, E2A, and PAX5 are well characterized "master" transcription factors crucial for pro-B cell development, BCL6 for GC reaction and antibody affinity maturation, and PRDM1 (Blimp1), interferon regulatory factor4 (IRF4), and XBP1 for PC differentiation. Understanding how these transcription factors regulate and are regulated has important implications for understanding not only normal B-cell development, but also autoimmunity and lymphomagenesis. Our laboratory has studied the patterns of genes expressed by spontaneous B lymphomas in NFS.V⁺ congenic mice. One particular gene, IRF8, was found to be preferentially upregulated in GC-derived tumors [12]. Our continuing work has revealed that IRF8 plays important roles at several checkpoints during B-cell development [12–14].

25.2 IRF8

The IRF family of transcription factors comprises nine members that are predominantly expressed in immune cells. IRF8, otherwise known as interferon consensus sequence binding protein (ICSBP) [15], is a 48-kDa protein containing a DNA binding domain (DBD) in the *N*-terminal half of the protein and an IRF association domain (IAD) in the *C*-terminus. The DBD forms a helix-turn-helix structure and interacts with DNA through five tryptophan residues. To date, four conserved DNA motifs have been found to correspond to IRF8 binding sites. They are the interferon stimulated response element; the ETS/IRF response element; the IRF/ETS composite element, and the ETS/IRF composite element (Fig. 25.2).

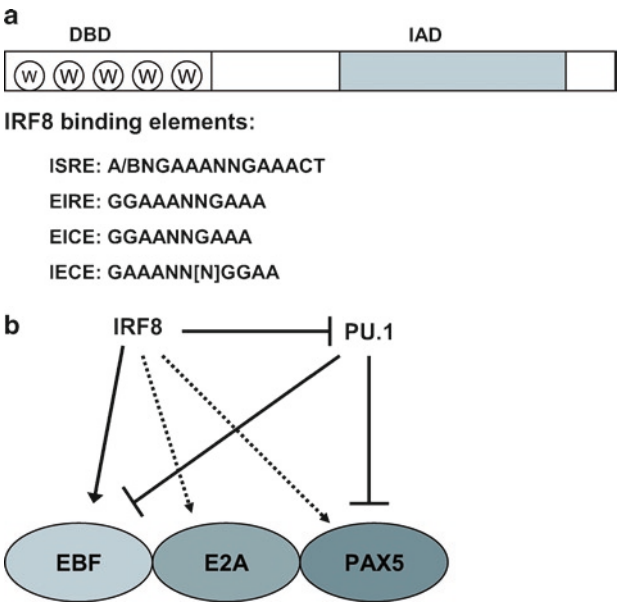

Fig. 25.2 The structure of IRF8 and nature of its target sites (**a**) and proposed regulatory activities of IRF8 as part of the transcriptional complex affecting B cell commitment and early differentiation (**b**). The known IRF8 responsive elements are connected by solid lines. Dashed lines indicate potential indirect regulation by IRF8

The IAD of IRF8 is responsible for heterodimerization with other transcription factors, including members of the ETS family (PU.1, TEL) [16,17] and IRF family (IRF1, IRF2, and IRF4) [18–20], as well as E47 [21], NFATc1 [22] and MIZ1 [23]. Stable complexes of IRF8 and its partners can repress or promote expression of a large number of target genes involved in cytokine signaling (*Il12*), cell cycle regulation (*Cdkn2b*), and host defense (*Slc11a1* [Nramp1] and *Nos2* [iNOS]), and differentiation (*Prdm1* [BLIMP1]), among many other activities.

IRF8 is expressed constitutively in B cells, macrophages, and CD11b$^-$ DCs and is further upregulated by IFN-γ in macrophages and by TCR stimulation in T cells [24]. The levels of IRF8 protein are regulated in part by CBL-mediated ubiquitylation and subsequent proteasomal degradation [25]. More recently, the transcriptional activity of IRF8 has been shown to be modulated by the E3 ubiquitin ligase, TRIM21 [26]. Following ubiquitylation, the transcriptional activity of IRF8 for the *Il12p40* promoter is significantly enhanced [26].

25.3 IRF8 Promotes B-Cell Lineage Specification at the Pre-pro-B Stage

25.3.1 B-Cell Lineage Specification and Commitment

Lineage specification refers to a process by which multipotent precursors develop a gene program specific for a certain lineage with restricted potential for developing into other lineages. B-cell lineage specification starts from the ELP stage, where expression of RAG1 and RAG2 genes is initiated [27]. These cells also begin to express EBF, a transcription factor crucial for early B-cell development, and terminal-deoxynucleotidyl transferase (TdT), an enzyme that induces nucleotide insertion during Ig V-D-J rearrangement and amplifies antigen receptor diversification [27]. A progeny population of ELP, termed CLP, exhibits more restricted T- and B-cell developmental potential. CLPs are a major source of B-lineage cells. Upon culture with OP9 stromal cells and B cell-permissive cytokines, Flt3L and IL-7, CLPs give rise to mostly B cells. The development of B220$^+$CD24^{lo}AA4.1$^+$CD19$^-$ pre-pro-B cells from CLPs marks the first stage of the B-cell lineage pathway; however, the pre-pro-B cells are plastic in that they can be induced to differentiate into myeloid and T cells when stimulated appropriately [2]. Expression of CD19 in pro-B marks a firm commitment to the B-cell lineage.

25.3.2 The Role of IL-7 In B-Cell Specification

One unique marker that distinguishes CLPs from other multipotent progenitors is the IL-7R. The IL-7R is composed of a γc chain and the IL-7Rα chain. The γc chain is a common γ chain for receptors of IL-2, IL-4, IL-9, IL-15 and IL-21. Early B cells including pre-pro-B and pro-B cells also express the IL-7R. The development of CLPs appears to be independent of IL-7R signaling, as mice deficient for *Il7r*, which encodes IL-7Rα, exhibited normal frequencies of CLPs [28,29]. In addition, the survival of CLPs and subsequent differentiation into pre-pro-B cells requires IL-7 [28]. A similar defect in B-cell differentiation has also been shown in IL-7-deficient mice [30–32].

Il7-deficient CLPs express reduced levels of EBF, suggesting that IL-7 signaling may regulate expression of the *Ebf1* gene, which encodes EBF [29]. Overexpression of EBF in IL-7Rα-deficient HSCs partially restored B cell development [33]. The transcription factor STAT5 is a critical downstream molecule in the IL-7 signaling pathway, and its absence impairs early B-cell development, similar to the phenotype observed in *Il7r*$^{-/-}$ mice [34,35]. Consistent with this observation, a constitutive active form of STAT5 can rescue B-cell development in *Il7r*$^{-/-}$ HSCs [36]. Together, these studies suggest that IL-7 plays a critical role in B-cell lineage specification.

25.3.3 A Transcriptional Network That Regulates B-Cell Lineage Specification and Commitment

Analysis of genetically mutant mice bearing targeted alleles of PU.1 [37,38], IKAROS [39,40], PAX5 [41], EBF [42], and E2A (E12 and E47 subunits) [43,44] have revealed a severe blockade of pro-B-cell development. New transcription factors with similar functions are continuing to emerge. For example, PBX1 has been found to regulate the development of CLPs, and its absence causes a severe deficiency in B cell numbers [45]. FOXO1 is essential for expression of IL-7Rα. Early deletion of FOXO1 impairs B-cell development at the pro-B-cell stage [46]. Our recent work has shown that IRF8-deficient mice have significantly reduced numbers of pre-pro-B cells, indicating that IRF8 may also participate in B-cell lineage specification and commitment.

The effects of PU.1 and IKAROS on B-cell lineage development are profound at the CLP stage. Mutation of *Ikzf1*, the gene that encodes IKAROS, interrupts development of CLPs and subsequent development of the B- and T-cell lineages [47]. CLPs are not detectable in embryos of *Sfpi1*$^{null/null}$ mice

(*Sfpi1* encodes PU.1) [38] and adult BM of *Sfpi1* conditional deletion mice *(,4849)*. PU.1 directly binds to a regulatory region of *Il7r* gene and may control its expression [50]. Ecotopic expression of *Il7r* in PU.1$^{-/-}$ progenitors partially rescues B-cell development, arguing that PU.1 has other targets important for development of CLPs and early B cells [50]. The molecular mechanisms involved in IKAROS effects at this stage of B-cell development remain to be determined.

EBF, E2A, and PAX5 play nonredundant but overlapping roles in B-cell lineage specification and commitment. Mutation of *Ebf1* and *E2a* genes blocks B-cell development at the earliest pro-B cell stage before the onset of D to J_H rearrangement [42–44]. By contrast, PAX5 is not -required for B-cell lineage specification, as the pre-pro-B cell compartment is largely intact in *Pax5*$^{-/-}$ mice [41]; however, PAX5 is indispensable for B-cell lineage commitment. *Pax5*-deficient mice fail to generate Fr. C or late pro-B cells [41]. Intriguingly, *Pax5*-deficient pro-B cells exhibit a remarkable multidevelopmental potential that can give rise to all major hematopoietic cell types except for B cells [51]. Furthermore, ablation of *Pax5* by Cre-mediated deletion in mature B cells leads to their dedifferentiation into progenitors with the ability to become T cells and macrophages [52]. These findings firmly establish a role of PAX5 in the maintenance of B-cell identity.

In addition to promoting directly B-cell lineage commitment by regulating B-cell-specific genes such as *Cd19*, *Blnk*, and *Mb1*, some of these master transcription factors, like PAX5, also suppress B-cell lineage-inappropriate genes such as *Csf1r* and *Notch1* [53]. *Csf1r* (encoding M-CSFR) is essential for myeloid fate specification, whereas NOTCH1 is critical for T-cell fate specification. Recently, Allman and Singh's groups reported that ecotopic expression of EBF in MPPs prevents their differentiation into T lineage cells [54]; however, introducing PAX5 into MPPs failed to block their T cell developmental potential [54]. This finding indicates that EBF also plays a role in B-cell lineage specification and commitment.

25.3.4 IRF8 Deficiency Impairs Early B-Cell Development

In *Irf8*$^{-/-}$ mice, the pre-pro-B compartment is reduced by 60% to 80%, based on Hardy's and Kincade's nomenclatures [14]. In contrast to the reduced number of early B cells, the number of myeloid progenitors is dramatically increased [14,55,56], an indication of myeloid bias at the CMP and CLP stages. Analysis of BXH2 mice bearing a point mutation, R294C, in the IAD domain of IRF8 revealed similar defects in generation of early B cells [57]. Both *Irf8*$^{-/-}$ and BXH2 mice develop myeloproliferative syndromes [58,59]. Consistent with a role of IRF8 in early B-cell development, aged individuals exhibit decreased production of B cells but increased production of myeloid cells, which has been associated with diminished expression of a handful of genes including IRF8 [60,61].

25.3.5 IRF8 Regulates PU.1 Expression

The expression levels of *Sfpi1*, the gene that encodes PU.1, appear to be critical for lymphoid and myeloid lineage specification. Singh and colleagues have shown that low levels of PU.1 guide lymphoid lineage specification, whereas high levels of PU.1 facilitate myeloid lineage commitment [62]. Consistent with this notion, overexpression of PU.1 in hematopoietic precursors interrupted B-cell development and accelerated macrophage differentiation in addition to the induced development of erythroleukemia [63]. siRNA-mediated knockdown of *PU.1* in ES cells induced expression of *Ebf* and *Pax5*, which correlated with enhanced B cell developmental potential [64].

Several transcription factors have been shown to stimulate expression of PU.1. These include PU.1 itself; Oct factors; GATA-1; POU2AF1 (BOB1 or OCAB), and SPIB [65–68]. It remains unclear how a low level of PU.1 is maintained. We have recently shown that IRF8 directly binds to a consensus sequence at the promoter region of *Sfpi1*. Functional analyses suggest that IRF8 may suppress PU.1 expression [14]. First, knockdown IRF8 with siRNA in NFS202 B lymphoma cells increased expression of PU.1 at both RNA and protein levels. Second, overexpression of IRF8 in NFS203 B lymphoma cells reduced the levels of PU.1 proteins. Third, introducing IRF8 into HSC-derived progenitors decreased the number of *Sfpi1* transcripts. Fourth, the levels of *Sfpi1* transcripts in *Ifr8*$^{-/-}$ MPs, CLPs, and pre-pro-B cells were increased, compared with those in wild-type cells. Together, these data suggest that IRF8 fine tunes the expression of PU.1 and modulates lymphoid and myeloid fate specification. This model is consistent with the observation that development of myeloid progenitors is enhanced in *Irf8*$^{-/-}$ mice [14].

25.3.6 IRF8 Regulates Expression of EBF, E2A, and Pax5

The decreased commitment of CLP to the pre-pro-B and pro-B stages of differentiation in IRF8-deficient mice was associated with reduced expression of *E2a*, *Ebf1*, and *Pax5* [14]. We have identified three IRF8 target sequences in the promoter region of *Ebf1*. Chromatin immunoprecipitation analysis suggests that IRF8 is physically associated with this segment. Mutation of these binding motifs substantially reduced the promoter

activity of the *Ebf1* gene [14]. Interestingly, studies using a reporter assay demonstrated that IRF8 synergizes with PU.1 in activating EBF expression [14].

How *E2a* is regulated by IRF8 remains unclear. There is no obvious IRF8 target sequence in the promoter region of *E2a*; however, gene regulation can occur in a distal region far away from the promoter or in an intron. Alternatively, IRF8 may regulate an unknown intermediate factor that is responsible for activation of *E2a* expression. Although no IRF8 binding site has been found in the *Pax5* promoter, the reduced expression of *Pax5* in IRF8-deficient pre-pro-B and pro-B cells could be secondary to downregulated expression of *Ebf1* and *E2a*.

25.3.7 A Model of IRF8 in B Lymphoid Lineage Priming

Based on the above discussion, we propose a model to incorporate IRF8 into a transcriptional network that is responsible for lymphoid lineage priming (ref. 57 and Figure 2B). Among this network, E2A, IKAROS, PU.1 and EBF together control the expression of B-cell lineage-specific genes such as those encoding CD19, Igβ, λ5, V_{preB}, and BLNK. IRF8 modulates PU.1 expression and maintains PU.1 at the low level required for B-cell lineage commitment. The activity of IRF8 also prevents overproduction of myeloid progenitors, because high levels of PU.1 drive myeloid cell differentiation. Ironically, IRF8 also synergizes with PU.1 to activate EBF expression. Moreover, IRF8 may regulate expression of *E2a* and *Ikzf1* [69] directly or indirectly. Finally, feedback regulation between each of these elements also exists to maintain the signaling strength at a certain level suitable for B-cell lineage development. For example, IRF8 has been found to be a target of PAX5 [70], whereas PAX5 is regulated by EBF [5,71], which itself is transcriptionally regulated by PAX5 and PU.1 [5,72].

25.4 IRF4/8 Regulate Pre-B-Cell Differentiation

The pre-B-cell stage is a key checkpoint during B-cell differentiation. Expression of the pre-BCR is an important step to test the quality of productive μH chains. Not all productive μH chains can associate with the surrogate L chain (SLC) and be expressed as a pre-BCR on the cell surface [73,74]. About 50% of productive μH chains cannot form pre-BCRs [74]. Analysis in V_H12 expressing pre-B cells indicates that approximately 95% of V_H12 pre-B cells are lost at the transition from pro-B to pre-B [75,76]. This is due to a failure of the majority of V_H12 H chains to associate with the SLC and form pre-BCRs [76]. Pre-B cells without a functional pre-BCR die by apoptosis.

Pre-B cells are hypersensitive to pre-BCR-induced proliferative signals, which drive proliferation of up to five divisions [77]. Each division dilutes the membrane pre-BCR density. The loss of pre-BCR signaling strength leads to cell-cycle cessation, allowing small pre-B-cell differentiation and subsequent L-chain gene rearrangement. Recent studies in IRF4/8 double-deficient mice have shed new light on understanding the role of IRF4 and IRF8 in pre-B-cell development. In IRF4/8 double-knockout (KO) mice, pre-B cells failed to downregulate pre-BCRs and exit the cell cycle [78]. Mice with a less severe defect, deficient only in IRF8, exhibited increased expression of pre-BCRs in large pre-B cells and increased numbers of cycling pre-B cells [14]. Introducing IRF4 or IRF8 into IRF4/8-deficient pre-B cells restored cell-cycle arrest [69], indicating that IRF4 and IRF8 facilitate small pre-B-cell development. Further analysis identifies IKAROS and AIOLOS as critical mediators of IRF4/8-induced growth control [69]. On one hand, pre-BCR signaling induces expression of IRF4, which promotes expression of IKAROS and AIOLOS. On the other hand, IKAROS and AIOLOS suppress the expression of the SLC [69,79,80]. Clearly, IRF4, IRF8, AIOLOS, and OCAB form a feedback inhibition loop to regulate negatively expression of the pre-BCR. This ultimately leads to attenuation of pre-BCR signaling and induces cell-cycle exit. Moreover, IKAROS also negatively regulates cell-cycle progression at the G1-S transition [81].

Whether IRF4 and IRF8 directly regulate the cell-cycle machinery in pre-B cells remains unclear. Previous studies in myeloid cells have shown that IRF8 directly induces expression of *Prdm1* and *Etv3*, which suppress expression of *Myc*, a gene responsible for cell growth [82]. Moreover, IRF8 directly promotes expression of *Cdkn2b*, a gene encoding an inhibitor for the cyclin-dependent kinase $p15^{Ink4b}$ [83]. Consistent with these findings, our previous studies showed that overexpression of IRF8 in HSC-derived progenitors reduced cell-cycle progression at the S phase and induced apoptosis [14]. It would be of considerable interest to determine the target genes of IRF4/IRF8 that are responsible for growth inhibition in pre-B cells.

In addition to regulation of cell-cycle progression, IRF4 has been found to regulate IL-7 signaling whose attenuation is crucial for initiation of L-chain gene rearrangement. Using IRF4/IRF8 double KO mice, Singh and colleagues demonstrate that IRF4 promotes expression of CXCR4 in pre-B cells, which allows pre-B cells to be positioned away from IL-7-expressing stromal cells *via* a CXCL12-mediated chemotaxis [84]. This step, together with the effect of IKAROS and AIOLOS, is thought to terminate pre-B-cell proliferation and initiate L-chain gene rearrangement [84].

25.5 IRF8 Together with IRF4 Regulates Immunoglobulin Light Chain Gene Rearrangement

Accessibility of the κ L chain locus to the RAG1 and RAG2 rearrangement machinery is regulated by a series of epigenetic mechanisms. In pro-B cells, both Igκ alleles are located at a central region of the nucleus, away from heterochromatin domains [85]. In small pre-B cells, one Igκ allele is silenced by association with heterochromatin protein-γ and IKAROS, while the other allele is positioned away from heterochromatin, which renders the Igκ locus accessible to factors such as RelB, responsible for DNA methylation, and to DNA cleavage induced by RAG1 and RAG2 [85,86]. At present, the molecular mechanisms responsible for *Igκ* gene positioning are not clear. Recent studies have demonstrated that IRF4 plays a role in generating an Igκ allele that is open for rearrangement [84]. In addition, IRF4 and IRF8 directly engage with an Ets-IRF composite binding site located at the 3′ enhancer of Igκ (3′Eκ) and promote expression of Igκ [84]. A similar function of IRF4/IRF8 has been observed for the enhancer of Igλ [84].

25.6 IRF8 Regulates MZ and FO B Cell Fates

In the spleen, the fate of a developing transitional B cell is determined by cell-intrinsic factors, such as the structure of the CDR3 region of the BCR, and cell-extrinsic factors including the environmental niche. Transitional 1 (T1) B cells, which represent newly migrated BM immature B cells, can differentiate into pre-MZ B cells [originally described as T2 by Carsetti and colleagues [87]] and MZ B cells, or pass through the T2-T3 pathway to become FO B2 cells [88]. The commitment to an MZ or FO B-cell fate is dependent on the signaling strength of the BCR. Hayakawa and colleagues have generated elegant models to support this view. B cells bearing a transgenic BCR specific for a self antigen, Thy1, are selected into the MZ B cell pool in the presence of low levels of antigen, while B cells expressing the same BCR differentiate into FO B cells in the absence of the antigen [89]. This model, together with an earlier finding that B1-cell development requires high concentrations of antigen [90] and a strong BCR signal [91], establishes a general dogma of BCR signal strength in peripheral B-cell maturation: the dependence of BCR signal strength for B-cell fate specification is ranked as B1a > MZ > FO.

This model is consistent with the view that MZ B cells are selected based on their autoreactivity, which could deliver relatively strong BCR signals [92]. Increasing the copy numbers of an Ig transgene can achieve the same outcome, as indicated by studies showing that the MZ B-cell compartment is expanded in mice expressing more copies of the transgene than those with low copy numbers [93]. In addition, NOTCH signaling is essential for mature B-cell fate specification. Overexpression of NOTCH1 completely blocks B-cell development, whereas deletion of *Notch2* specifically impairs MZ B-cell development [94]. There thus seems to be an element of cross-talk between BCR and NOTCH2 signaling, as first suggested by Moran et al, who showed that NF-κB1/p50, a downstream effector molecule of the BCR, synergizes with NOTCH2 to regulate MZ B cell development [95].

Several transcription factors have been found to affect MZ B-cell fate (Table 1). Loss of function mutations of EGR1, ETS1, RELB, and NF-κBp50 selectively reduced MZ B-cell development, whereas deficiency of Oct2 and BOB.1 caused defects in MZ B cell, as well as total B-cell numbers. Mutations of *Id2* and *Ikzf3*, which encode AIOLOS, result in reduced generation of MZ B cells, but enhanced development of FO B2 cells. In contrast, mice deficient in FLI1, E2A, and NF-κBp100 exhibited biased development of MZ B cells. These findings suggest that different transcription factors have distinct functions in controlling fate decisions of MZ and FO B-cells. We have recently found that IRF8 controls cell fate choice in MZ and FO B cells. In *Irf8*$^{-/-}$ mice, the MZ B cell compartment is enlarged, whereas the FO B-cell pool is reduced [13]. Because of the significant expansion of myeloid cells in these mice, which may alter the microenvironmental cue required for MZ B cell development, we have generated IRF8 conditional KO mice by employing CD19-Cre-mediated excision of exon 2 of *Irf8*, which encodes the DBD. Our preliminary data indicate a significant bias toward MZ B-cell development in these mice (unpublished data).

25.7 IRF8 Regulates GC B-Cell Programs

The GC is a specialized region of the B-cell follicle where mature B cells undergo a series of cellular and molecular changes driven by antigen and T-cell factors. Histologically, the GC consists of a dark zone where antigen-stimulated B cells termed centroblasts proliferate vigorously, and a light zone in which cells termed centrocytes undergo positive selection, SHM, CSR, and early plasmacytoid differentiation. These apparently distinct developmental programs are regulated by an unknown number of transcription factors. Several genes have been shown to be critical to the full development of the GC gene program, including *Bcl6*, *Aicda*, which encodes AID; *Hoxc4*, *Pou2f2*, which encodes Oct2; *Pou2af1*, which encodes OCAB; *Irf8*, and *Pax5*.

AID induces deamination at cytosine residues, resulting in generation of uracils in the single-stranded DNA of Ig V and

Table 25.1 Transcription factors with a function on development of MZ and/or FO B cells

Transcription factor	Targeted allele	Phenotype of mutants	Ref No.
Egr-1	Null	The MZ B cell compartment was reduced by 75%, while FO B cells remain unchanged	[110]
Fli-1	Truncated protein	The numbers of MZ B cells were increased by 2-fold while the numbers of FO B cells were decreased. The expression of CD23 was downregulated	[111]
Ets-1	Hypomorphic allele	Very few MZ B cells. The FO B cells expressed increased levels of CD23 and other activation markers	[112]
Oct2	Null	Reduced total splenic B cells. MZ B cells were lacking	[113]
BOB.1/OBF.1	Null	Reduced total splenic B cells. MZ B cell were lacking	[114]
Id2	Null	The number of FO B2 cells was increased 60% while the number of MZ B cells was decreased by 40%	[115]
Aiolos	Null	The number of FO B2 cells was slightly increased but the MZ B cells were markedly diminished	[116,117]
E2A	Hypomorphic allele	The frequency of FO B cells was decreased while the number of MZ B cells was increased by 4-fold.	[118]
NF-κB p100	Null	The numbers of MZ B cells were increased 5-fold, while the FO mature B cells remain unchanged	[119]
NF-κB p50	Null	Young mice had reduced number of MZ B cells, but aged mice (6-months) had normal numbers of MZ B cells	[120,121]
NF-κB RelB	Null and chimeras	Markedly reduced MZ B cells but the FO B cells were normal	[122]
NFATc2/c3	Null	Dramatically enlarged FO B2 cell compartment with little effect on MZ B cell numbers	[123]

switch regions. Subsequent repair of the uracil residues causes hypermutation of the V region and class switch recombination of Cμ with downstream constant region loci including Cγ, Cα, and Cε. A deficiency in *Aicda* completely blocks somatic hypermutation and Ig-class switch recombination [96,97]. Expression of AID is predominantly restricted to GC B cells. A high expression of AID is found in dark zones [98]. The activity of AID is influenced by oligomerization with self or other regulatory factors [99,100] as well as phosphorylation at Ser38 and Tyr184 of the AID protein [101,102].

HOXC4 is a member of the Hox transcription factor family that plays crucial roles in embryonic development. HOXC4 is expressed in B-lineage cells and particularly activated B cells [103,104]. Stimulating signals through CD153, IL-4, or TLR4 significantly upregulates expression of HOXC4, as well as AID [103–105], arguing that AID may be regulated by HOXC4. Indeed, HOXC4 is found to complex with Oct1, Oct2, and OcaB to activate expression of *Aicda* through binding to a sequence 5′ATTTGAAT3′ localized at the promoter region of *Aicda* [103]. Oct2 and OcaB function closely in regulating GC formation and CSR. Mice lacking Oct2 [106,107] or OcaB [108,109] exhibit similar defects in GC formation and production of IgG antibodies.

Our recent data suggest that IRF8 is preferentially expressed in centroblasts of human B cells and in dark zones of GCs [12], consistent with the expression pattern of AID and OcaB. By binding to an IRF consensus binding site at the promoter regions of *Bcl6* and *Aicda*, respectively, IRF8 may directly activate expression of these two genes, as suggested by a luciferase reporter assay [12]. Moreover, IRF8 also binds to a promoter region of *Mdm2* and activates its expression in a reporter assay (J. X. Zhou et al, manuscript submitted). MDM2 is actively involved in suppression of p53-dependent and independent pathways of apoptosis. In GC B cells of IRF8-deficient mice, the expression of MDM2 was greatly downregulated at both transcript and protein levels. This parallels decreased expression of transcripts of the p53 target gene *Pmaip1* (*Noxa*) and slowed proliferation of *Irf8*$^{-/-}$ GC cells (J. X. Zhou et al, manuscript submitted). Because DNA breaks are frequently generated during the GC reaction, our data suggest that IRF8 may regulate GC B cells to tolerate physiologic DNA breaks that otherwise would trigger growth arrest and apoptosis.

25.8 Conclusions

The results of these studies suggest a model in which IRF8 regulates B-cell development at multiple stages (Fig. 25.3). By controlling expression of PU.1 and EBF, IRF8 regulates B-cell lineage specification and commitment. We believe that IRF8 facilitates B-cell development at the expense of myeloid differentiation at progenitor stages. IRF8 control of myeloid progenitor differentiation is most likely through suppression of their clonal proliferation [14]. It will be interesting to determine the molecular mechanisms of IRF8 activities in lymphoid-myeloid lineage selection.

At the pre-B-cell stage, IRF8, together with IRF4, regulates cellular proliferation, cell-cycle exit, and L-chain gene rearrangement. At the immature B-cell stage, IRF8 modulates a gene program determining the MZ and FO B-cell fate.

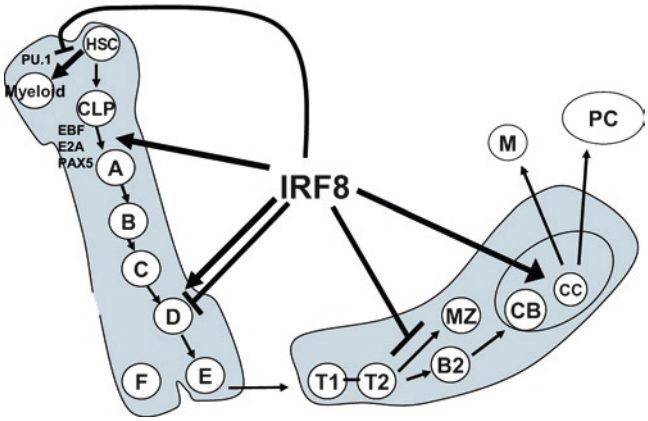

Fig. 25.3 IRF8 regulates B-cell development at multiple checkpoints. B-cell commitment requires functional IRF8, possibly through repression of PU.1 and activation of EBF, E2A, and PAX5. By suppressing myeloid progenitor differentiation, IRF8 controls the flow rate of lymphoid and myeloid cell differentiation from lymphomyeloid progenitors. By associating with IRF4, IRF8 restricts expansion of cycling pre-B cells and promotes Igκ gene rearrangement. In the periphery, IRF8 suppresses development of MZ B cells but facilitate FO B-cell maturation. In GC, IRF8 regulates several key genes of GC reaction and modulates production of memory B cells and plasma cells

During a T-cell-dependent immune response, IRF8 actively participates in regulation of key GC factors such as AID, MDM2, and BCL6 and modulates GC B-cell gene programs.

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Chapter 26
Laboratory of Molecular Immunology

Chemokines in Lymphocyte Biology

Joshua M. Farber

26.1 Introduction

The laboratory's earliest experiments were designed to identify novel proteins important in the host's response to infectious diseases. These experiments resulted in the discovery of the mouse and human CXCL9 (Mig) chemokine [1, 2], as well as the mouse CXCL10 (IP-10/Crg-2) chemokine [3]. Our subsequent findings that CXCL9 was a T-cell chemotactic factor, particularly for activated and/or memory cells, caused us to concentrate on the role of the chemokine system in lymphocyte biology and led to our molecular cloning of STRL22/CCR6 [4], STRL33/CXCR6 [5], and the A and B forms of CCR9 [6]. The discussion below will focus on some of these discoveries with regard to where they have led in our own work, as well as the work of other laboratories. It will touch on our efforts to address questions germane to the chemokine system as a whole within the context of lymphocyte physiology. Our laboratory's work is aimed at understanding not only how the individual chemokines and receptors are organized to contribute to the chemokine system's overall function, but also the broader processes in lymphocyte biology, such as recall/memory responses and lymphocyte migration that are relevant for developing new vaccines and for treating inflammatory disease.

26.2 MIG/CXCL9

CXCL9, 10 and 11 are IFN-γ-inducible CXC chemokines, whose human genes are clustered on chromosome 4, and which are produced by a wide range of hematopoietic, as well as parenchymal cells [7, 8]. These ligands share their only known receptor, CXCR3, which is expressed on subsets of T, B, NK and dendritic cells (reviewed in [9]). On peripheral-blood human CD4 cells, CXCR3 is preferentially expressed on the Th1 subset, concordant with the induction of its ligands during type 1 responses. Work in our laboratory has included the discovery of the mouse and human CXCL9/Mig and the mouse CXCL10/Crg-2/IP-10 as IFN-γ-inducible chemokines [1–3, 10]; the demonstration that the genes for CXCL9 and CXCL10 form a "mini-cluster" on human chromosome 4 [11]; the biochemical and functional characterization of the multiple forms of the CXCL9 protein [12]; the demonstration that CXCL9 and CXCL10 share a receptor on human lymphocytes [12]; and the demonstration and characterization of the induction of CXCL9 and CXCL10 in a variety of cell types and tissues during experimental infections of mice [13]. We have collaborated with other laboratories in the demonstrations of biological activities for CXCL9 and CXCL10 in a number of contexts. These include defense against vaccinia virus [14] and mouse hepatitis virus [15]; anti-tumor activity in mouse models [16–20]; anti-angiogenesis activities *in vitro* and *in vivo* [16–19, 21]; promotion of liver regeneration [22], and suppression of growth of CFU of human myeloid progenitors [23].

Using CXCL9$^{-/-}$ mice that we made in collaboration with Paul Love of NICHD, in experiments done in collaboration with Karen Elkins of CBER, FDA, we found that CXCL9 made a contribution to the antibody responses to the bacterial pathogen, *Francisella tularensis* LVS, now of particular interest because of the possible use of *F. tularensis* as a biological weapon. *F. tularensis* LVS elicits a strong type-1 response in mice, where the antibody produced is primarily of the IgG2a isotype, and the knockout mice showed a significant decrease in the anti-bacterial titer of IgG2a (and of total IgG) at three to four weeks after infection, as compared with responses in the wild type mice [24]. No differences were seen between knockout and wild type mice in the antibody responses to defined T-independent or T-dependent antigens. We presume that CXCL9 and CXCR3 have a role in optimizing B-cell/T-cell/dendritic-cell interactions involved in generating antibody responses against the bacterial pathogen. This presumption was strengthened by our observations that CXCR3 can be expressed on CD4^{+} T cells around and within

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germinal centers, and that we found CXCL9 expressed in a ring pattern at the margins of some germinal centers in inflamed human tonsils and in lymph nodes from an HIV-1-infected individual [25]. Recent data from a collaborator working with a model of immune complex glomerulonephritis showed that CXCL9$^{-/-}$ mice were relatively resistant to disease due to diminished production (and deposition) of antibodies [26]. Together, our mouse and human data support a broader view of the roles of so-called "inflammatory" chemokines and their receptors, suggesting that functions for these ligands and receptors are not limited to peripheral inflammatory sites, but that they also act in immune responses within lymphoid organs.

A recent search of PubMed for MIG/CXCL9 revealed more than eight hundred relevant publications since we reported the discovery of the gene in 1990. Just within the last year, work from many laboratories have implicated CXCL9 in a broad range of biology related to health and disease, including roles in protection against liver fibrosis [27]; promoting inflammatory responses to vascular injury [28]; enhancing cancer vaccines [29]; measuring responses to vaccines in humans [30]; protection against anthrax [31]; and the development of cerebral malaria [32]. We can anticipate that additional research into the activities of MIG/CXCL9, the related chemokines IP-10/CXCL10 and I-TAC/CXCL11, and their receptor, CXCR3, will suggest new ways to promote health and treat disease.

26.3 STRL33/CXCR6

In experiments to identify new chemokine receptors in activated lymphocytes, we cloned a sequence encoding a seven-transmembrane domain orphan receptor related to chemokine receptors, which we named STRL33 [5]. In collaboration with Edward Berger's laboratory in NIAID, we discovered that STRL33 had broad activity in cell-cell fusion assays used to detect HIV-1 co-receptors. We showed STRL33-expressing cells showed fusion with cells expressing HIV-1 envelope proteins from T cell line-tropic, macrophage-tropic, and dual-tropic HIV-1s. We showed that STRL33 was expressed on activated primary T cells, and together with Keith Peden of CBER, FDA, that STRL33 could function as a coreceptor on STRL33-transfected cell lines, to support productive infection with some strains of HIV-1 [5]. The demonstration that STRL33 had coreceptor activity for HIV-1 led to our testing with SIVs as well, particularly since at that time CCR5 was the only known SIV coreceptor, and it was clear that there were others. We found that STRL33, like CCR5, was a highly active coreceptor for both macrophage-tropic (SIVmac316) and T-cell line-tropic (SIVmac239) strains of SIV [33]. Coincident with our work, the laboratory of Dan Littman also identified the STRL33 sequence as an SIV/HIV co-receptor, and gave it the name BONZO [34]. Two groups identified the ligand for STRL33 as a novel transmembrane chemokine, CXCL16 [35, 36], whereupon STRL33/BONZO/TYMSTR was renamed CXCR6.

The discoveries that chemokine receptors function together with CD4 as obligate coreceptors for the entry of HIV-1 into cells have contributed to an understanding of multiple phenomena: CD8 T-cell suppression of HIV-1 infection *in vitro*; the differences in cyto-tropism among HIV-1 strains; strain-specific restrictions in HIV-1 transmission between individuals; changes in viral cyto-tropism associated with disease progression; and the resistance of some individuals to HIV-1 infection, among others [37]. The most significant consequence of the discoveries of the coreceptors to date is the recent approval of an agent to treat HIV infection by targeting the receptor CCR5 and thereby blocking viral entry [38]. It is clear that CCR5 and, to a lesser extent, CXCR4, are critical coreceptors in HIV-1-infected individuals. A number of studies have demonstrated the ability of some primary isolates of HIV-1, as well as SIV strains and isolates of HIV-2, to be able to use CXCR6 as a coreceptor, including studies showing CXCR6-using HIVs that have been transmitted from mother to infant [39, 40] and that are found preferentially in tissue vs. blood [41]. In addition, our unpublished studies, together with Keith Peden, have shown that HIV-1 viruses which use CXCR6 poorly, initially, are able to adapt to use CXCR6 more efficiently in culture *in vitro*. Nonetheless, a role for CXCR6 in HIV infection *in vivo*, like roles for the other (at least nine) "alternate," non-CCR5, non-CXCR4 coreceptors, is not established. One could imagine that alternate coreceptors might become important during therapeutic blockade of CCR5 and CXCR4, but, fortunately, the data to date do not indicate that coreceptor switching is a common mechanism of escape from CCR5 blockade [38]. Additional clinical experience with blockade of CCR5 (and perhaps CXCR4) will reveal whether or not HIV-1 can broaden its coreceptor repertoire *in vivo* to include CXCR6 and/or other alternate receptors.

With regard to activities unrelated to HIV/AIDS, CXCR6 has been described as expressed preferentially on, but not limited to, Th1 [42] cells, and recently, by our laboratory, on Th17 [43] cells. It has also been found to be highly expressed on T cells at inflammatory sites [42]. CXCR6 has been reported to be important in the trafficking of activated T cells in Peyer's patches [44], and in the trafficking and function of NKT cells *in vivo* [45–47]. CXCR6 has also been implicated in inflammatory diseases, including rheumatoid arthritis [48], atherosclerosis [49], and chronic obstructive pulmonary disease [50]. A number of recent reports have implicated CXCR6 and CXCL16 in cancer [51–55], and unpublished work from our laboratory suggests that CXCR6 and CXCL16 may be important in cross-talk between leukocytes and pre-malignant

and malignant cells in prostate cancer. We are still early in the study of the biology of CXCR6 and CXCL16, and additional important roles in immunology and immunopathogenesis are bound to emerge.

26.4 STRL22/CCR6

In addition to STRL33, our screening for novel chemokine receptors in activated T cells led to the discovery of a gene we named STRL22 [4], which we and others subsequently reported encoded CCR6, a receptor that is found on various populations of T, B, and dendritic cells, and that signals in response to the chemokine MIP-3α-CCL20, [56–60]. We described that CCR6-mediated chemotaxis of human B cells could be significantly enhanced by activation of cells through surface immunoglobulin without changes in levels of CCR6, revealing regulation of chemokine-receptor signaling through independent effects on downstream pathways, and establishing CCR6 as a highly efficacious receptor on activated B cells [61]. Studies by others have shown a contribution of CCR6 to numbers of antigen-specific IgA-producing cells in the gut after oral immunization, and to titers of intestinal IgA made against rotavirus in mice [62]. Additional studies in mice have suggested roles for CCR6 in dendritic cell-T-cell interactions at epithelial surfaces [63, 64], and in models of inflammation in skin, lung, and bowel [65–70].

Data on expression of CCL20 and CCR6 in humans has implicated the ligand and receptor in autoimmune disease in the skin, joints, and gut [71–73]. The last several years have seen an explosion of data on a newly recognized lineage of helper T cells: Th17 cells, whose signature cytokine is IL-17. Th17 cells have been suggested to be critical effector cells in mediating tissue damage in autoimmunity [74]. We and others have recently reported a unique relationship between CCR6 and the Th17 lineage, namely that all human Th17 cells (as well as the small number of IL-17-producing $CD8^+$ T cells) can be found within the $CCR6^+$ subset of effector/memory T cells [43, 75–77]. These data establish analogous relationships among three chemokine receptors, CXCR3, CCR4, and CCR6, and the three lineages of effector/memory helper cells, Th1, Th2, and Th17, respectively. Consistent with an important role for CCR6 in diseases caused by type-17 inflammation, CCR6 is essential for the inflammatory reactions in mouse models of rheumatoid arthritis [78] and multiple sclerosis [79], and, in unpublished work from our laboratory, in a model of autoimmune skin disease. Together, these data suggest that a CCR6 antagonist might be an effective drug in treating a number of Th17-mediated diseases, and it is likely that groups in both academia and industry are actively investigating the therapeutic applications of these findings.

26.5 Conclusion

Work in the Inflammation Biology Section has contributed to the discovery and characterization of members of the families of chemokines and their receptors. Starting with the genes and proteins that we initially described, we have moved to studying not only the functions of individual family members, but also the way that the chemokine system as a whole fits within the immune system, particularly within the physiology and pathophysiology of effector/memory T cells. Experiments along these lines have shed light on the mechanisms of differentiation of human Th cells *in vivo*, revealing early branching pathways to produce highly heterogeneous populations, including effector-capable cells with nearly naïve surface phenotypes [80]. We anticipate that additional investigations of the complexities of T-cell subsets, viewed from the perspective of the chemokine system, will help in understanding how effector/memory populations arise, and how the many T-cell subsets contribute to host defense, immunological memory, and inflammatory disease.

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Part VIII
Immunology: The Role of Receptors

Chapter 27
Structure and Function of Immunoreceptors

Peter D. Sun

27.1 Structure and Ligand Recognition of NK Cell Receptors

Natural killer (NK) cells constitute an important branch of host innate immunity against tumors and viral infections. The activation of NK cells is controlled by the balance of activating and inhibitory receptors. Inhibitory NK receptors, such as members of Killer Immunoglobulin-like Receptors (KIR) and lectin-like CD94/NKG2 receptors, contain signaling ITIM motifs and recognize major histocompatibility-complex (MHC) antigens. Unlike MHC-recognizing T-cell antigen receptors, the inhibitory NK receptors are germline encoded and have fewer numbers compared to their MHC counterparts. They recognize self MHC molecules and exhibit allotype specificity instead of allele restriction. To investigate the mechanism of these largely nonpolymorphic receptors to recognize polymorphic MHC ligands, yet still retain their pathogen sensitivity, we have determined the crystal structures of human CD94 [1], KIR2DL2 and its complex with a class I MHC ligand, HLA-Cw3 (Fig. 27.1a) [2,3]. Our results show that the inhibitory KIR receptor binds to the polymorphic α-helices of MHC in a similar mode as TCR.Unlike TCR, however, KIR receptors recognize conserved, rather than polymorphic residues, on MHC and have only minimum contacts with the eighth residue of the MHC-bound peptide, thus alleviatingthe peptide-MHC restriction of TCR.

Allotype specificities of KIR are controlled by hydrogen bonds between KIR and MHC. Major activating NK receptors include NKp30, NKp44, NKp46, and NKG2D, which signal through the ITAM motif containing CD3ς, DAP12, or DAP10. The ligands for NKG2D have been identified as members of MHC-like molecules, including MICA/B, ULBPs, and RAE-1 like transcripts (RAET). While many of NKG2D ligands are stress induced, they share little sequence homology among them. To investigate the molecular basis defining ligands of NKG2D, we determined the crystal structure of NKG2D in complex with its ligand ULBP3 [4,5](Fig. 27.1b). The structural study showed that while the position and identity of the interface residues vary among the ligands, the critical interactions, both hydrogen bonds and hydrophobic interactions, are preserved. Different ligands are accommodated by NKG2D through adjustment of the interface side-chain conformations. In addition to the activating NK receptors, triggering receptors expressed on myeloid cells (TREM) form a family of activating receptors that also signal through the ITAM-containing DAP12 molecule. Although not expressed on NK cells, TREM receptors appeared to participate in innate inflammatory responses and to contribute to septic shock, in response to microbial-mediated infections [6]. We have expressed and refolded the extracellular domains of human TREM-1 and -2 and determined the crystal structure of TREM-1 to 2.6 Å resolution. The overall fold of the receptor resembles that of a V-type immunoglobulin domain with differences primarily located in the *N*-terminal strand (Fig. 27.1c) [7].

Through these structural works, we have gained great insight into how NK cells recognize their ligands. However, much remains unknown. In particular, ligands for NK natural cytotoxicity receptors (NCR), despite recent publications of several candidates, remain largely unresolved. Finding ligands for NCR may be the key to our understanding of the anti-viral and anti-tumor activity of NK cells. Toward this aim, we have expressed all three NCR genes using recombinant bacteria systems and have determined the crystal structure of the extracellular ligand binding domain of human NKp46 (Fig. 27.1d) [8]. In addition to the structural study of NCR, we established a solution-based direct-binding assay to evaluate cell-surface antigens as potential ligands of NCR. We intend to combine the binding-based solution ligand screen with cell-based lytic assays to identify ligands defining the NCR-specific NK cytotoxicity. Once the ligands for NCR are identified, we will further attempt to characterize the structural details of NCR and its ligand complexes, in order to reveal the principles of tumor and viral recognition by NK cells.

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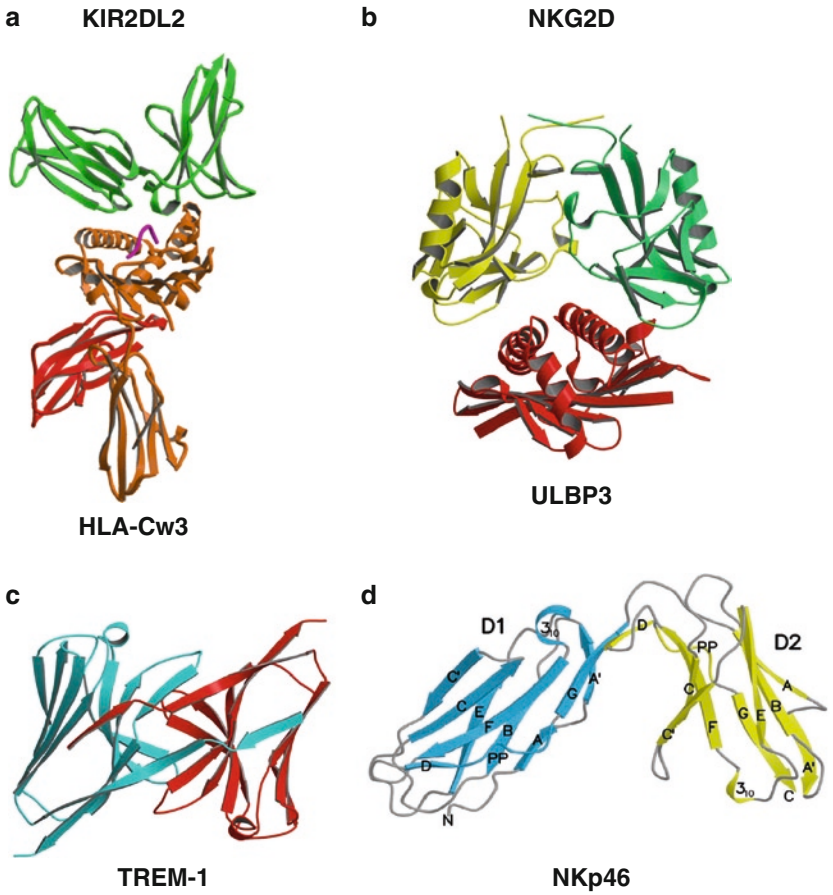

Fig. 27.1 (**a**) The structure of HLA-Cw3 bound to KIR2DL2 (green). The β2m domain, HLA-Cw3 heavy chain and peptide are colored in red, orange and magenta, respectively. (**b**) Structure of human NKG2D-ULBP3 complex. The two subunits of the NKG2D homodimer are shown in yellow and green and ULBP3 is colored red. (**c**) TREM-1 dimer with the two monomers colored in cyan and red. (**d**) The crystal structure of NKp46 D1D2. Shown are secondary structure elements of domains 1 (blue) and 2 (yellow), with regions of coil in gray

27.1.1 Structure and Function of B-Cell Co-Receptors

B-cell antigen receptor (BCR) plays a critical role in all stages of B-cell development and function. It is a complex structure consisting of two principal components: antigen-binding and signaling subunits. The antigen-binding subunit is a membrane-bound form of immunoglobulin (mIg) with a short cytoplasmic tail lacking any signaling motifs. Through non-covalent interactions, it associates with a disulfide-linked ITAM-containing heterodimer of Igα (CD79a) and Igβ (CD79b) [9,10]. Aggregation of BCRs is initiated by their crosslinking with multivalent antigens. Subsequently, the ITAM motifs of Igα and Igβ are phosphorylated by Src-family kinases, primarily by Lyn that, in turn, starts signaling cascades in B cells. While the stoichiometry of BCR in complex with Igα/Igβ has been established, there is no structure available on any component of BCR or on the BCR/Igα/Igβ assembly. To understand the molecular mechanism of BCR assembly and signaling, we carried out the structure and binding studies of the assembly between BCR and its co-receptor, Igα/Igβ.

Using recombinant bacteria expression systems, the extracellular portions of Igα and Igβ from both human and mouse were expressed as inclusion bodies and reconstituted individually *in vitro*. The refolded Igα and Igβ bound their respective antibodies with high affinities. The constant domains of BCR regions Cμ2-Cμ4, from both human and mouse, were expressed using a recombinant CHO cell-expression system in a pcDNA 3.1 plasmid vector. The refolded Igβ is presented in both monomeric and disulfide-bonded dimeric forms. Both forms were crystallized. The structures of the murine Igβ monomer and dimer were determined to 1.7 Å and 3.1 Å resolution, respectively (Fig. 27.2). While the higher resolution monomeric Igβ structure presented a better view of the overall structure, the structure of the homodimer allowed us to determine the disulfide bonding pattern in the BCR signaling chain, and to build a model for the Igα/Igβ heterodimer. In addition, we also carried out surface plasmon resonance (SPR)-based binding studies to show that both Igβ

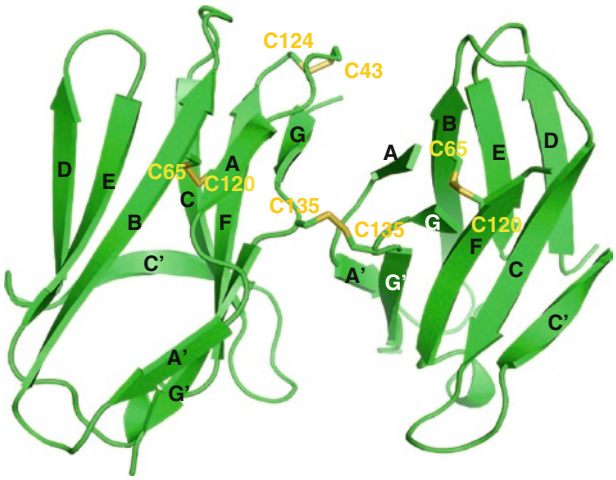

Fig. 27.2 Structure of the Igβdimer All secondary structure elements are labeled. Disulfide bonds are shown in stick representation

and Igα extracellular domains bound to the Cμ2-Cμ4 regions of BCR. The structural work, together with surface plasmon resonance measurements, allowed us to predict the potential BCR binding sites on the co-receptors. While the structures of individual Igα and Igβ are informative, much of the BCR activation hinges on the interaction between the co-receptors and BCR, and thus requires the solution of the co-crystal structure of BCR and its Igα/Igβ receptor assembly.

27.1.2 Fc Receptor Recognition by Pentraxins

Pentraxins (PTX) are a family of highly conserved proteins with characteristic pentameric ring structures. The classic short pentraxins include C-reactive protein (CRP) and serum amyloid P component (SAP), which are major acute-phase plasma proteins [11]. Both CRP and SAP recognize various pathogenic bacteria, fungi, and yeasts and, by doing so, activate the classical complement pathway through C1q. More recently, members of the pentraxin family were found to interact with Fcγ receptors (FcγR) [12], although the mechanism of this recognition was unresolved. Binding of CRP-and SAP-opsonized pathogens to FcγR activated receptor-mediated phagocytosis. We have been interested in the structural mechanism of antibody-mediated Fc receptor activation and published the crystal structure of a human FcγRIII in complex with IgG1-Fc [13–15]. The question is whether pentraxins and antibodies share similar mechanisms in Fc-receptor recognition, despite the lack of sequence homology between them. In addition, studies in mice indicated that both SAP and CRP could induce protective immune responses [16,17]. To understand the molecular mechanism of pentraxins-mediated Fc-receptor activation and its role in regulating immune responses, we carried out structure and function studies on pentraxins and their complexes with Fc receptors. In particular, we determined the crystal structure of human SAP in complex with FcγRIIa to 2.8 Å resolution (Fig. 27.3a) [18]. The 1:1 receptor-SAP recognition is predominantly mediated through the interactions of the ridge helix from two separate SAP protomers with the D1 and D2 domains of the Fc receptor. The structure provides a basis for SAP-opsonized pathogens to activate phagocytosis through Fcγ receptors. SAP and CRP display similar structural folds. Both have the characteristic ridge helix and assemble into pentamers. We proposed the SAP/FcγRIIa complex structure to be a prototypic model for pentraxin-Fcγ receptor recognition. Among the Fcγ receptors, FcγRIIb is most homologous to FcγRIIa, with nearly identical interface residues, suggesting that SAP can recognize both the activating and inhibitory Fc receptors. To test if the SAP/ FcγRIIa structure serves as a model for CRP recognition by Fc receptors, we generated several single mutations in the putative receptor binding site on CRP, including the putative salt bridge-forming residue His 38, and two residues on the ridge helix, Tyr 175 and Leu 176. The H38A and L176A mutations of CRP each resulted in a two-fold decrease in the solution-binding affinity to FcγRIIa, as measured by BIAcore experiments, while the Y175L mutant displayed a four-fold loss in FcγRIIa binding. These mutational results indicate that the Fc receptor binding mode is conserved between SAP and CRP.

To define further the specificity and affinity of the recognition between pentraxins and Fcγ receptors, we carried out a series of solution-binding experiments between the three human pentraxins (SAP, CRP, and PTX3) and four Fcγ receptor isoforms. The results showed that SAP, CRP, and PTX3 bound to different FcγRs with very different affinities. SAP bound the tightest to FcγRIIa; CRP displayed similar affinities to FcγRI, FcγRIIa, FcγRIIb, and FcγRIII, while PTX3 bound only to FcγRIII. Unexpectedly, the SAP binding site on FcγRIIa overlaps partially with the IgG binding site on the receptor (Fig. 27.3c), predicting a potential competition between antibodies and pentraxins in accessing Fc receptors. The solution-based binding experiments confirmed that SAP and CRP competed against IgG for the binding to FcγRs (Fig. 27.4). Moreover, soluble SAP and CRP inhibited significantly the immune complex-mediated phagocytosis, suggesting a regulatory function for this family of plasma proteins in antibody-mediated Fc receptor activation.In addition to activating Fc receptor-mediated phagocytosis, we showed that SAP binding to Fc receptors on monocytes resulted in the secretion of cytokines, such as IL-6, IL-8, and IL-10 (Fig. 27.3) [18]. The structural solution of SAP in complex with FcγRIIa illustrated how a classical complement-activation component, like SAP, can activate and regulate Fc receptor signaling.It suggests that the complement

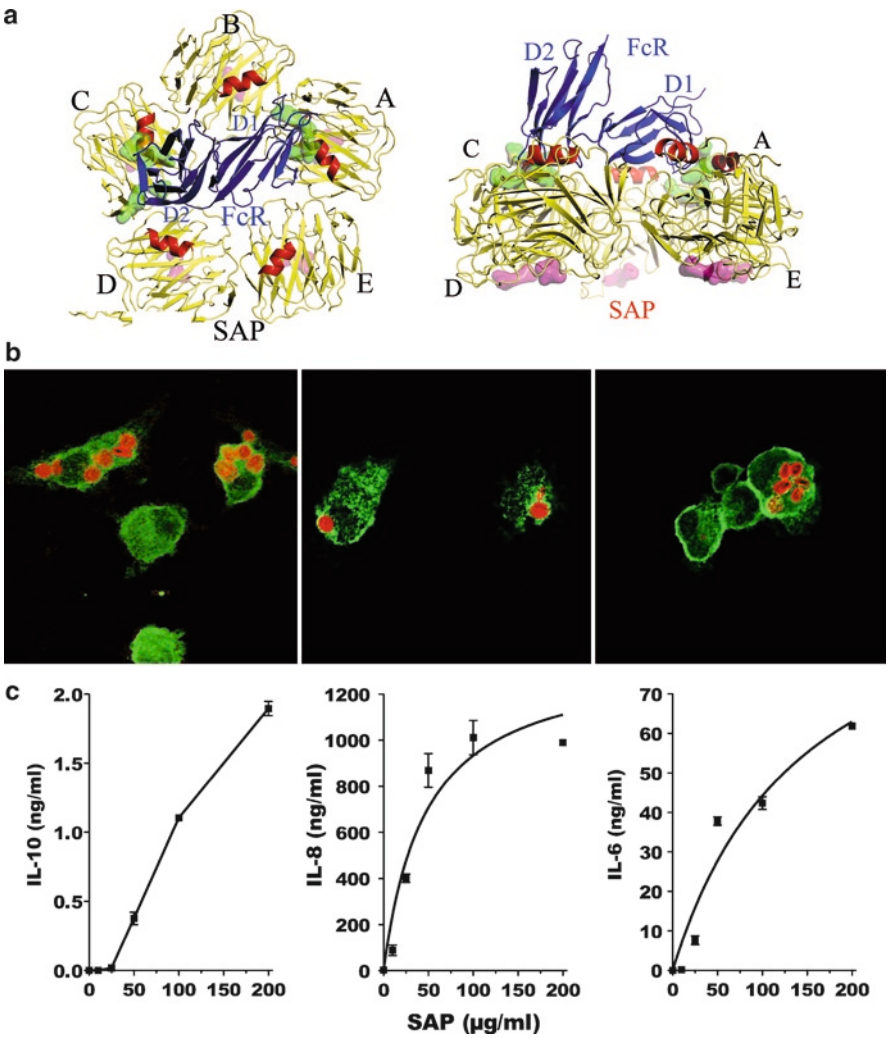

Fig. 27.3 (**a**) Crystal structure of SAP-FcγRIIa complex, viewed from the face and side of the SAP pentamer. The five SAP subunits are shown in yellow with ridge helices in red, and FcγRIIa is colored in blue. The FcγR contact regions on SAP A and C subunits are represented by molecular surface in green. The calcium and ligand binding sites on SAP are highlighted in magenta. (**b**) SAP- and CRP-opsonized zymosan are phagocytosed by human macrophages through FcγRIIa. MDM incubated with Texas red, labeled zymosan opsonized with (from left to right) SAP, CRP, and IgG. Fixed and permeabilized cells were stained with mAb to FcγRIIa and AF488 anti-mouse IgG. (**c**) Pentraxin activation of FcγR results in opsonization and cytokine release. The production of IL-10, IL-8, and IL-6 by $CD14^+$ monocytes in response to different concentrations of aggregated SAP

and Fc receptor pathways are not isolated but, rather, interact with each other, an example of cross-talk and regulation between innate and humoral immunity.

27.1.3 Structural Study of TGF-β Recognition of Its Receptors

TGF-β is a pleiotropic, multifunctional cytokine with potent immunoregulatory properties. Nearly all cells of leukocyte lineage express the cytokine. The function of TGF-β has been established in many processes, including inflammatory response, carcinogenesis, and oral tolerance as a primary inhibitory cytokine. TGF-β treatment leads to cell-cycle arrest at the late G1 phase through the expression of a cyclin-dependent kinase inhibitor p27 molecule. The TGF-β receptor consists of two chains, type I and II (TβRI and TβRII); both are receptor serine/threonine kinases. The binding of TGF-β to the type II receptor recruits and activates the type I receptor, which in turn activates the Smad signaling pathway, leading to the regulation in gene expression. To probe the molecular mechanism of TGF-β receptor activation, we have solved the structures of TGF-β2, as well as the extracellular domain of its type II receptor [19–21]. However, the structure of the ternary complex that would provide

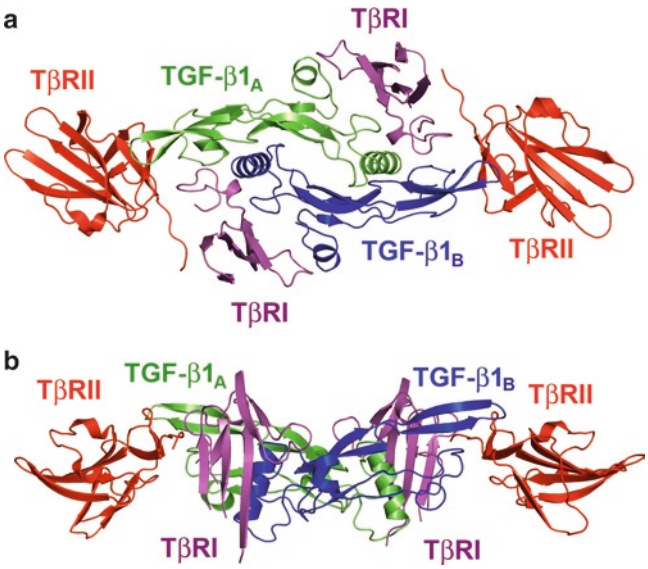

Fig. 27.4 TGF-β1 ternary complex viewed from top (**a**) and side (**b**). TGF-β1 monomers $TGF\beta1_A$ and $TGF\text{-}\beta1_B$ are colored in green and blue, respectively. TβRI and TβRII are colored in magenta and red, respectively

much-needed insight into the molecular activation is not available to date. A significant technical difficulty in structural studies of TGF-β is to produce functional recombinant TGF-β for crystallization. The failure to obtain properly folded TGF-β1 and TGF-β2 from a bacteria expression system led us to explore the possibility of using a mammalian pcDNA3.1-based CHO cell-expression system. To ensure the expression of a fully active mature TGF-β1, we subcloned the entire latency peptide into a modified pcDNA3.1 vector, in which the neomycin gene was replaced with a glutamine synthetase gene, to allow amplification of the plasmid copy number (Fig. 27.1). To maximize the expression and simplify the purification, we replaced the TGF-β1 signal peptide with that of rat albumin, introduced a six-histidine-tag at the N-terminus of the latency peptide for efficient purification of the latency protein, but removal upon activation of the mature TGF-β1.We also removed an unpaired cysteine in the TGF-β1 propeptide region to prevent disulfide mispairing. Stable clones were selected with the expression level of ~20 mg /liter of spent media [22]. The high expression level of TGF-β1 has made it possible to assemble the ternary complex between TGF-β and both of the receptors for crystallization. Recently, we have solved the crystal structure of a ternary complex between human TGF-β1 and the extracellular domains of its type I and type II receptors at 3.0 Å resolution (Fig. 27.4). The overall assembly of the TGF-β1 ternary complex is very similar to that of the TGF-β3 complex, with TβRII bound identically to both isoforms of TGF-β. The docking of TβRI on TGF-β1 is ~10° different from that of TGF-β3. The structural analysis suggests that the lower TβRII binding affinity to TGF-β2 is the result of replacement of both Arg 25 and Arg 94 in TGF-β1 and TGF-β3 with lysines in TGF-β2. Comparison between the TGF-β and BMP-2 ternary complexes revealed that predominantly hydrogen bonds mediate binding of the cytokines to their high-affinity receptors (TβRII and BMPR-IA, respectively), whereas hydrophobic interactions dominate their contacts to the low-affinity receptors (TβRI and ActRII, respectively). This suggests the importance of hydrogen bonding in determining the receptor preference for these cytokines.In addition, the solution-binding studies unexpectedly revealed significant binding of TGF-β2 and TGF-β3 to TβRI. While all three isoforms of TGF-β assembled their ternary complexes equally well, the preference for the type II receptor varied with TGF-β1 being the most TβRII dependent and TGF-β2 being the least. The equally energetic contributions of TβRI and TβRII to the assembly of the TGF-β2 signaling complex suggest a simultaneous, rather than sequential, receptor-recruitment model for TGF-β2. The differences in the receptor recruitment provide a potential mechanism for non-overlapping forms of three TGF-β isoforms that are probably coordinated with the cellular and tissue-dependent receptor expressions.

27.2 Molecular Recognition of DC-SIGN and other Carbohydrate Receptors by HIV gp120

HIV-1 infections of macrophage and T cells require both CD4 and chemokine receptors. While binding to CD4 provides viral attachment to host cells, the interaction of the viral envelope protein gp120 with chemokine receptors initiates the conformational changes that lead to the fusion and entry of the virus [23]. However, additional receptors have also been found to interact with HIV-1 viral protein and to facilitate viral infection.In particular, HIV-1 envelope protein gp120 is heavily glycosylated, which results in high-affinity binding to C-type lectins, such as DC-SIGN. Our laboratory has been focused on the carbohydrate-mediated binding between HIV-1 envelope gp120 and cell surface lectins, as well as the influence of these interactions on viral infection and host immune response.

27.2.1 Structural and Functional Study of DC-SIGN/R

DC-SIGN, a dendritic cell-surface C-type lectin receptor, has been proposed to enhance HIV-1 infection to T cells *in trans*[24]. The physiological role of DC-SIGN was thought to bind adhesion molecules, ICAM-2 and ICAM-3, to initiate

a dendritic-cell and T-cell contact to facilitate the T-cell activation and priming by dendritic cells [25]. To understand the pathogen specificity of DC-SIGN and its physiological function, we carried out a structural and biochemical study of the receptor and its ligand recognition. We have expressed various extracellular truncations of soluble DC-SIGN and DC-SIGNR and showed that (i) DC-SIGN/R forms a tetramer through the extracellular repeat region; (ii) the receptor-gp120 binding affinity depends on the oligomerization of the receptor; (iii) DC-SIGN/R recognition of gp120 is pH dependent such that the affinity reduced drastically under the endosomal pH condition, and (iv) DC-SIGN recognizes ICAM-3 with 100-fold weaker affinity than it does to gp120 [26]. These results suggest that DC-SIGN most likely releases HIV-1 upon internalization and is thus unlikely to promote the viral infection *in trans*. The low affinity to ICAM-3 suggests that DC-SIGN functions most likely as an antigen-capture receptor rather than an adhesion receptor. To understand the mechanism differentiating DC-SIGN binding to gp120 and ICAM-3, we crystallized a monomeric DC-SIGNR in complex with mannose and modeled the structure of the tetrameric intact extracellular receptor (Fig. 27.5a, b) [27]. The structure shows that the receptor assumes a typical C-lectin fold. The requirement of the tetramer for high-affinity ligand recognition imposes limitations on the receptor ligand selection. Specifically, ligands carrying multiple glycosylations capable of simultaneously engaging the multimeric DC-SIGN/R would be preferred by the receptor. Based on the DC-SIGN tetramer model, we formulated a glycan density-based scheme to evaluate potential ligands of DC-SIGN/R. When applied to both the human and viral non-redundant genomic sequences for potential ligands of DC-SIGN, in addition to ICAM-3, other adhesion molecules;cell-surface receptors; mucin-like proteins; members of CEA family, and lysosomal associated proteins (LAMP) also appear to be able to bind DC-SIGN. Some of the potential cellular ligands are tumor antigens, suggesting a potential role of the receptor in capturing tumor antigen and thereby contributing to the host immune recognition of tumors. The search against viral genome sequences showed that most of the enveloped RNA and DNA viruses appear to express potential ligands for DC-SIGN, suggesting a role of the receptor in capturing enveloped viruses for antigen presentation.

27.2.2 Structure and Function Studies of Siglecs

Sialic acid binding **I**mmuno**g**lobulin-like **Lec**tins (Siglecs) are a recently discovered and rapidly expanding family of receptors that specifically recognize sialylated glycans, including mucins. So far, there are over a dozen human Siglecs genes identified. Most of them are expressed on the surface of myeloid lineage cells or lymphocytes. They have diverse functions, including the promotion of cell-cell recognition and adhesion and the regulation of leukocyte trafficking; lymphocyte homing; cell proliferation; apoptosis, and inflammation [28]. Examples of the involvement of Siglec receptors in viral infection have been reported, such as the involvement of Siglec-1 in a porcine respiratory syndrome virus infection to alveolar macrophages [29]. However, the relevance of Siglec receptors in viral and other microbial infections has not been extensively studied. We proposed to study the pathogenic involvement of this class of carbohydrate receptors by a combination of structural and biochemical methods. Specifically, we proposed to express and purify the recombinant human Siglec receptors for crystallization and solution-binding studies, to define both the mechanism and the specificities of their ligands. To date, out of thirteen human Siglecs and nine mouse Siglecs identified, the structural information is only available for the V-set ligand-binding domains of a mouse sialoadhesin and human Siglec-7 [30,31]. Early structural data on mouse sialoadhesin, in complex with α(2,3)-sialyllactose, showed that the ligand recognition occurs primarily through interactions with the terminal sialic acid moiety. Among the three commonly observed linkages, the α(2,3)-, α(2,6)-, and α(2,8)-linkages between the terminal sialic acid and a subterminal carbohydrate, different Siglec receptors often exhibit linkage-dependent binding specificities. The structural mechanism of this linkage-dependent Siglec binding specificity remains largely unresolved. To gain insight into the function of Siglec receptors, we have expressed and purified the two *N*-terminal domains of human Siglec-3 (CD33) and Siglec-5 (CD170), using a recombinant bacteria expression system. We have crystallized both the native Siglec-5 and two sialylated carbohydrate complexes to 2.8 Å resolution (Fig. 27.5c, d) [32]. The native structure revealed an unusual conformation of the CC′ ligand-specificity loop and a unique inter-domain disulfide bond (Figure 8). The α(2,3)-sialyllactose- and α(2,6)-sialyllactose-complexed structures showed a conserved sialic acid recognition motif that involved both Arg 124 and a portion of the G-strand in the V-set domain forming β-sheet-like hydrogen bonds with the glycerol side chain of the sialic acid. Only a few direct protein contacts with the subterminal sugars were observed and mediated by the highly variable GG′ linker and CC′ loop. The lack of direct interactions between the receptor and the subterminal galactose and glucose suggests that Siglec-5 is promiscuous in its linkage specificity. To support the structural observation, we also performed a series of solution-binding experiments between the recombinant Siglec-5 and the linkage-specific sialyllactoses. The results showed that Siglec-5 bound similar to α(2,3)- and α(2,6)-sialyllactose, with dissociation constants of 8.7 and 8.0 mM, respectively. The affinity

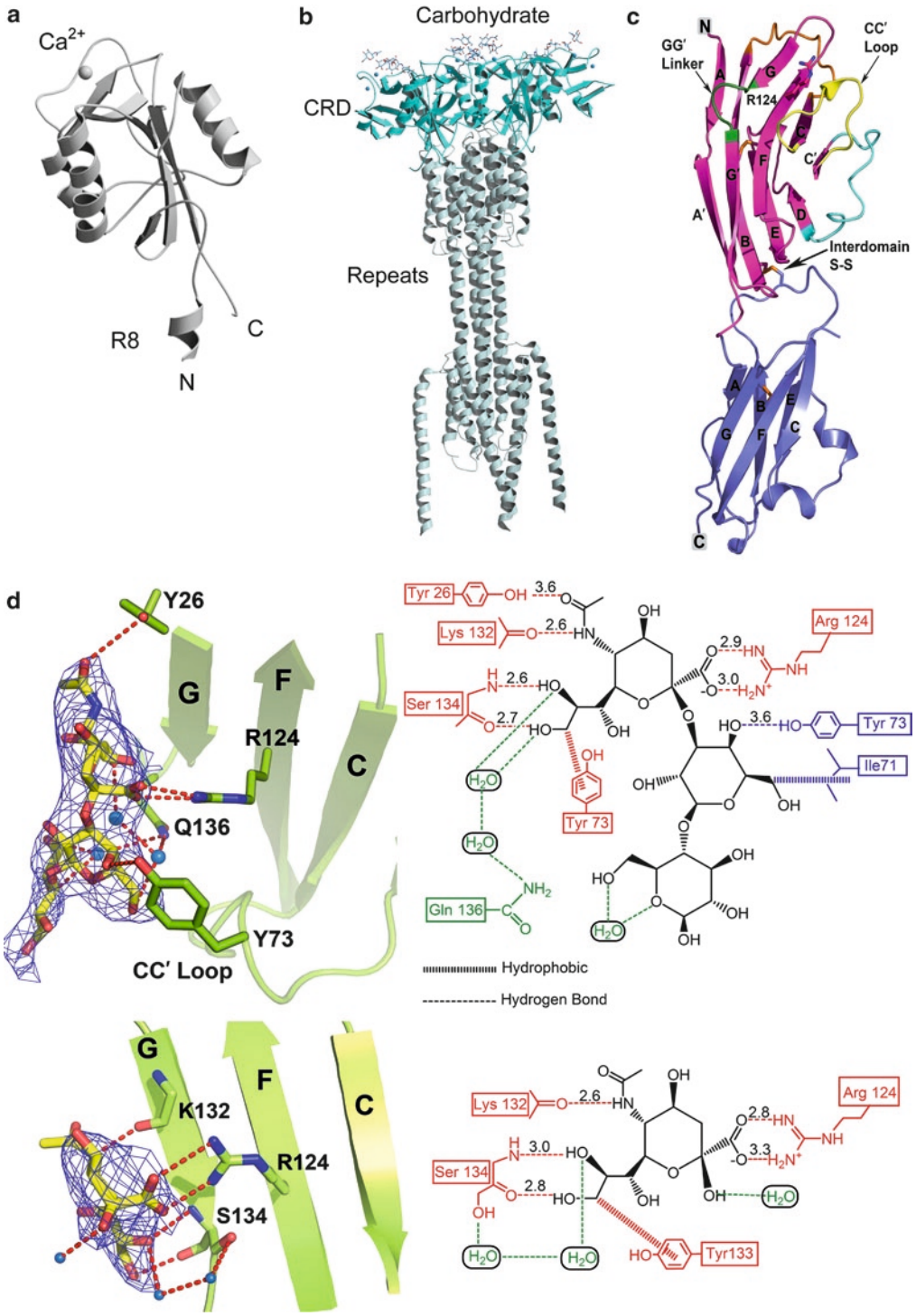

Fig. 27.5 (**a**) Structure of the DC-SIGNR R8 and (**b**) a model of the receptor tetramer. (**c**) Ribbon diagram showing the native Siglec-5 structure with V-domain in magenta and C2-domain in blue. The sialic acid coordinating Arg 124 and the disulfides are shown with sticks. The specificity-determining GG′ and CC′ regions are depicted in green and yellow, respectively. (**d**) Siglec-5 in complex with (upper) α(2,3)- and (lower) α(2,6)-sialyllactose. The left panels show the omit density maps contoured at 2σ with the carbohydrates colored in yellow. The right panels illustrate the interactions between the respective receptor and its carbohydrate at the binding site. Direct protein and ligand interactions are shown in red and blue, respectively. Water-mediated interactions are colored in green

of Siglec-5 to α(2,8)-disialic acid was slightly lower, with a K_D of 25 mM. These structural and binding studies provided mechanistic insights into the linkage-dependent Siglec-5 carbohydrate recognition and suggest that Siglec-5 and other CD33-related Siglec receptors are more promiscuous in sialo-glycan recognition than was previously understood.

It would be very interesting to see if the Siglec receptor-mediated carbohydrate recognition is involved in viral entry and pathogenesis.

27.3 Summary

The goal of our research is to understand the function of immuno-receptors through structural and functional studies. There are two main research themes: the receptors involved in lymphocyte functions and the receptors involved in HIV-1 pathogenesis. Our approach is to characterize the structures of the immuno-receptors and their ligand complexes by X-ray crystallography. Based on the structural results, we then propose functional studies using a combination of mutational analyses, solution-based BIAcore binding experiments, FACS and Confocal analyses, to define the specificity and the hot spots of the receptor-ligand interactions. Toward understanding the structure and function of lymphocyte receptors, we have been focusing on the receptors on NK and B cells, as well as Fc receptors on macrophages. In HIV-receptor biology, we have been interested in the carbohydrate receptors, such as DC-SIGN and Siglecs, and their role in HIV-1 infection and host immune response. The results of these studies not only helped us to understand the condition under which these receptors are activated, but also revealed potential new ligands of the receptors, as in the case of DC-SIGN.

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Chapter 28
Role of the NKG2D Receptor in Health and Disease

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28.1 Introduction

Natural-killer (NK) cells are inflammatory cytokine-producing and/or cytotoxic lymphocytes that comprise a major component of the innate immune system. NK cells express a large variety of activating receptors dedicated to accomplishing this task, one of which is NKG2D. This receptor is also expressed on subsets of T cells, where it functions to transmit co-stimulatory signals. The fact that viruses and tumors have evolved strategies to evade NKG2D-mediated immune recognition highlights the importance of this receptor in immunity. This article describes the molecular properties of NKG2D; the diverse array of ligands with which it interacts; the biological mechanisms that regulate its cell-surface expression and, very importantly, the role that NKG2D plays in a variety of disease pathologies.

28.2 NKG2D Receptor Complex

NKG2D is a type II transmembrane, C-type lectin-like receptor that can activate NK cells and co-stimulate T cells (reviewed in [1–4]). NKG2D is expressed on the cell surface as a homodimer [5], in association with an adaptor molecule DAP10 (DAP10 and DAP12 in mice) that is essential for NKG2D signaling and cell-surface expression [6–9]. NKG2D is encoded by *Klrk1,* a gene that is located in the NK gene complex on chromosome 12 (chromosome 6 in mice). NKG2D is expressed on NK, γδ T cells, as well as on subsets of $CD4^+$ and $CD8^+$ T cells [10]. The NKG2D homodimer assembles on the cell surface with two dimers of DAP10, thereby forming a hexameric structure, which probably serves to reduce the signaling threshold for NKG2D effector function [5]. *Klrk1* shares about 20% nucleotide sequence homology with other members of the NKG2 family and, in contrast with other members of the NKG2 family, does not associate with CD94 [11].

The DAP10 and DAP12 adaptor molecules are type I transmembrane proteins that contain aspartic acid residues in their transmembrane domains that interact with a positively charged arginine residue in the transmembrane region of NKG2D. While both human and murine NKG2D can pair with DAP10, only the alternatively spliced form of NKG2D (NKG2D-S), expressed by activated mouse, but not human, NK cells can pair with DAP-10 or DAP-12 *in vivo* [6,12,13]. DAP10 contains an intracellular YxxM motif that is responsible for initiating signal transduction by the NKG2D/DAP10 complex. Receptor ligation promotes tyrosine phosphorylation within the YxxM motif of DAP10, which leads to activation of the PI3K/Grb-2-Vav pathway [7,14,15].

NKG2D ligands are usually absent or expressed at very low levels on healthy cells, but are often induced in cells under stress; hence, they are often referred to as stress-induced antigens. Up-regulation of NKG2D ligand expression by infected or malignant cells can promote their destruction by NKG2D-bearing NK cells. On the other hand, inappropriately elevated NKG2D ligand expression by "normal" host cells can have deleterious effects, including the induction of autoimmune responses.

28.3 NKG2D Ligands

28.3.1 MICA and MICB

The ligands for NKG2D are structurally diverse and are the subject of intense research [16]. The first to be described were the MHC class I-chain related proteins A and B (MICA and -B), each capable of activating NK cells [3,17]. The human *MICA* and *MICB* genes have been mapped to the MHC class I region, close to the *HLA-B* locus [18]. MICA and MICB are highly glycosylated proteins that consist of α1, α2 and α3 domains with MHC class I folds, and though structurally similar, they share low sequence homology (18% to 30%) to HLA-A, -B or -C [19]. Unlike classical class I MHC molecules, MICA/-B do not bind peptides; nor do they associate with β_2-microglobulin [10,20]. MICA and MICB are very polymorphic, with more than 50 MICA and 13

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MICB alleles so far reported [21]. Some alleles have been associated with diseases such as Behcet's disease [22], ankylosing spondylitis [23] psoriasis vulgaris [24, 25] and Kawasaki's disease [26].

Recently, compelling evidence has revealed that MICA and MICB expression are influenced by specific microRNAs [27, 28]. Interestingly, the microRNAs responsible for MICA/B down-regulation were found to be over-expressed in tumors and may represent a novel mechanism of tumor evasion by dampening NKG2D ligand expression. One of these microRNAs, *miR-520b,* is induced by IFNγ, a cytokine produced by NKG2D$^+$ NK and CD8 T cells [28]. It seems feasible that microRNAs for stress-inducible ligands serve to protect healthy cells from unwanted NKG2D-mediated attack, but may be exploited by tumors and viruses to thwart immune attack [29].

28.3.2 ULBP

Another NKG2D ligand, termed UL16 binding protein (ULBP)-1, was named because of its interaction with UL16, a protein encoded by the human cytomegalovirus (HCMV) [30]. Subsequently, ULBP-2, -3 and -4, were identified and found to interact with NKG2D [31, 32]. ULBP 1–3 are expressed on a wide variety of tissues, whereas ULBP-4 exhibits a more restricted tissue distribution, being expressed predominantly in the skin [32]. Recently, functional splice variants of ULBP-4 reactive with human NKG2D have been identified in various tumor cell lines [33]. Like MICA/-B, ULBPs also contain MHC class-I related α1 and α2 domains, but lack the α3 domain, and except for ULBP-4, are expressed as GPI-anchored proteins [32]. Like MICA/-B, they do not associate with β2 microglobulin.

28.3.3 RAE-1, H60 and MULT1

In mice, the NKG2D ligands include members of the retinoic acid early (RAE) family of proteins [34], the minor histocompatibility antigen H60 [35] and the murine ULBP, like transcript 1 (MULT1). A recent report has identified two more functional members of the H60 ligand family, termed H60b and H60c [36]. H60b was described as a transmembrane protein with a wide tissue distribution, whereas H60c is a GPI-anchored protein and exists mainly in the skin. RAE-1, H60 and MULT1 share low sequence homology, when comparing members of the different families, and have weak homology to classical MHC class I molecules in the α1 and α2 domains, and like ULBPs lack the α3 domain [37]. RAE-1 proteins are GPI-anchored within the plasma membrane, whereas H60 and MULT1 are transmembrane [19]. They do not bind peptides and do not appear to associate with β_2- microglobulin. Mouse ULBP-like transcript (MULT1) appears to be the murine equivalent of the human ULBPs [38].

28.4 Signal Transduction

Ligation of the NKG2D-DAP10 receptor complex leads to phosphorylation of the tyrosine within the Tyr-Ile-Asn-Met (YxxM) motif in the cytoplasmic tail of DAP10 by a src family tyrosine kinase(s) [39]. Activation of the PI3K pathway occurs through the binding and subsequent activation of the p85 subunit of the lipid kinase PI3K by tyrosine phosphorylated DAP10 [7]. T-cell signaling through CD28, which also carries an YxxM motif in its cytoplasmic tail, is initiated in a similar fashion [40]. PI3K recruitment leads to the phosphorylation of phosphoinositide 4,5 bisphopshate (PIP2), creating phosphoinositide 3,4,5 trisphosphate (PIP3), a bioactive second messenger. PIP3 functions, in part, to recruit selectively to cell membranes various pleckstrin-homology (PH) domain-containing signaling effectors, including phosphoinositide-dependent kinase-1 (PDK1), Akt (PKB), and Vav1. Vav1 is a guanine exchange factor that activates small GTPases, such as RhoA, Rac and Cdc42 [41]. GTPase activation leads to cytoskeleton re-organization, granule polarization and release, and gene activation [39, 42]. Further downstream, activation of the PI3K pathway can lead to activation of the serine/ threonine kinases MEK and ERK that induce expression of genes required for NKG2D-mediated cytotoxicity [43–45]. Overexpression of the tyrosine kinase Itk reduces NKG2D effector functions, including cytotoxicity and granule release, implying a negative role for this kinase in NKG2D signaling [46].

Upshaw et al [14] showed that both p85 and Grb2 can directly bind phosphorylated DAP10. Grb2 binds directly to Vav1, which leads to phosphorylation of Vav1 and subsequent phosphorylation of PLCγ2 and SLP76 [14]. Shaw and colleagues have shown that the PI3K docking domain within DAP10 is also necessary for the formation of an NKG2D immunological synapse [47]. They conclude that Grb-2 is recruited to the synapse via the PH domain within the SOS adapter protein.

The ligation of NKG2D/DAP10 on freshly isolated human NK cells by anti-NKG2D mAb alone is insufficient to trigger cytotoxicity and cytokine secretion [48, 49]. However, stimulation by NKG2D ligation apparently can only act by synergy with other receptors on freshly isolated NK cells [49]. Such NK cells activated with cytokines, such as IL-2, become responsive to stimulation with anti-NKG2D mAb alone, in that they can both produce cytokines and exhibit cytotoxic effector functions [3, 50]. In mouse models, it has been shown that freshly isolated NK cells are able to kill target

cells in an NKG2D-dependent manner [51, 52]. However, it was not shown if other receptors are involved in the target-cell killing. As in humans, activation of murine NK cells increases their ability to kill target cells [52]. The requirement for priming of NK cells by cytokines, namely IL-15 and IL-18, to achieve full NK cell activation, has recently been emphasized [53,54]. Related to this are the recent findings of Medzhitov and colleagues [55], that propose a coupling of the IL-15 and NKG2D signaling pathways. They show that IL-15 can induce activation of Jak3, leading to phosphorylation of DAP10. This supports the notion that specific cytokine signals are required to achieve 'full strength' NKG2D signaling, following ligand binding. Such cytokine priming may reduce inappropriate NKG2D-mediated activation of NK cells when exposed to its ligands alone. In contrast, anti-NKG2D-mediated stimulation of freshly isolated, resting murine NK cells, alone, leads to profound cytotoxic capabilities and cytokine production

In human $CD8^+$ T cells, NKG2D plays a costimulatory role for activation, in a manner similar to CD28 [2,4]. The basis for this cell-type specific role for NKG2D in lymphocyte activation is not yet clear. The difference could be due to the existence of different activation thresholds, cell-specific signaling cascades for NKG2D and/or differences in response to the local cytokine environment.

Studies from our lab have shown that inhibitory signals, in this case mediated by CD94/NKG2A, can inhibit NKG2D-mediated activation and, in particular, Vav1 phosphorylation [56]. Likewise, inhibitory Ly49 receptors can over-ride and negatively regulate murine NKG2D signaling, if both receptors are co-engaged [57]. Nonetheless, it has been shown that tumor cells expressing Rae1 are rejected in an NKG2D-dependent mechanism, despite the presence of MHC class-I molecules that can interact with inhibitory receptors present on NK cells [35, 58]. This supports the notion that the result of receptor ligation *in vivo* is ultimately determined by the balance of activating and inhibiting signals being received by the NK cell from its corresponding target cell [59].

28.5 Regulation of Expression

Because NKG2D-mediated activation of NK and $CD8^+$ T cells plays an important role in mediating immunity towards viral infections and tumors [60], it is important to determine the factors that regulate cell-surface expression of NKG2D. Very little is known about NKG2D regulation at the transcriptional level. Human *Klrk1,* the gene that encodes NKG2D, has multiple transcriptional start sites, with some *Klrk1*transcripts initiating within exon IV of the upstream adjacent *NKG2F* gene, and others starting closer to exon I of *Klrg1* [48, 61, 62]. The existence of multiple alternative transcripts of *Klrk1* is reminiscent of other genes within the NK complex, e.g. the *CD94* [63] and *NKG2A* genes [64], and may indicate the existence of multiple promoters. As mentioned, for NKG2D to be expressed on the cell-surface association with adapter proteins, DAP10 or DAP12, is required [6–9,65]. We recently described the existence of multiple *DAP10* gene-transcriptional start sites and defined the basic promoter, showing that it is active in NK and CD8 T cells, but not CD4 T cells [66].

As summarized in fig. 28.1, cell-surface expression of NKG2D can be both positively and negatively regulated by cytokines. Both human and murine $CD8^+$ T-cells stimulated with IL-2 up-regulate NKG2D and DAP10 expression [4,67,68]. IL-15, a cytokine that drives both proliferative and effector functions of both CTL and NK cells, can induce NKG2D expression and help activate NK cells to clear the cryptosporidium intestinal protozoa [69,70]. The homeostatic cytokines IL-7 and IL-15 have been shown to sustain NKG2D expression [4] by stimulated naïve CD8 T cells. On human activated CD4 cells, the combination of IL-15 stimulation plus TNFα has been shown to induce *de novo* and expand $NKG2D^+CD4^+$ T-cells. Such cells have been described in patients with rheumatoid arthritis [71]. It has also recently been shown that IL-12, a cytokine produced mainly by APC, which helps activate NK and T cells, can also upregulate NKG2D transcript and protein expression and enhance NKG2D effector functions [72,73]. Recent studies from our laboratory have shown that stimulation of human CD8 T cells with anti-CD3 plus IL-2 can result in up-regulation of DAP10 protein levels, with a corresponding increase in NKG2D cell-surface expression [66]. The mechanism of the observed TCR-mediated increase in DAP10 expression in CD8 T-cells was shown to be partially mediated through the AP-1transcription factors. Functional AP-1 sites were found to exist in the DAP10 promoter, as confirmed by over expression of c-fos and c-jun and chromatin immunoprecipitation analyses [66]. Therefore, inhibitors of the AP-1 signaling pathway may have therapeutic value for the regulation of DAP10/NKG2D expression, at least in CD8 T cells. Another receptor that has been shown to increase the expression of NKG2D on human CD8 T cells is 4-1BB. Using artificial APCs, it has been demonstrated that 4-1BB costimulation is superior to CD28 costimulation in expanding *ex vivo* CD8 T-cells and in increasing their cytotoxic potential, in part due to increased expression of NKG2D [74].

IL-21, which is secreted by activated $CD4^+$ T-cells, has been shown to activate NK and $CD8^+$ T-cells, and can cause tumors to regress, via an NKG2D-dependent mechanism [75). However, we showed that IL-21 *in vitro* stimulation of human NK and $CD8^+$ T-cells is able to down-regulate IL-2-induced expression of NKG2D [76]. This was not observed with murine NK cells [75]. IL-21 was shown to inhibit DAP10 mRNA expression, as well as *DAP10* gene-promoter activity [75]. The observed differences in IL-21-mediated regulation of NKG2D expression in human versus murine

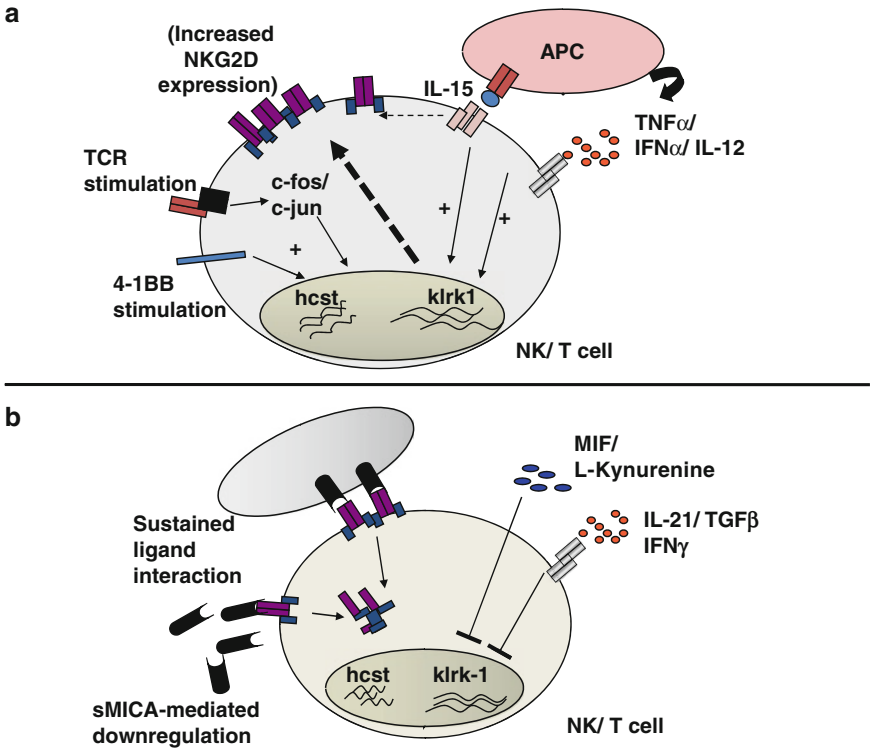

Fig. 28.1 Positive and negative mechanisms regulating NKG2D/DAP10 receptor expression in T and NK cells. (**a**) Activation of the transcription factors c-jun and c-fos, following CD3 crosslinking in T cells, can upregulate *DAP10* transcription and induce NKG2D/DAP10 cell surface expression. Signals emanating from the 4-1BB have also been reported to induce NKG2D expression. In addition, pro-inflammatory cytokines such as IL-12/15 and IFNα can elevate NKG2D cell surface expression. When the DAP10 adapter protein is not present (as in DAP10 knockout mice), or through transcriptional down-regulation NKG2D does not efficiently reach the cell surface (dashed lines). (**b**) Down-regulation of NKG2D expression can be mediated by overexposure to both soluble and membrane bound NKG2D ligands. The cytokines IL-21, TGFβ and IFNγ have been reported to inhibit NKG2D/ DAP10 expression. Hcst *ED* and *kirk-1* are the DAP10 and NKG2D genes, respectively

NK cells may be due, at least in part, to the ability of murine NKG2D to pair with DAP12, whose expression—unlike DAP10—may not be influenced by IL-21.

The inflammatory cytokine IFN-γ is produced in large amounts by NK cells in the early stages of infection and acts as an autocrine factor in promoting NK-cell activation and cytotoxic functions. However, Zhang et al [77] observed an inhibitory effect of high concentrations of IFNγ on NKG2D expression, with a resultant decrease in cytotoxicity towards NKG2D-ligand expressing targets. In the same study, *in vitro* culturing of NK cells with IFN-α had the opposite effect, in that it induced NKG2D expression. The fact that IFN-γ can downregulate NKG2D expression may imply that there is a negative feedback loop in which IFNγ can 'dampen' NKG2D-mediated NK-cell activation.

TGF-β1 is a cytokine that has been reported to downregulate cell-surface expression of NKG2D ,leading to a reduction in NKG2D-mediated cytotoxicity [78]. TGF-β1-mediated NKG2D downregulation is suggested to occur via a smad-dependent mechanism. Indeed, elevated serum levels of TGF-β1, secreted by tumors, have been identified in cancer patients and may represent a mechanism by which tumors evade NKG2D cytotoxicity [79,80]. Regulatory T cells, present in tumors, have also been demonstrated to deliver an inhibitory, membrane-bound TGF-β1 signal to NK cells, resulting in NKG2D downregulation [81]. TGF-β1-mediated NKG2D downregulation and reduced NKG2D-mediated cytotoxicity can be restored by the addition of blocking anti-TGF-β1 mAb, as well as by targeted RNA interference to TGF-β1 [82]. Recently, it has been shown that culturing of NK cells with IL-2 and IL-18, along with TGF-β, can inhibit the ability of TGF-β to downregulate NKG2D in a JNK-dependent manner [83]. Also, it has recently been shown that co-expression of 4-1BB by cytotoxic CD8 T cells, a co-stimulatory receptor, can protect NKG2D from TGF-β1-mediated down-modulation [84]. In addition to TGF-β1, another soluble cytokine, macrophage-migration inhibitory factor—frequently overexpressed by tumors—is also capable of inhibiting NKG2D transcript and protein expression. This results in decreased NKG2D recognition of tumors, contributing to immune evasion of ovarian tumors [85].

A recent study has shown that L-kynurenine, a by-product of tryptophan metabolism catalyzed by the enzyme indoleamine 2, 3-dioxygenase (IDO), can inhibit cytokine-induced surface expression of NKG2D [86]. Addition of L-kynurenine

to cell cultures, at mM levels, could specifically abrogate cell-surface NKG2D-expression normally seen in IL-2-conditioned media, which results in a corresponding decrease in NKG2D-mediated cytotoxic function. The effect of L-kynurenine appears to be reversible, as NKG2D transcript and cell-surface expression levels can be restored following extensive washing. Whether the tryptophan metabolite L-kynurenine has a physiological role in regulating NKG2D expression remains to be determined.

Another described mechanism for NKG2D downregulation is attributed to overexposure to NKG2D ligands. It [87] has been shown that tumor-derived, soluble MICA/B ligands (sMICA/B), generated by proteolytic shedding, mediate the downregulation and subsequent degradation of NKG2D/DAP10 on tumor-infiltrating lymphocytes (Fig.28.1). Elevated sMICA levels have been identified in the serum of patients with various types of cancer [88,89], and may represent a diagnostic marker in patients with suspected malignancies [90]. Some cancer patients have been reported to have an increased frequency of a rare $NKG2D^{+}CD4^{+}$ subtype T-cell population, despite the presence of elevated serum sMICA levels [91]. In fact, sMICA drives the expansion of these $NKG2D^{+}CD4^{+}$ cells while, at the same time, inhibiting the proliferation of $NKG2D^{-}CD4^{+}$ cells, by a mechanism that involves soluble FasL [91]. In pregnant individuals, placenta-derived sMICA/B has been shown to be responsible for NKG2D downregulation, with a corresponding reduction in NKG2D-mediated cytotoxic capacity [92]. In this study, sMICA levels were elevated throughout pregnancy in maternal blood compared to non-pregnant controls. The findings suggest a possible role for sMICA in promoting fetal survival through suppression of NKG2D-mediated cellular activation. The prevalence and functional impact of soluble NKG2D ligands have recently been reviewed [93].

Recent reports have described that chronic exposure to cell-surface bound NKG2D ligands is also capable of mediating NKG2D downregulation, as well as reducing NKG2D-mediated cytotoxicity [94,95]. The study by Courdert et al [95] revealed that sustained signaling by the murine NKG2D ligand H60 was able to abrogate murine NKG2D expression, which correlated with a reduction in the expression of the DAP10 and DAP12 adapter proteins. This observation that chronic exposure to membrane bound NKG2D ligands reduces NKG2D expression may perhaps imply a self-regulatory mechanism to prevent uncontrolled NKG2D-mediated $NK/CD8^{+}$ T-cell activation.

Thus, there appears to be a variety of mechanisms (depicted in Fig. 28.1) by which NKG2D expression is regulated: (i) by availability of the adapter proteins DAP10 and/or DAP12; (ii) differential regulation by cytokines and soluble factors (IL-2/15/21, TNF-α, IFN-γ, IFN-α, TGF-β1 and L-kynurenine); (iii) receptor-induced signals on T cells, e.g. TCR and 4-1BB, and (iv) via chronic exposure to soluble or membrane-bound NKG2D ligands.

28.6 Regulation of the Immune Response

NK cells have been shown to "edit" maturing dendritic cells through non-NKG2D activating receptors [96]. That NKG2D may also be involved in such processes was recently demonstrated in studies showing that human NK cells kill resting, but not activated, microglia via NKG2D plus NKp46-mediated recognition [97]. Similarly, human NK cells have been shown to kill LPS-activated macrophages via NKG2D [98]. On the other hand, there is evidence that NK cells can potentiate the immune response through recognition of NKG2D ligands on other immune cells. Kloss et al [99] showed that human monocytes, upon TLR triggering, upregulate MICA expression, which can stimulate the NK-cell mediated IFN-γ production that promotes the innate response to infection. In a similar fashion, poly I:C-stimulated human uterine NK cells have been shown to interact with autologous uterine macrophages and produce IFN-γ in an NKG2D-dependent manner [100]. Others have shown that myeloid–derived suppressor cells that accumulate in cancer patients and tumor- bearing mice, which potently suppress T-cell activation can, on the other hand, activate NK cells. The myeloid-derived suppressor cells express ligands for NKG2D, which apparently promote their NKG2D-dependent elimination [101].

28.7 NKG2D in Disease

28.7.1 Tumor Immunity

Transformation of normal cells into tumor cells often involves the induction of NKG2D ligands that serve as cellular stress signals [102]. Human tumor cells have been shown to be susceptible to NKG2D-mediated cytolysis, although binding of a co-receptor is usually required [103]. NK- and CTL-cell activation through NKG2D can contribute to immune-mediated tumor clearance. Following a study that implicated NKG2D in tumor immunity, through the recognition of specific ligands on tumor targets [35], *in vivo* tumor transfer studies in mice elegantly showed that NKG2D expression on NK and $CD8^{+}$ T-cells can mediate tumor clearance [58]. A variety of *in vitro* studies has also shown that induced expression of NKG2D ligands can markedly enhance the sensitivity of tumor cells to NK cell-mediated lysis [3,50]. In agreement with these observations, it has recently been

shown that the intracellular retention of MICA by human melanomas confers immune privilege and prevents NK cell-mediated cytotoxicity [104].

Strid et al [105] showed that the upregulation of NKG2D ligands by tumors may not always have straightforward consequences. They showed that epidermis-specific upregulation of Rae-1 induced rapid, coincident and reversible changes in the organization of tissue-resident Vγ5Vδ1 TCRγδ$^+$ intraepithelial T cells and Langerhans cells, swiftly followed by epithelial infiltration by unconventional αβT cells. Whereas local Vγ5Vδ1$^+$ T cells limited carcinogenesis, Langerhans cells unexpectedly promoted it. These results indicate that upregulation of NKG2D ligands may alone initiate a rapid tissue reorganization with pleiotropic effects.

Recently, defective tumor surveillance has been reported in the first characterization of NKG2D-deficient mice, reported by Raulet and colleagues [106]. In this study, NK-cell development from NKG2D-deficient mice was normal, but in well established tumor disease models, NKG2D-ligand bearing tumors that escaped immunity were more frequent than their wild-type controls. In contrast, a study of DAP10-deficient mice showed enhanced activity against melanoma tumors [107]. The basis for this surprising observation is thought to be due to hyperactive NKT-cell-effector function, as well as impaired activity of T regulatory cells in DAP-10-deficient mice.

While this ligand-receptor interaction is important in the clearance of tumor cells, sustained ligand expression or shedding into the plasma may result in NKG2D down-regulation, thereby impairing NKG2D-mediated cytotoxicity [87,94]. The presence of sMICA/B appears to be a common occurrence for tumors expressing these NKG2D ligands [87,90,108–111]. Thus, the shedding of sMICA/B may be a common mechanism within the tumor-cell population to avoid immune recognition. Recent investigations have shed some light on the mechanisms involved in NKG2D-ligand shedding [112]. A recent study by Spies and colleagues revealed that plasma-membrane-bound MICA can associate with endoplasmic reticulum protein 5 (ERp5), which functions in regulating protein folding. Pharmacological inhibition of ERp5 resulted in decreased shedding, suggesting that the complex formed with MICA and ERp5 folds MICA into conformations that allow efficient proteolytic shedding.

Others have shown that exosomes, produced by various cancer-cell lines *in vitro*, and that can be isolated from pleural effusions of mesothelioma patients, express ligands for NKG2D, as well as TGF-β1. These exosomes trigger down-regulation of surface NKG2D expression by NK cells and CD8$^+$ T cells. This decrease was rapid, sustained, and resulted from direct interactions between exosomes and NK cells or CD8$^+$ T-cells. Other markers (CD4, CD8, CD56, CD16, CD94, or CD69) remained unchanged, indicating the selectivity and non-stimulatory nature of the response. Exosomal NKG2D ligands were partially responsible for this effect, as down-modulation of NKG2D was slightly attenuated in the presence of MICA-specific Ab. In contrast, TGF-β1-neutralizing Ab strongly abrogated NKG2D down-modulation, suggesting exosomally expressed TGFβ as the principal mechanism [113].

The chronic exposure of NK cells to NKG2D ligands appears to have a pleiotropic effect. Coudert et al [114] found that sustained NKG2D engagement induced cross-tolerization of several unrelated NK-cell activation receptors. They showed that receptors that activate NK cells via the DAP12 and DAP10 signaling adaptors, such as murine NKG2D and Ly49D, cross-tolerize preferentially the NK-cell activation pathways that function independent of DAP10/12, such as antibody-dependent cell-mediated cytotoxicity and missing-self recognition. Conversely, DAP10/12-independent pathways are unable to cross-tolerize unrelated NK-cell activation receptors such as NKG2D or Ly49D. The general suppression of the NK cells' cytolytic function may be needed to limit tissue damage.

Tumor patients treated with a therapeutic antibody directed to block cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) function, or those vaccinated with lethally irradiated autologous tumor cells, engineered to secrete granulocyte-macrophage colony stimulating factor, have increased titers of anti-MICA antibodies [115]. The increase in anti-MICA Ab results in a reduction of circulating sMICA, and an augmentation of NK and CD8$^+$ T-cell cytotoxicity, thereby enabling increased tumor clearance in patients. The manipulation of the NKG2D pathway for the benefit of tumor immunotherapy is now being realized. A recent study in mice provides promising data, wherein murine T cells expressing a chimeric NKG2D receptor are able to reject ovarian tumors and establish a tumor-specific memory response, thus leading to improved survival rates for mice [116].

Others [117,118] have recently reviewed the role of NKG2D ligands in tumor immunity.

28.7.2 Autoimmunity

A common feature of autoimmune diseases is the selective targeting of a cell-type, tissue or organ by a certain population of autoreactive immune cells. Examples of such diseases include multiple sclerosis (MS), celiac disease and rheumatoid arthritis (RA). Recent research, discussed below, reveals that dysregulated expression of both NKG2D

receptor and its ligands may also play a significant role in autoimmune diseases.

Celiac disease, a gluten-induced, intestinal-inflammatory disorder, results in epithelial-cell destruction in the small intestine, by both innate and adaptive immune responses [119]. The inflammation associated with celiac disease results in elevated levels of the pro-inflammatory cytokine IL-15, which in turn can induce the cell-surface expression of NKG2D on intraepithelial lymphocytes (IEL) [44]. MICA, which is expressed at low levels on epithelial cells, can be markedly upregulated in celiac patients. Activation of NKG2D following MICA ligation results in activation and costimulation of IEL, leading to an innate-like cytotoxicity toward epithelial cell targets and tissue damage, one of the major complications of celiac disease.

Current literature now suggests that NKG2D and its corresponding ligands play an important role in the pathogenesis of RA [71,120]. The pathogenesis of RA is characterized by excessive production of pro-inflammatory cytokines and chemokines, leading to the recruitment and inappropriate activation of inflammatory leukocytes, ultimately resulting in cartilage- and bone-destruction [121]. In particular, increased levels of the cytokines TNF-α and IL-15 are often observed in the serum and inflamed synovial joints that are characteristic of RA patients [71]. These altered cytokine levels induce an unusual subset of $CD4^+$ T cells that express NKG2D, but not CD28. In addition, MICA and MICB are expressed on synovial tissue in RA patients and can activate autoreactive, autologous T cells in an NKG2D-dependent manner [71]. Abundant levels of sMICA have also been found in RA patients that, intriguingly, fail to down-modulate NKG2D—possibly due to upregulation of NKG2D by pro-inflammatory cytokines, TNF-α and IL-15. The net effect is prolonged co-stimulation of autoreactive T cell, via the NKG2D receptor from its corresponding ligands. This increased expression of NKG2D and its corresponding ligands appears to promote some of the pathology that occurs in RA.

Recent evidence suggests a potentially deleterious role for NKG2D in Crohn's disease, an inflammatory bowel disease caused by an excessive inflammatory response to gut microflora [122]. Conclusions from this study found that NKG2D ligands were significantly elevated on intestinal epithelial cells in Crohn's disease patients. A rare $CD4^+NKG2D^+$ subset, which expresses perforin and elicits a Th1 cytokine response when engaged with MICA, was also identified.

NKG2D has also been implicated in the pathogenesis of type I diabetes mellitus. This multifactorial disease is characterized by the destruction of insulin-producing β-cells by autoreactive lymphocytes in the pancreas. Non-obese diabetic (NOD) mice, which have a defect in NK cell-mediated function, are widely used for the study of type 1 diabetes [123]. Genomic linkage analysis in NOD mice has revealed several insulin-dependent diabetes loci [124]. The gene(s) conferring susceptibility to diabetes in NOD mice have been mapped to the NK complex region [125], where a cluster of genes preferentially expressed by NK cells is contained. Rae-1 expression on pancreatic β cells in NOD mice and NKG2D on the autoreactive $CD8^+$ cells infiltrating the pancreas led to the study on NKG2D and its ligands in diabetes studies. One such study showed that NKG2D antibody blockade efficiently prevented the onset of the autoimmune disease [123]. In this study, treatment with a non-NK-cell depleting anti-NKG2D mAb during the pre-diabetic stage completely prevented disease by impairing the expansion and function of autoreactive $CD8^+$ T cells. This exciting finding highlights the promising therapeutic potential of NKG2D blockade for autoimmune diseases where aberrant NKG2D recognition is known to participate.

28.7.3 Infection

NK cells are one of the most important effector components in conferring immunity to viral infection, and NKG2D usually plays an important role in this process [126]. For example, a recent study in mice has shown that NKG2D is required for optimal NK-cell-mediated resistance to mousepox, in order to prevent disease and lethality [127]. In addition, another study has demonstrated increased HBV liver titers in mice, following NKG2D neutralization with a mAb [128]. On the other hand, evidence has emerged that blockade of NKG2D signaling in NKT cells can have beneficial effects for hepatitis B viral infections [129]. Hepatitis often occurs through an acute immune response to HBV, resulting in tissue damage, likely via induction of NKG2D ligands in an HBV-dependent mechanism.

The prominent roles that NKG2D plays in immunity to viral infections are highlighted by the numerous mechanisms viruses have evolved to elude NKG2D-mediated recognition and clearance. Downregulation of the cell-surface expression of NKG2D ligands is a potent mechanism utilized by many viruses to inhibit NKG2D-mediated antiviral functions. Kaposi's sarcoma-associated herpesvirus downregulates NKG2D ligands, as well as the NKp80 ligand AICL, through expression of K5, which acts as an ubiquitin E3 ligase to target substrate cytoplasmic tail lysine residues [130]. Both human and murine CMV encode proteins which negatively regulate the cell-surface expression of NKG2D ligands and thus compromise the

efficacy of NK and T-cell responses [131]. The HCMV viral glycoprotein UL16 has been shown to sequester MICB, ULBP1 and ULBP2 in the endoplasmic reticulum, causing a reduction in ligand cell-surface density [132–134], while UL142, another HCMV-encoded protein, has been shown to downregulate MICA expression [135]. Downregulation of MICA by UL142 is observed in the full-length MICA, but not for the prevalent truncated allele of MICA that contains a frameshift mutation in the transmembrane region. This mutant allele was suggested to be the result of evolutionary selection for promoting resistance to HCMV infection [135]. Like HCMV, MCMV encodes proteins that inhibit NKG2D-mediated NK and CTL antiviral activity by downregulating NKG2D ligands. Downregulation of RAE-1 by m152 [136,137], H60 by m155 [136,138] and MULT-1 by m145 [139] has also been reported. Recent evidence suggests that HCMV has also evolved the use of specific miRNAs (miR-UL112) to down-modulate MICB expression and, therefore, evade NKG2D-mediated recognition [140].

The viral Fcγ receptors (vFcγRs) are expressed on the surface of infected cells [141]. MCMV expresses such a vFcγR, encoded by the m138/fcr-1 gene that selectively binds IgG via its Fc domain [142]. A novel viral strategy for down-modulating NK-cell responses, which highlights the impressive diversity of Fc receptor functions, has been established by Lenac et al [131]. In this study, they show that the MCMV molecule fcr-1 targets the down-modulation of NKG2D ligands MULT-1 and H60, from the cell surface to proteolytic degradation by lysosomes. It was shown that the *N*-terminus within the fcr-1 ectodomain, in conjunction with the fcr-1 transmembrane domain, was critical for NKG2D-ligand degradation. Furthermore, the authors concluded that specific deletion of the m138/fcr-1 gene from the MCMV genome attenuated viral replication *in vivo*. Presumably, this viral strain cannot abrogate NKG2D-ligand expression and, thus, can be eliminated by $NKG2D^+$ NK cells. However, exactly how a viral protein with apparent Fcγ-receptor function can mechanistically downregulate NKG2D ligands that lack Fc domains remains to be understood fully.

Viral interference of NKG2D ligand has been discussed in this review, but a study by Carayannopoulos and colleagues has revealed that certain orthopoxviruses can actually encode proteins, termed orthopoxvirus MHC class I-like proteins, that act as high-affinity antagonists of NKG2D [143]. These viral proteins—as their name suggests—resemble MHC-class I, and represent a further novel mechanism of viral evasion of NKG2D-mediated immunity.

NKG2D also appears to play a role in dealing with certain bacterial infections. *P. aeruginosa* has been shown to be a potent inducer of NKG2D ligands by pulmonary epithelial cells, and NKG2D-receptor blockade inhibited pulmonary clearance of *P. aeruginosa* in mice [144]. IFN-γ by the activated NK cells appears to promote enhanced resistance to *P. aeruginosa* [145].

28.8 NKG2D in Grafts and Transplantation

Evidence has emerged to suggest that NKG2D interaction with its ligands can have functional consequences for organ transplants. Recent data suggest that successful solid-organ transplantation correlates with the expression of MICA/B proteins on grafts [146]. Renal and pancreatic grafts in patients with evidence of both acute and chronic rejection have been shown to express MICA/B proteins, and serum from these patients contains anti-MICA/B antibodies [147]. The presence of sMICA has also been correlated to a lower incidence of graft rejection [148]. In a murine model of bone-marrow transplantation, NKG2D/RAE-1-mediated activation of NK cells results in the rejection of bone-marrow grafts. Significantly, NKG2D neutralization, using specific antibodies, allowed transplant engraftment of bone marrow [149]. In addition, a recent study has also correlated the heightened expression of NKG2D mRNA to acute and chronic nephropathy after kidney transplantation [150]. These studies highlight the therapeutic use of NKG2D-antibody blockade and its promising potential in transplantation.

28.8.1 Concluding Remarks

NKG2D is a primary activation receptor expressed by NK cells, that can also provide co-stimulatory signals for CD8 T cells. NKG2D has no identifiable signaling motifs; rather, it attains signaling capability through association with DAP10, which contains an YxxM motif that can activate the PI3K signaling pathway. The ligands for human NKG2D are the MHC class I chain-related proteins A/B (MICA,-B) and the UL16-binding proteins (ULBPs). NKG2D ligands are usually absent on normal cells, but are often upregulated by cancerous and stressed cells, and can promote NKG2D-mediated tumor clearance. On the other hand, aberrant NKG2D signaling and inappropriate activation of NK and T cells by NKG2D have been implicated in various human diseases, including celiac disease and rheumatoid arthritis; and for diabetes in NOD mice (summarized in Table 28.1). Thus, the modulation of NKG2D-receptor expression may have therapeutic implications for a variety of diseases. Consequently, our goal is to determine factors that regulate NKG2D/DAP10 expression, in hope of ultimately being able to manipulate receptor expression.

Table 28.1 Examples of the implications of NKG2D and NKG2D-ligand expression in disease pathogenesis

Disorder	Type	Responsible factors	Outcome	Ref
Tumors	epithelial malignancy	a) expression of NKG2D ligands	a) NKG2D-mediated tumor clearance	[35,52,102]
		b) NKG2D ligand shedding	b) NKG2D downregulation impairing natural cytotoxicity	[87,88,90,108]
Autoimmunity	celiac disease	a) IL-15 induced expression of NKG2D	a, b) inappropriate T-cell response	[44,119]
		b) increased expression of ligands		
	rheumatoid arthritis	a) high levels of pro-inflammatory cytokines	a) increased NKG2D expression	[71,120]
		b) increased expression of ligands	b) increased NKG2D-mediated co-stimulation of autoreactive T cells	
	diabetes (mice)	expression of NKG2D ligands	elimination of insulin-producing cells by auto-reactive T cells	[123]
Infection	CMV	downregulation of NKG2D ligands	evasion of NKG2D-mediated recognition	[131–133,135]
	HCMV	upregulation of miRNA directed to MICB	reduced NKG2D-ligand expression	[140]
	Zoonotic orthopoxvir uses	Encode proteins that are NKG2D antagonists	Blockade of NKG2D function	[143]

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Part IX
Allergy

Chapter 29
G-Protein-Evoked Signaling Mechanisms in Asthma and Allergic Disease

Kirk M. Druey

Asthma, which is characterized by chronic and reversible airways obstruction, leads to shortness of breath, cough, wheezing, and occasionally death, due to respiratory failure [1]. Asthma is extremely common, affecting 5–8% of the U.S. population [2]. This disease accounts for substantial morbidity and mortality, and over $18 billion in direct (e.g., hospitalizations) and indirect (loss of income or productivity due to illness) health care dollars each year in the United States [3]. Despite the recognition, over the last twenty years, that asthma is often accompanied by chronic inflammation and an accordant shift in treatment strategies to include the use of anti-inflammatory agents [4], the incidence of asthma and of mortality due to asthma continue to rise [2–3].

A certain percentage of asthmatics are also atopic (estimated to be anywhere from 25–60%, depending on the population studied) [5], which means lung inflammation may be caused by an abnormal immune response, mediated by IgE antibodies. Atopic (allergic) individuals generate IgE to normally innocuous environmental substances (allergens), such as tree and grass pollens, foods, and dust mites. Allergen-specific IgE binds to high-affinity IgE receptors on tissue mast cells, and subsequent cross-linking of these receptors by allergen upon re-exposure incites the allergic inflammatory cascade [6]. Mast-cell mediators, including leukotrienes and cytokines, generate a lung milieu characterized by inflammatory cell infiltration, increased mucus production and collagen deposition, and bronchial epithelial sloughing. Collectively, these abnormalities contribute to increased bronchial smooth-muscle contraction, hypertrophy, and hyperplasia (termed airway hyperresponsiveness "AHR") [7,8].

Despite considerable advances in our understanding of allergic inflammatory mechanisms, the molecular defect(s) underlying AHR in asthma remain unknown. Because reversible airways obstruction is sometimes observed in the absence of much lung inflammatory infiltrates or detectable allergen-specific IgE [9], intrinsic abnormalities in airway smooth muscle (ASM) contractile properties and/or growth may contribute to AHR [10]. In addition, airways of chronic asthmatics have both increased myocyte cell number (hyperplasia) and cell volume (hypertrophy) [11,12]. Both ASM hyperplasia and hypertrophy have been postulated to contribute to *fixed* airway obstruction in a certain percentage of moderate-to-severe asthmatics—that is, bronchoconstriction resistant to standard bronchodilator therapy [8]. Thus, an understanding of pathways leading to ASM contraction and growth is vital to proper disease treatment.

The primary signaling routes mediating ASM contraction and relaxation in normal airways and in the presence of allergic inflammation are evoked by G-protein-coupled receptors (GPCRs) [13]. These receptors, which have a distinct seven-transmembrane-spanning (heptahelical) structure, are by far the most common type of cell-surface receptor [14] and are the predominant targets of anti-asthmatic therapy. Mast mediators, such as histamine, cysteinyl leukotrienes (CysLT, LTD4), endothelin-1, and bradykinin, act on procontractile GPCRs coupled to $G_{\alpha q}$ to induce bronchoconstriction by promoting actin-myosin interactions [13]. A requirement for $G_{\alpha q}$ signaling in the lung has been reported for muscarinic receptor-dependent airway responses in a mouse model of allergic asthma [15]. By contrast, $G_{\alpha s}$-mediated signaling induced by βARs leads to ASM relaxation. Antagonists of CysLT receptors or βAR agonists are currently mainstays of asthma treatment.

In fact, GPCRs regulate most functions of mammalian cells and are targeted by 50% of all pharmaceutical agents currently in use for treatment of a wide array of diseases [16]. Unlike many growth-factor receptors, GPCRs use an intermediary to transduce intracellular signals—the heterotrimeric G protein, which consists of α, β, and γ subunits [17]. In the absence of a receptor ligand, these elements associate in complex, in which the α subunit is bound to GDP. Upon GPCR stimulation, Gα exchanges GTP for GDP, and temporarily dissociates from the βγ dimer. Each of these activated components interacts with distinct effectors, inducing an array of cellular responses, ranging from morphological change to gene transcription [18]. The catalytic cycle, which is extremely rapid, is terminated by the intrinsic GTPase activity of the α subunit, which promotes Gα re-association with βγ to form an inactive heterotrimer. Although βγ initiates a distinct set of cellular processes, the α subunit distinguishes

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effectors activated by a particular GPCR. There are four major subfamilies of α subunits: α_i, α_s, α_q, and $\alpha_{12/13}$, encoded by 20 α genes.

Negative regulation of G proteins is mediated in part by the family of Regulators of G-protein Signaling (RGS) proteins, which number greater than 30 in mammalian cells, and can be subdivided into subfamilies on the basis of characteristic domains [19]. All RGS proteins contain the characteristic 120 amino acid RGS domain, which mediates binding to Gα subunits and GTPase accelerating (GAP) activity. RGS GAP activity promotes the hydrolysis of GTP by Gα by stabilizing an intermediary structure of the reaction without directly contributing, catalytically [20]. The net result is the formation of inactive (GDP-bound) Gα, which facilitates rapid termination of the signaling pathways. In a cell, the principal function of RGS proteins may be to mediate adaptation to or recovery from a stimulus. Although a number of determinants of RGS expression and activity have been elucidated over the past 14 years, many physiological function(s) of RGS proteins are incompletely defined. Most cells often express several RGS proteins [19], and it is unclear whether one RGS family member may compensate for another, or whether an individual RGS protein regulates a unique subset of GPCRs within a cell. Although early studies suggested little discrimination between Gα subunits by RGS proteins [21], recent systematic crystallographic and NMR studies indicate that each individual RGS may have subtle structural differences, that allow selective pairing with cognate Gα substrates [22]. In the lung, the physiological function(s) of RGS proteins is by and large unknown. We are examining which RGS proteins are expressed in specific cell types in the lung to discern their functions in normal lung function, as well as in disease processes such as asthma. These studies will elucidate mechanisms and eventually lead to new treatment approaches.

We detected RGS2, 3, 4, 5, and 10 in primary-cultured mouse and human-bronchial smooth-muscle cells (unpublished data). As has been found for a number of cell types, the transcription of *RGS* genes in ASM appears to be highly regulated. Growth factors such as platelet-derived growth factor (PDGF), which is mitogenic for ASM, induce expression of RGS4. In contrast, chronic treatment of primary ASM cultures with β-adrenergic receptor agonists such as isoproterenol, which are used clinically to achieve bronchodilation, may be accompanied by reduced quantities of both RGS4 and RGS5. Although the mechanism(s) underlying these variations in RGS quantities and the physiological consequences of such changes are as yet unclear, we are investigating whether they alter ASM contractility and/or growth.

In prior studies of *RGS* knockout mice or RGS-deficient cell lines, the amplitude and/or durability of GPCR-induced signaling pathways are increased in the absence of the negative regulator [23]. In our preliminary studies, signaling responses to several GPCR ligands, including thrombin and bradykinin, are augmented in ASM cells expressing RGS5-specific siRNA. These responses include elevations in intracellular Ca^{2+} and phosphorylation of myosin light chain, which leads directly to ASM fiber shortening [13]. Thus, under physiological conditions, RGS5 may limit GPCR-evoked excitation-contraction coupling in ASM by hastening G-protein deactivation. To investigate this hypothesis further, we are measuring the contraction of precision-cut human-lung slices (PCLS) [24]. These slices allow direct visualization of changes in the diameter of small airways, which are thought to represent the primary anatomical site of AHR in asthma [25], upon exposure to specific ligands. Both overexpression and knockdown [siRNA or short hairpin RNA (shRNA)] strategies should clarify how individual RGS proteins control both contraction and relaxation of ASM in these slices, in response to specific GPCR ligands. Finally, to determine how RGS4 and RGS5 regulate the contraction of ASM in normal lung and in asthma, we are examining contractility of both excised tracheas and PCLS from *Rgs4-/-* and *Rgs5-/-* mice. Tracheas are connected to a pressure transducer in a physiological salt solution bath, which allows measurement of tension at baseline and after agonist stimulation [26]. AHR in these mice is also assessed by plethysmography, after allergen sensitization and challenge.

In addition to airway smooth-muscle changes, chronic inflammation of the lung induces airway remodeling, such as epithelial proliferation and goblet-cell differentiation; subepithelial fibrosis; angiogenesis; collagen deposition, and nerve proliferation [8]. In asthma and other inflammatory diseases of the airway, epithelial cells upregulate mucin genes like *MUC5AC*, resulting in differentiation into mucous-producing cells, most likely through interactions with allergens or infectious pathogens [27]**.** For example, lipopolysaccharide (LPS) from gram-negative encapsulated bacteria induces mucous metaplasia through toll-like receptor (TLR)-dependent stimulation of epithelial cells, which in turn leads to extracellular ATP secretion [28]. ATP acts on $P2Y_2$ GPCRs, which induces *MUC5AC* expression. Hyperplasia and hypertrophy of these epithelial cells results in mucous overproduction, which ultimately contributes to airway narrowing. Small, interfering RNA (siRNA) to RGS4 enhanced both LPS- and ATP-induced *MUC5AC* expression in human airway epithelial cells, whereas expression of either RGS2 or RGS4, but not RGS3 or RGS5, inhibited this response. Tracheas from RGS4-deficient mice also showed increased LPS- and ATP-elicited *MUC5AC* expression, and systemic treatment of these mice with LPS induced overproduction of thick mucous in the airways. Prior studies also demonstrated expression of RGS2 in bronchial epithelial cells. RGS2 mRNA and protein was detected in ciliated airway epithelial cells [29]. Purinergic receptors on these cells stimulate ciliary beat frequency (CBF) in response to ATP, through

activation of phospholipase Cβ by Gαq and subsequent increases in cytosolic Ca^{2+} concentration. Knockdown of endogenous RGS2 in epithelial cells increased CBF induced by ATP, while overexpression of RGS2 reduced the response. Thus, both RGS2 and RGS4 may regulate physiological functions of airway epithelial cells, such as pathogen clearance and mucous production.

Another cell type that may contribute to airway abnormalities in asthma is the type II pneumocyte. TIIP are structural cells that secrete surfactant, which is composed of several phospholipids, including phosphatidylcholine [30]. Surfactant reduces alveolar surface tension at the air/liquid interface. In addition, there are four surfactant proteins, which facilitate exocytosis of surfactant-containing lamellar bodies in TIIP in a Ca^{2+}-dependent manner. Abnormalities in TIIP numbers and function may be present in asthma [31], and surfactant or surfactant proteins may have a function in acute respiratory viral infection [32]. RT-PCR analysis indicated expression of *Rgs9-1* and *Rgs11* in mouse lung. Our immunohistochemistry studies identified the presence of RGS9-1 in alveolar type II pneumocytes (TIIP) in unchallenged mouse lung sections, based on characteristic morphology of the cells and co-localization of RGS9-1 staining with pro-surfactant proteins (Druey, unpublished data). The function of these RGS proteins in TIIP is currently under investigation.

Finally, in addition to regulating the function of lung structural cells, RGS proteins may control the extent to which inflammatory cells infiltrate the lung parenchyma and/or airway lumen after allergen challenge. Chemokines orchestrate coordinated movement of T lymphocytes through lymphoid organs and promote their migration to sites of inflammation [33]. Distinct patterns of chemokine receptors are expressed in naive and activated T-cell populations, and gene-targeting experiments have implicated a role for specific chemokines and their receptors in T-cell activation [34]; differentiation into T_H1 or T_H2 subtypes [35,36], and inflammatory responses mediated by either of these subpopulations [37,38]. Asthma is a T_H2-mediated disease in which $CD4^+$ T-lymphocytes migrate to the lung parenchyma upon allergen exposure and secrete proinflammatory cytokines (IL-4, -5, and -13), leading to recruitment and activation of eosinophils [6]. In murine asthma models, T_H2 migration to the lung may be dependent upon, among others, CCR3-6, CCR8, and CXCR4 receptors [37], with CCR4 being particularly important [39].

The signaling pathways that mediate chemokine-induced trafficking immediately downstream of the GPCR are incompletely defined. Although chemokine receptors couple to $G\alpha_i$ and $G\alpha_q$, chemotaxis is not evoked by receptors coupled exclusively to $G\alpha_q$ although there is evidence of a Gq-mediated alternative pathway leading to chemotaxis in some leukocytes [40,41]. Thymic emigration, transendothelial migration of lymphocytes into lymph nodes, and Ag-induced recruitment of lymphocytes to the lung are blocked by pertussis toxin (PTX), which inactivates $G\alpha_i/G\alpha_o$ proteins [42]. Consistent with a role for $G\alpha_i$ signaling in T-cell activation, $G\alpha_{i2}$-deficient T lymphocytes produce higher levels of proinflammatory cytokines in the gut, which is associated with inflammatory colitis [43]. Whether this abnormality is related directly to $G\alpha_i$ function in T lymphocytes is unknown.

Because they directly bind and inactivate $G_{\alpha i}$ and $G_{\alpha q}$ subunits, RGS proteins may regulate chemokine-mediated migration of T lymphocytes. We have demonstrated that T lymphocytes express several RGS proteins, including RGS2 and RGS16 [44]. *RGS16* mRNA is increased in human T cells after IL-2 stimulation, whereas *RGS2* mRNA is downregulated by IL-2. RGS overexpression inhibits chemotactic and proadhesive responses in lymphocyte cell lines *in vitro* [45]. RGS16 is expressed in both T_H1 and T_H2 T cells from the mouse, and T lymphocytes from transgenic (Tg) mice that overexpress RGS16 in both the $CD4^+$ and $CD8^+$ populations showed reduced migration to the lung, in response to allergen challenge [46]. In a model of allergic airway inflammation, T_H2 ($CD4^+CCR3^+$) lymphocyte numbers were increased in the spleen of transgenic mice, but reduced in bronchoalveolar lavage fluid from these animals. In contrast, RGS16 overexpressing T cells proliferated more and produced more cytokines to Ag stimulation in recall assays, which correlated with increased serum eotaxin levels and airway hyperreactivity (AHR). The molecular mechanisms underlying these observations remain to be determined, but we hypothesized that reduced chemotactic and haptotactic responses of T cells overexpressing RGS16 to T_H2-associated chemokines interrupted their normal circulation pattern in an inflammatory disease challenge.

In mice, targeted deletion of *Rgs2* in mice resulted in impaired T-lymphocyte proliferation and IL-2 production, in response to T-cell receptor (TCR) or PMA stimulation [47]. $Rgs2^{-/-}$ mice also had delayed and reduced responses to lymphocytic choriomeningitis virus infection, which is a model of T_H1-dependent inflammation. These early studies suggested a function of several RGS proteins in adaptive T-cell immune responses in mice. Our current studies examine T-cell activation and migration to an inflammatory stimulus *in vivo* in $Rgs16^{-/-}$ mice, using models of allergic inflammation. Preliminary data show that these mice have numbers of both B and T lymphocytes in thymus and secondary lymphoid organs, comparable to wild-type, suggesting that RGS16 does not play a central role in lymphocyte development or homeostasis (Druey, unpublished data). This observation is consistent with the upregulated expression of RGS16 in activated lymphoid cells. We are currently studying the response of these mice to immunization with T-dependent antigens and to allergen induction of allergic pulmonary inflammation. Cellular infiltration in BALF and lung-tissue preparations are analyzed in OVA-sensitized and

challenged mice, and *in vivo* AHR is assessed by methacholine responsiveness. These studies will provide a greater understanding of how RGS16 affects migration and activation of T lymphocytes and the importance of these cells to the development of allergic airway inflammation.

Summary/Future Perspectives

RGS proteins appear to represent an important regulatory component of the intracellular signaling pathways induced by GPCRs. The role of RGS GAP activity in controlling GCPR signaling is reflected by the hyperresponsiveness of cells deficient in one or more RGS proteins [48]. A drug that selectively inhibits RGS4 activity has shown initial promise *in vitro*. Such compounds could be more specific than receptor-targeted drugs or even potentiate current therapies, since RGS activity may increase GPCR agonist potency up to 10-fold [49]. In the broadest sense, an understanding of how RGS expression or activity levels modify the development or course of asthma may reveal the importance of a given GPCR in disease pathogenesis. In addition, such studies may one day allow modulation of G-protein signaling downstream of the receptor, creating drugs with better efficacy or fewer side effects.

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Chapter 30
Mast Cell Precursors and Signaling Pathways

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30.1 Introduction

Mast cells are primary effector cells in allergic inflammation, where they cause symptoms related to mediators produced and released after mast cell activation. Understanding mast cell growth and differentiation is critical for deciphering the pathophysiology of allergic inflammation and developing new therapies [1]. Mastocytosis is a condition characterized by a pathologic accumulation of mast cells in tissues. It offers a unique opportunity to study the growth and differentiation of mast cells and their contribution to various pathologic processes. The molecular pathways regulating the proliferation and survival of mast cells are strikingly similar between normal mast cells and mast cells involved in mastocytosis. KIT, a transmembrane receptor for stem cell factor (SCF) is critical for normal mast cell growth and differentiation, and yet activating mutations in *c-kit* are often implicated in the etiology of mastocytosis [2].

The research of the Mast Cell Biology Section (MCBS), Laboratory of Allergic Diseases (LAD), has provided a better understanding of growth, differentiation and lineage relationships of mast cells and has characterized systemic mastocytosis as myeloproliferative, a disorder of hematopoietic progenitor cells. As a major referral center for mastocytosis, researchers in the MCBS have discovered novel surrogate markers of disease activity in mastocytosis, identified novel mutations and polymorphisms influencing the course of the disease, and evaluated new mast cell targeting therapies. We also contributed to a consensus document on the classification and diagnostic criteria for mastocytosis that was adopted by the World Health Organization (WHO) [3]. In addition to the important discoveries of the etiology, pathogenesis and treatment of mast cell disorders, and the molecular and cellular mechanisms of mast cell growth, differentiation, activation and survival, the MCBS has contributed important research methodologies and tools for mast cell research.

30.2 Mast Cell Ontogeny and Development

30.2.1 Development of human mast cells from CD13⁺/CD34⁺/CD117⁺ pluripotent progenitor cells

Murine mast cells are derived from a pluripotent bone marrow stem cell. In humans, the corresponding pluripotent cell is included in the $CD34^+$ bone marrow population. It was once thought that human mast cell progenitors existed in the bone marrow, arose from a common stem cell, and matured in tissue depending on the presence of cytokines and other factors in the physiological setting. To determine whether human mast cells arise from pluripotent $CD34^+$ human-progenitor cells found in human bone marrow, bone marrow cells were grown under different culture conditions. Mast cells and basophils continued to appear in cultures when T cell, B cell, macrophage, and eosinophil-committed progenitor cells were removed, but were not seen in cultures from which $CD34^+$ cells were removed, suggesting that they did not arise from committed T cell, B cell, eosinophil or macrophage progenitors [4]. Purified $CD34^+$ progenitor cells cultured in the presence of IL-3 gave rise to large numbers of basophils and smaller numbers of tryptase-positive mast cells [4]. After three to six weeks in culture, mast cells exhibited morphologies of mature human mast cells found in tissue [4] (Fig. 30.1a). Overall, these results were consistent with the concept that committed mast cell progenitor cells arise in bone marrow from $CD34^+$ pluripotent progenitor cells and mature in tissue.

Based on these results, we conducted studies to determine the effects of IL-3 and SCF on the appearance of mast cells and basophils from $CD34^+$ progenitor cells. Purified $CD34^+$ cells, cultured with IL-3 and SCF, proliferated and differentiated into mast cells and basophils, along with macrophages, eosinophils and neutrophils. The presence of SCF with IL-3 potentiated the growth of mast cells and basophils, compared to growth in the presence of IL-3 alone.

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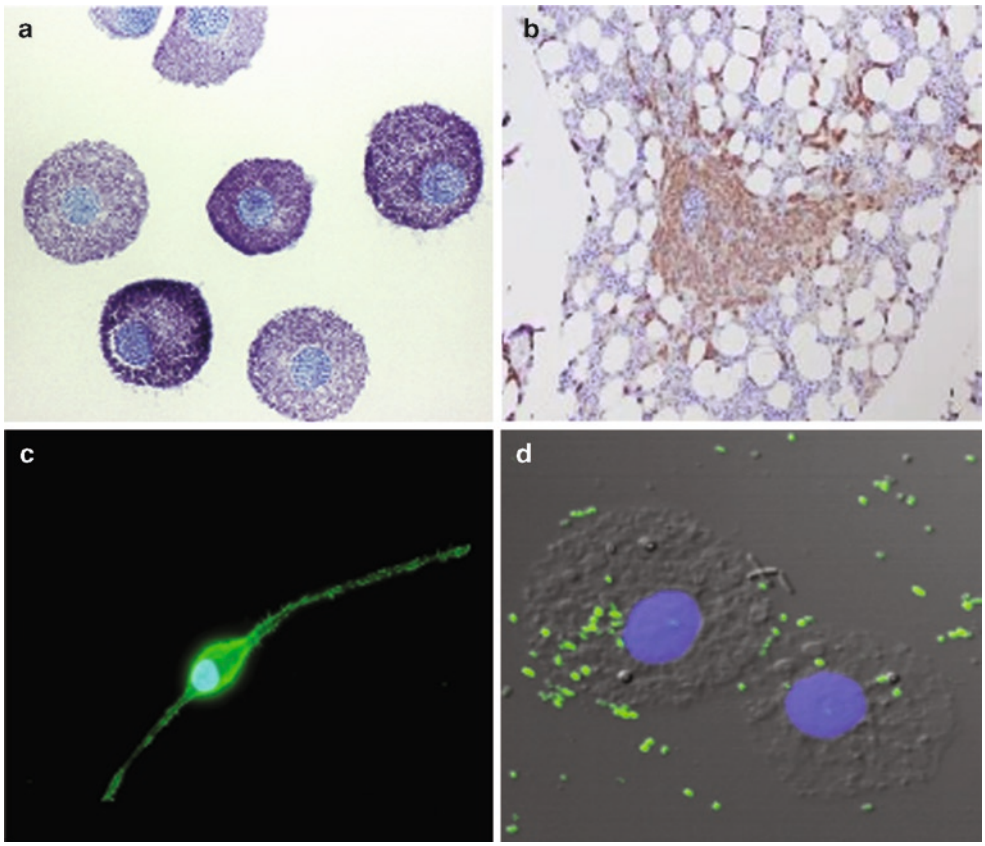

Fig. 30.1 Images of mast cells. (**a**) Human CD34⁺-derived mast cells stained with toluidine blue. (**b**) Tryptase-stained mast cell aggregrate in the bone marrow of a mastocytosis patient. (**c**) Human mast cell stained with phalloidin for F-actin following treatment with serotonin. (**d**) Phagocytosed fluorescent *Escherichia coli* particles in mast cells

This demonstrates that SCF is a human mast cell growth factor that promotes the differentiation and maturation of mast cell progenitor cells from CD34⁺ pluripotent progenitor cells [5].

Having shown that mast cells arise from CD34⁺ pluripotent progenitor cells from bone marrow, we attempted to identify the mast cell progenitor in human peripheral blood by sorting CD34⁺ cells and culturing in SCF and IL-3. As circulating CD34⁺ cells in peripheral blood, these cells have KIT and FcγRII, but not FcεRI (CD34⁺/KIT⁺/FcεRI⁻). In culture, they rapidly become CD34⁻ and by six weeks are largely mast cells with KIT and FcεRI, and granules which contain histamine and tryptase [6]. As shown with human bone marrow CD34⁺ cells, the presence of SCF and IL-3 in culture produces substantial numbers of mast cells. After six weeks in culture, other cell types are largely absent [6]. In this study, we also noted that CD34⁺ cells from patients with aggressive mastocytosis produced more mast cells than normal subjects or patients with indolent mastocytosis [6]. This study offered evidence that mast cells from normal subjects, as well as mastocytosis patients, arise from CD34⁺/KIT⁺ (CD117⁺)/FcεRI⁻ peripheral blood cells. Another outcome of this study was the development of a reliable method for the growth of human mast cells from bone marrow or peripheral blood, an important tool for mast cell research [7].

In subsequent studies, we characterized the CD34⁺ progenitor population that gives rise to mast cells by sorting using FACS analysis for CD34, KIT, and CD13, a cell surface aminopeptidase known as a myeloid marker expressed at several stages of differentiation. CD34⁺/KIT⁺/CD13⁺ progenitor cells, cultured in SCF alone and in combination with selected growth factors, resulted in a preferential expansion of mast cells, as well as a smaller number of monocytes, depending on the growth factors present in the culture media [8]. No other cell types were evident, regardless of the culture conditions and growth factors added [8]. These results suggest a lineage relationship of mast cells and monocytes, consistent with clinical data from some mastocytosis patients. Taken together, the results of this study identify CD34⁺/KIT⁺/CD13⁺ progenitor cells as containing the precursors for human mast cells and a subset of monocytes.

To explore further the cell lineage of mast cells, we employed a biologic signature based on the D816V *c-kit* progenitor. In an earlier study, we screened several mastocytosis patients and control subjects for a point mutation in *c-kit* transcripts expressed in peripheral blood mast cells, and

identified the D816V mutation in patients with mastocytosis with an associated hematologic disorder [9]. Based on the presence of the *c-kit* mutation, screening for the D816V mutation in myelomonocytic cells, B cells and T cells from peripheral blood, and bone marrow from mastocytosis patients, would provide a trackable genetic marker to assess hematopoietic lineage relationships. In one study, mRNA samples showed the mutation in the peripheral blood and bone marrow, lineage-committed cells with differentiation markers for lymphoid and myeloid cells, although flow cytometric analysis showed no surface expression of KIT, and therefore, no likely biologic consequence from the activating mutation [10]. These results suggested that the mutation in *c-kit* occurs in a pluripotential hematopoietic progenitor cell, but the exact lineage relationships and the frequency of cells derived from the progenitors required additional analysis. It was not clear whether the mutation occurred in a hematopoietic progenitor cell committed solely to the mast cell lineage. We examined the occurrence of the D816V *c-kit* mutation in genomic DNA of individually sorted peripheral blood T cells, B cells and monocytes in patients with indolent systemic mastocytosis. Single-cell mutational analysis enabled us to study the lineage relationship and extent of expansion of cells derived from the mutated clone, and to provide clear determination of the hematopoietic progenitor cell involvement. The D816V mutation was detected in varying frequencies in individual B cells, monocytes and bone marrow mast cells in patients with extensive disease [11]. In B cells, the immunoglobulin repertoire was polyclonal, indicating the mutation occurred before V(H)/(D)/J(H) recombination [11]. These results proved for the first time that mastocytosis is a disorder of pluripotential hematopoietic progenitor cells that give rise to B cells and monocytes, in addition to mast cells, and underscored the similarity of mastocytosis and other myeloproliferative disorders. In a subsequent study, using the laser-capture microdissection technique, we confirmed that the D816V mutation was detected in clusters of mast cells, B cells and T cells in the lesional areas of bone marrow in mastocytosis patients [12] (Fig. 30.1b). In addition, we found the B cells were oligoclonal, suggesting that clonal expansion is unlikely to be the cause of the clusters, and that preferential chemotaxis is possible, enhanced by the *c-kit* D816V mutation [13].

We also used the D816V mutation as a biologic signature to determine the lineage relationship of mast cells and basophils. Because of the similar morphologic and functional properties of mast cells and basophils, it was thought that they might share a bilineage (basophil/mast cell)-restricted progenitor. Peripheral blood and bone marrow aspirates were collected from mastocytosis patients, and blood basophils, monocytes and neutrophils were sorted. Analysis by restriction, fragment-length polymorphism of the genomic DNA and mRNA from sorted cells detected the D816V mutation in the basophils of five patients, who also had the *c-kit* mutation in monocytes, demonstrating multilineage involvement [14]. Single-cell analysis of the genomic DNA of one patient showed a similar degree of clonal expansion in basophils, monocytes, and neutrophils [14]. Mutated *c-kit* was expressed at the mRNA level in all five patients [14]. There was no difference in surface KIT-expression levels in basophils, and basophils carrying the D816V *c-kit* mutation in mastocytosis were detected only in the context of a multilineage involvement [14]. These results suggested that there is not a bilineage-restricted committed progenitor for mast cells and basophils.

30.2.2 Activating Mutations In KIT and Allergic Diseases

Molecular studies first reported from this laboratory have shown that more than 90% of adult patients with mastocytosis have the same acquired activating mutation (D816V) in the receptor tyrosine kinase, KIT. We found that the D816V mutation of *c-kit* is detectable in multiple hematopoietic lineages in patients with mastocytosis [9]. We next sought to identify the functional consequences of the presence of the mutation. Because SCF is a chemoattractant for KIT^+ cells, we considered that the D816V mutation may enhance chemotaxis of cells bearing the mutation. Constructs encoding wild-type KIT or KIT with the D816V mutation were transfected into Jurkat cells, and migration to SCF was assessed in the presence or absence of tyrosine kinase inhibitors. The chemotaxis to SCF was enhanced in D816V transfectants, compared to wild-type KIT transfectants [13]. Tyrosine kinase inhibitors inhibited the migration of both transfectants, but the D816V transfectants were more sensitive to inhibition [13]. $CD34^+/KIT^+$ circulating mast cell precursors obtained from mastocytosis patients showed preferential migration of mast cells with the D816V mutation [13]. These results demonstrated that the D816V KIT mutation enhances chemotaxis of $CD117^+$ cells, consistent with the increased number of mast cells observed in tissues of mastocytosis patients.

Anaphylaxis is a severe systemic reaction caused by release of mediators from mast cells and basophils. Although the common causes of anaphylaxis include food, drugs and stinging insects, a significant proportion of patients with recurrent anaphylactic episodes have no identifiable causative factor. Because mastocytosis is considered in the differential diagnosis of idiopathic anaphylaxis, we examined bone marrow mast cells from referred patients with a history of recurrent unexplained anaphylaxis who lacked the characteristic skin lesions of mastocytosis and had an apparently normal bone marrow histopathology. One-third of the patients had aberrant mast cells detectable in bone marrow, which displayed several diagnostic features of mastocytosis,

including abnormal morphology, surface expression of CD2 and CD25, and the *c-kit* D816V mutation [15]. These studies suggested that KIT mutations may be an underlying pathology in a significant number of patients with idiopathic anaphylaxis.

30.2.3 LAD2 Cell Line

A major limitation of the study of mast cells was the lack of an appropriate, long-term human mast cell line that exhibited normal tissue mast cell function. For many years, the only cell culture available to researchers which bore any resemblance to human mast cells was "human mast cell leukemia cells," designated HMC-1. The HMC-1 mast cell line was limited by several important deficiencies. HMC-1 cells did not require growth factor and exhibited inconsistent degranulation, in response to IgE stimulation [16].

During routine study of cells from bone marrow aspirates from a patient with mast cell sarcoma/leukemia in SCF-containing serum-free media, we observed cultures of mast cells with functional FcεRI and FcγRI receptors that continued to proliferate [16]. One of these unique cell cultures, now designated LAD2, has been separately maintained in culture, frozen and recovered successfully, and characterized at the cellular and molecular levels. LAD2 cells are the first reported human mast cell line that closely resemble primary cultures of $CD34^+$-derived human mast cells, respond to rhSCF, and have functional FcεRI and FcγRI receptors [16]. LAD2 cells have the ultrastructural features of human mast cells, and express FcεRI, CD4, 9, 13, 14, 22, 31, 32, 45, 64, 71, 103, 117, 132, CXCR4 (CD184), CCR5 (CD195); and intracytoplasmic histamine, tryptase and chymase. LAD2 cells release β-hexosaminidase, following FcεRI or FcγRI aggregation [16]. LAD2 cells survive in culture without SCF, and double in approximately three weeks in the presence of 100 ng/ml SCF [16]. The slower doubling time reflecting cell proliferation and death, in contrast to three to five days for some tumorigenic cell cultures, may allow LAD2 cells to exhibit a more mature phenotype. The addition of rhIL-3, rhIL-5, or rhIL-6 did not influence LAD2 cell numbers, while rhIL-4 reduced cell numbers [16]. The presence of functional FcεRI receptors is an important feature of the LAD mast cell cultures, with IgE-mediated β-hexosaminidase release of approximately 40%. Naive LAD2 cells also express FcγRI (CD64), which is upregulated in the presence of IFN-γ. HMC-1.2 cells have the *c-kit* D816V activating mutation, which is known to cause SCF-independent phosphorylation of CD117, and may contribute to the proliferation of these cells. LAD2 cells do not exhibit a *c-kit* activating mutation at codon 816 [16]. The lack of a mutation at codon 816 in LAD2 may explain the requirement for SCF for proliferation of these cells.

The availability of this novel human mast cell line offers an unparalleled circumstance to examine the biology of human mast cells, and facilitate research in human mast cell survival and proliferation studies, and in mast cell activation through FcεRI and FcγRI. This cell line, one of the most widely disseminated research tools from NIAID, is used by researchers in more than 125 laboratories in universities and non-profit research organizations. NIAID has made the cell line widely available to the research community since 2001, resulting in more than 60 publications from laboratories worldwide. Transfer of the LAD2 cell line to other laboratories exemplifies NIAID's commitment to conducting and supporting basic and clinical research on immunologic diseases and promotes NIH's mission of advancing research to improve global public health. The Federal Laboratory Consortium for Technology Transfer recognized the technology, "Mast Cell Line for Research on Allergies and Inflammatory Diseases," as the winner of a 2009 Award for Excellence in Technology Transfer.

30.3 Mast Cell Activation

Mast cell activation has long been understood to arise from antigen-mediated aggregation of the high-affinity receptors for IgE (FcεRI) at the cell surface. FcεRI aggregation initiates an intracellular signal cascade (discussed in the last part of this review), resulting in degranulation and the release of preformed mediators, such as histamine and tryptase, activation of cyclooxygenases and lipooxygenases and the generation of eicosanoids, such as prostaglandin D_2 and leukotriene C_4, and gene expression and the synthesis and secretion of cytokines, such as TNF-α [1]. In more recent years, through studies conducted by the Mast Cell Biology Section, LAD, and other laboratories, it is evident that there are IgE-independent mechanisms for activation of mast cells [17]. In addition, other studies have shown that the physiological setting for mast cells influences development, growth, differentiation, migration and survival, and, consequently, proliferation and susceptibility to activation (Fig. 30.1c). It is well known that SCF kinase promotes mast cell proliferation and maturation. Our laboratory also established that SCF is a survival factor for peripheral blood-derived human mast cells grown in culture and that its absence results in significant apoptosis [5]. The importance of SCF to the survival of mast cells led us to speculate that the presence of other growth factors and cytokines in physiological proximity to mast cell progenitors as they migrate and mature may also affect the phenotype. Factors that regulate mast cell growth and differentiation would be especially important to understand with regard to pathogenesis of diseases, such as mastocytosis and anaphylaxis, and identify opportunities for potential therapies.

30.3.1 Expression and Function of FcγRI on Human Mast Cells Following IFN-γ Treatment

Studies using animal models had shown IgE-independent mechanisms for mast cell activation in allergic reactions, including IgG-mediated anaphylaxis in IgE-deficient mice. We hypothesized that human mast cells might express IgG receptors, particularly when influenced by Th1 and Th2 cytokines and growth factors potentially present in the physiological setting of inflammation or infection. We cultured human mast cells from peripheral blood $CD34^+$ progenitors and treated with IL-4, IL-5, IL-10, GM-CSF, IFN-γ or NGF. Exposure of human mast cells to IFN-γ produced an increase in FcγRI mRNA expression when measured by RT-PCR, whereas untreated mast cells, or those treated with the other cytokines or growth factors, showed only low levels. Analysis by flow cytometry showed a 20-fold increase in expression of FcγRI at the cell surface, with no changes in expression of other Ig receptors (FcγRII, FcγRIII and FcεRI) [18,19]. Although flow cytometry showed surface expression of FcγRII, receptor aggregation did not trigger degranulation of IFN-γ-treated mast cells [19]. IFN-γ-treated human mast cells produced functional FcγRI that triggered degranulation and cytokine mRNA expression following receptor aggregation after incubation with mouse F(ab')2 anti-FcγRI and goat anti-mouse F(ab')2 fragments [18]. When FcγRI and FcεRI-mediated activation of the IFN-γ-treated human mast cells were compared, histamine release and eicosanoid production were similar, but mRNA expression for several cytokines (TNF-α, IL-1β, IL-5, IL-6, IL-13, IL-1R antagonist, GM-CSF) was significantly increased for mast cell activation through FcγRI than through FcεRI [20]. These results were confirmed by sensitizing IFN-γ-treated human mast cells with human IgG isotypes, followed by anti-IgG to aggregate the bound receptors [21,22]. After determining differences in FcγRI- and FcεRI-mediated responses in IFN-γ-treated human mast cells, we investigated the signaling events downstream from receptor aggregation to determine if differences existed in activation of signaling intermediaries. Although the FcγRI-mediated pathway was determined to be largely similar to the FcεRI-mediated pathway, some differences were revealed. Downstream phosphorylation of tyrosine kinases (Src, Syk) was similar, although Syk phosphorylation was greater in the FcγRI-mediated pathway, and tyrosine kinase inhibitors blocked degranulation, and the Src inhibitor, PP2 was much more potent for the FcγRI-mediated pathway than for FcεRI. Phosphorylation of MAP kinases (p38, JNK, ERK1/2) was also similar, and MAP kinase inhibitors blocked cytokine production. We also observed that PI3K was essential for FcγRI-mediated degranulation, but that FcεRI signals through both a PI3K-dependent and –independent pathway [23]. Simultaneous activation of FcγRI and FcεRI in IFN-γ-treated human mast cells showed additive degranulation responses at submaximal levels, but diminished responses near maximal levels, suggesting common intracellular pathways or possible differences in regulatory mechanisms [22].

These studies demonstrated that human mast cells may be activated through FcγRI when IFN-γ is present, suggesting mast cells may be activated in either a Th1 or Th2 setting, depending on the profile of cytokines and growth factors present and the effects on receptor expression and responsiveness. The results offer another role for the mast cell in inflammation and infection, and another possible mechanism to explore for IgE-independent allergic reactions.

30.3.2 Activation of Mast Cells through Environmental and Occupational Exposure: Silica and γ-Radiation

Clinical observations suggested that mast cells play a role in the development of occupational silicosis: an increase in mast cells in the lungs after exposure to silica dust [24], and an increase in mast cells staining for basic fibroblast growth factor from silicotic nodules of patients' lungs [25]. Because there were no studies of the effects of silica on mast cells, we examined the direct effects of silica exposure on mast cell activation, signaling mediation by mast cell surface receptors, and inflammation in mouse models.

Our studies showed that exposure of mast cells to silica produces several pro-inflammatory cytokines, the immediate production of reactive oxygen species (ROS) and protease release [26]. There was little effect on mast cell degranulation [26]. ROS and TNF-α production were at least partially dependent on the activation of scavenger receptors expressed on mast cells, because bone marrow-derived mast cells from mice deficient in the class A scavenger receptors, SR-AI/II and MARCO (macrophage receptor with collagenous structure) showed lower production of ROS and TNF-α when exposed to silica [26]. Inhalation of silica caused pulmonary inflammation and collagen deposition in control mice, but little or none in mice that are deficient in mast cells, demonstrating that in this mouse model, mast cells play a role in development of silicosis [26].

Our results showed that mast cells are activated by exposure to silica and produce inflammatory mediators enhanced by the activation of scavenger receptors [26]. Mast cells, which appear to have a significant role in the recruitment of inflammatory cells, leading to the development of silicosis, may offer an important therapeutic target for the treatment of silicosis.

Because there are few studies on mast cell exposure to γ-radiation, and reports differ on the effects, we conducted

studies to determine how γ-radiation affects human and mouse mast cell activation and survival. Our results demonstrated that mast cells are highly resistant to the cytotoxic and apoptotic effects of γ-radiation, but not UV irradiation, compared to lymphocytes [27]. Although exposure to γ-radiation alone did not cause mast cell degranulation, IgE-mediated degranulation was transiently impaired, following exposure to γ-radiation, and recovered after several hours [27]. Irradiated mast cells also retained responses to TLR ligands. ROS were generated immediately following irradiation [27].

Overall, immune cells, such as lymphocytes, are damaged by exposure to radiation and undergo apoptosis. However in this study, we demonstrated that mast cells are resistant to cell death after exposure to γ-radiation, and that they retain innate and acquired immune functions [27]. When normal host responses are severely compromised due to radiation exposure, mast cells may provide an important component of innate immunity.

30.3.3 Activation of Mast Cells Through TLR3 and Following Bacterial Exposure

In addition to mediating allergic responses, mast cells participate in innate immunity responses to bacterial infection. The physiological position of mature mast cells at interfaces with the external environment suggests that mast cells are one of the important contributors to host defense mechanisms. Mast cells have been shown to express Toll-like receptors (TLR) [28] and to respond to bacterial products such as lipopolysaccharide (LPS) and peptidogylcan (PGN), generating cytokines [29]. Less was known about mast cell responses to viral infection.

To explore the range of mast cell responses in innate immunity, the Mast Cell Biology Section conducted a set of studies to investigate the role of mast cells in viral infection. We identified that mature mast cells in culture express all TLR except TLR 8 and 10 [30]. When exposed to viruses (inactivated influenza, type 1 reovirus and respiratory syncytial virus (RSV)) or treated with a mimic for viral double-stranded RNA (polyinosine-polycytidylic acid, polyI:C), cultured human mast cells responded by producing IFN-α [30]. This effect of dsRNA was mediated through TLR3, because treatment with TLR3 antibodies inhibited dsRNA-induced IFN-α production, and antigen or other TLR ligands such as LPS, PGN and flagellin did not stimulate IFN-α secretion [30]. When enzyme inhibitors were employed in these studies, p38, JNK and NF-κB were implicated at least partially in the signaling pathway to gene transcription for IFN-α [30]. This was the first report of mast cell expression of TLR3 and generation of IFN-α, suggesting that mast cells play a role in innate immunity responses to viral infection and mobilization of other immune cells.

Our studies with polyI:C treatment also showed that TLR3-mediated activation of mast cells did not induce or affect mast cell degranulation. We extended our studies to consider other effects of TLR3 activation on cultured human mast cell responses, particularly the influence on IgE-mediated degranulation potentiated by adhesion to extracellular matrix proteins. Mast cell adhesion to fibronectin (FN) resulted in potentiated IgE-mediated degranulation [31]. Treatment with the TLR3 ligand polyI:C inhibited mast cell adhesion in a dose-dependent manner and also abrogated the adhesion-potentiated IgE-mediated degranulation [31]. This effect was shown to depend on inhibition of mast cell adhesion rather than decrease in FcεRI or KIT expression [31]. Taken together, these studies demonstrate a role in innate immunity for mast cell responses to viral infection. Regulation by TLR3 appears to promote this role through IFN-α production and inhibition of IgE-mediated allergic inflammation, representing a shift to host defense.

The apparent shift in mast cell responses to host defense stimulated by viral infection led us to consider the effects of bacterial infection on mast cells. Mast cells are known to recognize and respond to bacterial infection by phagocytosis and cytokine production (Fig. 30.1d). We began an examination of mast cell mechanisms for responding to bacteria by conducting DNA microarray analysis to identify patterns of gene expression following exposure to *Escherichia coli (E. coli)*. Mast cells showed a global response that included a number of genes that are upregulated over a period of 24 hours, such as chemokines, adhesion molecules, and cell surface receptors, and altered expression of cytokines [32]. Among chemokines that were upregulated, three (CCL-1, CCL-18, CCL-19) were notable as reported recruiters of T cells, monocytes, macrophage progenitors, and immature dendritic cells [32]. There was also up-regulation of adhesion molecules, particularly carcinoembryonic antigen-related cell-adhesion molecules (CEACAM), which are associated with binding and phagocytosis of *E. coli* [32]. Upregulated cell surface receptors included receptors for IL-4 and IL-9, which stimulate mast cell proliferation and cytokine release, and for IL-10, which inhibits FcεRI expression [32]. As seen in our studies with mast cell responses to viral infection, exposure to bacterial infection appears to regulate mast cell activity toward host defense through a switch from Th2-type to Th1-type response.

Because of our finding that bacterial exposure profoundly influences the profiles of gene expression in mast cells, with an apparent shift in responses toward host defense, we conducted additional studies to determine short-term and long-term effects on mast cells. Using LPS and PGN, known ligands for TLR, we examined the effects of long-term (six weeks) exposure on human mast cell progenitors cultured in the presence of SCF. Our results showed that PGN,inhibited human mast cell growth, while LPS had no effect [33].

Mature (eight week old) human mast cells exposed to PGN for 72 hours (short-term) decreased in number, whereas LPS treatment increased numbers somewhat [33]. The effects of PGN and LPS were reversed at higher concentrations of SCF (100 ng/mL) [33]. In long-term cultures of mature human mast cells, LPS, but not PGN, treatment resulted in small increases in expression of KIT, tryptase and chymase but a decrease in FcεRI. In short-term cultures, LPS treatment also reduced FcεRI expression [33]. LPS treatment resulted in increased protease expression in mature human mast cells cultured in lower, but not higher concentrations of SCF, effects that correlated to low KIT expression [33]. We also examined cytokine expression in mature human mast cells with short-term exposure to LPS or PGN, and found that while PGN had no effect, LPS-treated cultures showed increased levels of IL-1β, IL-6, IL-8 and IL-12 [33]. These results suggest that LPS, arising from bacterial infection, may stimulate protease and cytokine expression and decrease FcεRI expression to reorient mast cell activity to participate more in innate immunity and less in allergic inflammation responses.

30.3.4 Reactive Oxygen Species (ROS) in Activated Mast Cells

During the immune response, neutrophils and macrophages participate in bacterial killing through phagocytosis and the production of high levels of ROS and reactive nitrogen oxide species (RNOS). Mast cells also contribute to innate immunity responses, but the role and extent of ROS and RNOS production by mast cells in this response was not well known. ROS and RNOS are also known to act as intracellular signaling intermediates [34]. Our laboratory conducted studies to examine the generation of ROS and RNOS by mast cells and the signaling pathways and effects on mast cell function.

In three different mast cell models, rat peritoneal (RPMC), mouse bone marrow-derived (BMMC), and human $CD34^+$ blood cell derived (HuMC), we found that activation of FcεRI by IgE/antigen produced degranulation and intracellular ROS, but not NO [35]. Levels of LPS and IFN-γ that stimulated NO production in macrophages were not able to induce NO formation in the three mast cell types [35]. Based on our initial studies, we conducted experiments to determine the enzymes responsible for ROS generation by BMMC and HuMC. Using enzyme inhibitors, we demonstrated that FcεRI-dependent ROS generation by mast cells was primarily dependent on 5-lipoxygenase (5-LO) with a smaller contribution from cyclooxygenase-1 (COX-1) activation [36]. This dependence on 5-LO and COX-1 was confirmed in BMMC from 5-LO and COX-1 deficient mice [36]. We also conducted similar studies with NADPH oxidase inhibitors and mice deficient in NADPH oxidase subunits and demonstrated that FcεRI-dependent ROS generation in BMMC was independent of NADPH oxidase activation [36]. Enzymatic inhibition of FcεRI-dependent ROS production through 5-LO and COX-1 did not affect degranulation (β-hexosaminidase) or cytokine release (TNF-α and IL-6), but did reduce eicosanoid production (prostaglandin D_2 and leukotriene C_4), as expected [36]. Furthermore, we showed that mast cells also generate ROS, following FcεRI-independent activation following incubation with IgG-coated latex beads, also through a 5-LO and COX-1-dependent and NADPH oxidase-independent mechanism [36]. In summary, our studies demonstrate that mast cells generate internal ROS through a mechanism exclusively involving 5-LO and COX-1. The role of ROS generation in mast cell function remains to be explored further.

30.4 Mast Cell Signaling

Once resident in their target tissues, mast cells can be activated to release a variety of inflammatory mediators, including histamine, eicosanoids, and cytokines, which contribute to anaphylaxis and the pathogenesis of the allergic reactions associated with asthma [1]. Although these events are generally thought to be initiated following antigen-dependent aggregation of the high-affinity receptors for IgE (FcεRI) on the surface of the mast cell [1], we demonstrated that, following IFN-γ treatment, high-affinity receptors for IgG (FcγRI) could also be expressed on human mast cells [18]. Binding of aggregated IgG_1 to the FcγRI on these cells induced the release of a similar suite of inflammatory mediators to those released upon FcεRI aggregation [19]. It is also now evident that, at least under experimental conditions, other receptors expressed on the surface of mast cells can contribute to such reactions [37,38]. These receptors include TLR3 [30], TLR2 and TLR4 [39]; G protein-coupled receptors (GPCRs) including those for sphingosine-1-phosphate [40], adenosine [41], PGE_2 [42], and the complement component C3a [22]; and KIT [43]. Ligands for these receptors can either induce the release of specific or multiple groups of mediators, by themselves, or can synergistically enhance antigen/IgE-induced mediator release. It should also be noted that some of these particular ligands, including sphingosine-1-phosphate [40], SCF [44], and PGE_2 [45] can also promote mast cell chemotaxis. (Refer to Fig. 30.2 for a diagram of mast cell signaling from these receptors.)

These observations and reports led to the following specific aims of the signaling program within the Mast Cell Biology Section: the identification of key players in the integration of the signal transduction pathways initiated by the FcεRI and other receptors for the synergistic enhancement of mast cell

responses; the examination of how polymorphisms or alternatively spliced variants of these receptors or signaling proteins may produce a hyperactive phenotype; the identification of disease states/clinical populations where hyper-responsive and hypo-responsive mast cells exist and identify signaling defects; and the exploration of potential approaches for inhibiting these responses. Initially, we focused our efforts on investigating signaling processes regulating FcεRI and FcγRI-mediated activation of human mast cells. These studies later evolved to encompass the exploration of integrated signaling pathways initiated by KIT and GPCRs in conjunction with those initiated by FcεRI in the context of the synergistic activation of human and rodent mast cells.

30.4.1 FcεRI-FcγRI

The signaling cascade initiated by the FcεRI has been extensively described in the literature and the readers are referred

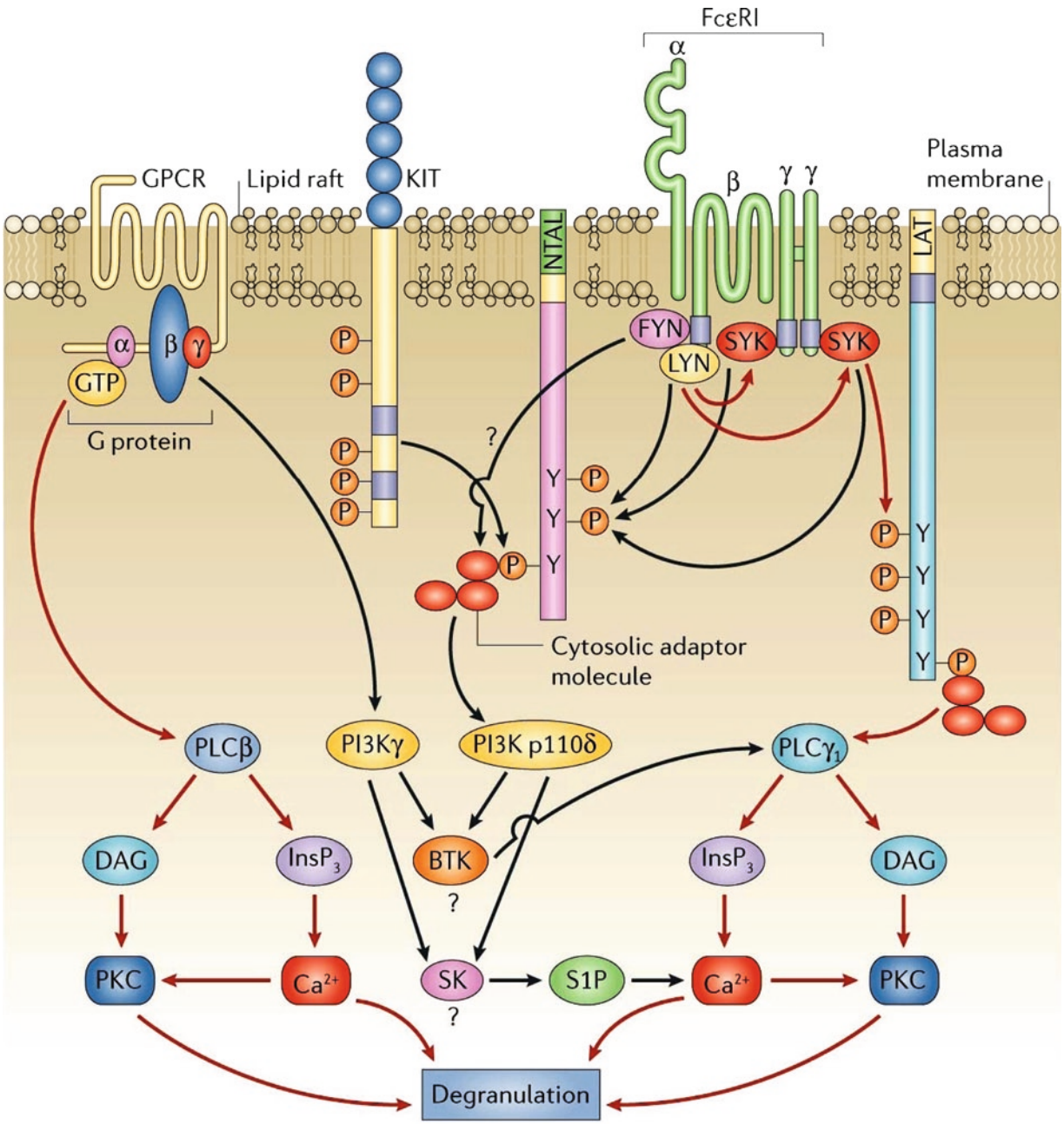

Fig. 30.2 Integration of the signaling pathways for FcγRI, KIT, and G protein-coupled receptors. Red lines represent principal signaling pathways arising from receptors. Black lines represent amplification pathways used by the receptors for mast cell degranulation [Figure originally published in *Nature Reviews Immunology* (37)]

to a number of reviews we have written on this topic for further details [37,46]. Although FcγRI possess the common FcRγ signaling subunit that is also part of the FcεRI, it was not clear whether the signaling pathways regulated by both receptors were similar. Indeed, the fact that there was significantly more TNF-α released following FcγRI aggregation, compared to following FcεRI aggregation, suggested differences may exist [19]. However, when we examined the signaling events elicited by both receptors, they were remarkably similar [23]. The only real difference that we could observe was that, whereas degranulation in response to FcγRI aggregation was completely dependent on phosphoinositide 3-kinase (PI3K) activation, degranulation in response to FcεRI aggregation was only partially dependent on PI3K. In further investigations conducted in human mast cells, we determined that the initial $PLC\gamma_1$–IP_3 dependent calcium signal required for mast cell activation, induced upon FcγRI aggregation, is independent of PI3K [47]. However, PI3K was observed to be required for maintenance of this signal. As discussed below, further studies led us to the conclusion that this PI3K-regulated response is part of an amplification signaling pathway that can be utilized by other receptors for the synergistic enhancement of mast cell activation.

30.4.2 Synergistic Activation of Mast Cells

The extent to which other receptors influence FcεRI-dependent, mast cell reactions *in vivo*, has yet to be determined. However, based on the obligatory role of SCF in mast cell survival [1], it is likely that FcεRI-mediated, mast cell activation may be significantly influenced by, at least, SCF-induced KIT activation in a physiological setting. We thus initiated a project to determine how the signaling cascades initiated by KIT and other receptors, in conjunction with the FcεRI, may influence mast cell development and function in normal and disease states.

Following the demonstration that SCF and antigen synergistically enhanced degranulation and cytokine production in human mast cells [48], we investigated the early point of integration of the signaling cascades induced by KIT and FcεRI. We observed that one of the most receptor-proximal events commonly initiated by KIT and the FcεRI in human mast cells was the tyrosine phosphorylation of a 25–30 kD protein which we identified as the transmembrane adaptor protein (TRAP) NTAL (LAB) now termed LAT2 [49]. Concurrent activation of both receptors resulted in enhanced phosphorylation of this protein. Interestingly, the closely related TRAP, LAT, which had been demonstrated to be phosphorylated following antigen challenge of BMMCs and which had also been demonstrated to be critical for antigen-mediated mast cell activation [50], was not phosphorylated following KIT activation. FcεRI-mediated degranulation, and the ability of KIT to enhance this response, was substantially reduced in human mast cells incubated with LAT2-targeted siRNA oligonucleotides [49] and in human mast cells where LAT2 had been stably knocked down using LAT2-targeted shRNA constructs [51]. Similar inhibition was also observed following inclubation of the cells with LAT-targeted siRNA oligonucleotides [48]. Based on these and other observations [49], we concluded that KIT requires LAT to be phosphorylated by FcεRI-dependent pathways to influence mast cell degranulation. However, once this occurs, the enhanced phosphorylation of LAT2 induced by both receptors regulates the synergistic activation of downstream signals, leading to the observed augmented degranulation.

The mechanisms by which FcεRI and KIT induced LAT2 phosphorylation and the implications of this response for downstream signaling were next examined. Following aggregation and phosphorylation, FcεRI and KIT were observed to migrate to lipid raft membrane fractions, and this was associated with LAT2 phosphorylation within these microdomains [51]. Whereas the FcεRI was observed to utilize the tyrosine kinases Lyn and Syk for LAT2 phosphorylation, KIT was determined to directly phosphorylate LAT2 [49,51]. It was further determined that these kinases phosphorylated distinctly different tyrosine residues on LAT2. In this respect, whereas Syk primarily phosporylated *N*-terminal binding sites for the cytolsolic adaptor molecule Grb2, Lyn and KIT primarily phosphorylated tyrosines outside of these motifs [51]. Furthermore, the sites phosphorylated by Syk were observed to bind indirectly to $PLC\gamma_{1,}$ which may provide a mechanism by which LAT2 helps regulate FcεRI-mediated degranulation [51]. The molecules binding to the sites phosphorylated by Lyn and KIT have, however, yet to be identified. Regardless, the data suggest that the differential phosphorylation of LAT2 by Lyn, Syk, and KIT may result in selective activation of specific downstream signaling molecules regulated by preferential recruitment of these molecules into the receptor-signaling complex.

As described, the D816V activating mutation in KIT has been associated with the mast cell hyperplasia associated with mastocytosis. As idiopathic anaphylaxis has been described to be associated with a subgroup of mastocytosis patients [15], as an extension to the above studies, we investigated whether the D816V KIT mutation may influence the aforementioned signaling events. Following transduction of 293T cells with LAT2 and KIT constructs containing the D816V point mutation, we observed a dramatic enhancement of both KIT and LAT2 phosphorylation above that observed with wild type KIT [52]. Furthermore, whereas wild type KIT selectively phosphorylated specific tyrosine residues of LAT2, D816V KIT induced global tyrosine phosphorylation of LAT2, implying that downstream signaling events would also be upregulated. Thus, these events may render the mast cells hypersensitive to antigen [52].

These, and the other aforementioned studies on the enhancement of antigen-mediated mast cell degranulation by SCF, led to the hypothesis that concurrent inhibition of KIT- and FcεRI-mediated signaling may represent a novel opportunity for targeting mast cell driven disorders [53,54]. Having these desired properties, we utilized the mixed acting tyrosine kinase inhibitor hypothemycin to explore this concept in both cell culture and *in vivo* [55]. Indeed hypothemycin was observed to not only block antigen-mediated degranulation and cytokine production *in vitro* and *in vivo* but also the ability of SCF to enhance this response when examined in both human and mouse mast cell in culture. These data provided support for the concept of a dual inhibitory strategy for targeting mast cells in specific disease states.

30.4.3 PI3K and Mast Cell Activation

As discussed above, we previously determined that PI3K regulates the maintenance of the calcium signal required for mast cell degranulation. We also observed that PI3K, particularly the P110δ isoform, was a key regulator of these downstream signaling events required for the SCF-mediated amplification of antigen-dependent mast cell activation [56]. This research also revealed that the p110δ isoform of PI3K was also critical for multiple mast cell responses, including cytokine generation, chemotaxis, adhesion, and growth and survival [56]. As the downstream targets of PI3K for the generation of these responses were relatively unknown, studies were initiated to identify these targets.

30.5 Btk

The Tec family tyrosine kinase, Btk, had been previously documented to be downstream of PI3K in various hematopoietic cells, including mast cells. In addition to confirming that Btk was phosphorylated, thus activated following FcεRI aggregation, we observed that Btk was phosphorylated in response to SCF [57]. The possibility that Btk, downstream of PI3K may be a link in the amplification pathway connecting LAT2 and PI3K to downstream events critical for the ability of KIT to potentiate FcεRI-induced mediator release was investigated using mast cells derived from the bone marrow of $Btk^{-/-}$ mice [57]. Indeed, we observed that the ability of SCF to enhance antigen-mediated degranulation was lost in the $Btk^{-/-}$ BMMCs. Furthermore, we observed that the synergistic enhancement of $PLC\gamma_1$ in response to antigen and SCF, which we had previously observed [48], was absent in the $Btk^{-/-}$ BMMCs. Hence, it appears that Btk may represent a critical signaling molecule linking PI3K to $PLC\gamma_1$, thus the required calcium signal, for the amplification of mast cell activation.

From these and other observations, we developed a model for dual pathways regulating mast cell activation in which we proposed that a principal pathway, regulated by LAT, and leading to the initial activation of $PLC\gamma_{1,}$ provides an initial rapid-calcium signal required to promote mast cell activation, and that PI3K and Btk support an amplification pathway for the enhancement and maintenance of the $PLC\gamma_1$-dependent calcium signal, leading to augmented degranulation. This latter pathway, which appears to be coordinated by LAT2, allows KIT to potentiate of antigen-mediated degranulation [46].

30.6 mTOR

Although our studies further revealed that the PI3K-dependent activation of Btk is also required for optimal eicosanoid production and generation of ROS in antigen-stimulated mast cells [58], we deduced that Btk could not account for all of the responses regulated by PI3K in mast cells. We thus explored the possible involvement of the mTORC1 cascade, another major signaling element downstream of PI3K, in these responses. SCF and antigen challenge of human and mouse mast cells was observed to induce the PI3K-dependent phosphorylation of the mTORC1 cascade components, tuberin, mTOR, p70S6 kinase, and 4E-BP1 [59]. The mTORC1 inhibitor, rapamycin, was observed partially to inhibit SCF-mediated chemotaxis, cytokine production and cell survival, suggesting that these responses were, at least in part regulated by mTORC1. However, mTORC1 appeared to have little role in antigen-induced or SCF-enhanced degranulation. Interestingly, the mTORC1 cascade was observed to be constitutively activated in human cancer mast cells, suggesting a possible role in mast cell tumorigenesis. This conclusion was supported by the ability of rapamycin to block the proliferation of the cells' lines.

30.6.1 G Protein-Coupled Receptors

We next examined whether the ability of GPCRs to enhance antigen-mediated mast cell degranulation was similarly regulated by a PI3K-dependent pathway. Although a number of such receptors has been previously demonstrated effectively to enhance antigen-induced mast cell activation, our results suggested that, of those examined, prostaglandin E_2 (PGE_2) had the most robust response, in terms of degranulation and cytokine production [60]. Therefore, we utilized PGE_2 to examine the integrated signaling mechanisms accounting for

these responses. From studies conducted in mouse BMMCs, we deduced that the PGE_2 receptor responsible for the enhancement was linked to G_i thus was likely to be the EP3 PGE_2 receptor [60]. Surprisingly, although PI3K was required for optimal antigen-mediated degranulation, it was dispensable for the ability of PGE_2 to enhance this response. This latter response, however, was determined to be a consequence of greater influx of calcium via store-operated calcium channels as mediated by a coordinated and synergistically enhanced activation of PLCβ and PLC$\gamma_{1,}$ leading to enhanced levels of IP_3.

Taken together, our studies suggest that multiple alternative mechanisms drive the integrated signaling cascades that mediate the synergistic responses observed upon co-activation of specific receptors in mast cells. We are now examining whether such synergy occurs in other mast cell responses such as growth, chemotaxis and cell survival. We are also examining whether activating mutations/polymorphisms in critical players of these pathways may play a role in specific mast cell related disorders. These studies may provide information, which may aid in the design of novel therapies for the treatment of mast cell related disorders.

30.7 Conclusions

In this chapter, we have described some of the key research conducted in the Mast Cell Biology Section over the last two decades. The endeavors of the clinical and research staff, postdoctoral and clinical fellows, and collaborators at NIH and other institutions have generated significant findings in the areas of mast cell development, growth, activation and signaling, which have advanced the field of allergy and immunology research. The group has also made valuable contributions to the research methods and tools that continue to be used by researchers at NIH, as well as across the world. Through clinical research and experience in treatment of patients at the NIH Clinical Center, investigators from the Mast Cell Biology Section have contributed expertise to the development of national and international standards and diagnostic criteria for allergic diseases. Future efforts of the laboratory and clinical programs will be directed to define the genetic basis of mast cell disorders; explore the signaling pathways that regulate the threshold and extent of mast cell activation; continue to translate the results of laboratory research to clinical application, and seek novel therapeutic approaches to treatment of allergic diseases.

Acknowledgements Due to space constraints, not all pertinent literature could be cited. This does not imply that studies not cited are of lesser merit. Research conducted in the authors' laboratory is supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Part X
Clinical Medicine: Infectious Diseases

Chapter 31
Prion Biochemistry and Therapeutics

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31.1 Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are caused by infectious agents that are unusually hard to decontaminate and can resist more heat and radiation than other pathogens. These characteristics led to proposals, during the 1960s, that they might lack nucleic-acid genomes. The first recognized TSE disease was scrapie, a widespread infection of sheep that can persist in pastures for years after removal of infected animals. Similarities between scrapie and human kuru, which was spread by cannibalistic mortuary feasts amongst the Fore tribe in Papua New Guinea, contributed to widespread interest in these fatal neurodegenerative diseases. Since then, the impact of TSEs has been greatly amplified by the recognition of more common human forms, such as Creutzfelt-Jakob disease (CJD), and the epidemics of bovine spongiform encephalopathy (BSE) and cervid chronic-wasting disease (CWD).

Prion diseases are now defined by the essential involvement of abnormal forms of prion protein (PrP) in transmission, susceptibility and pathogenesis (reviewed in [1]). The normal form of PrP, PrP^{sen}, is usually a glycoprotein that is monomeric, protease-sensitive, and linked to cellular membranes via a glycophosphatidyl-inositol (GPI) anchor. In prion-infected cells, mature PrP^{sen} is converted to a pathological protease-resistant form, PrP^{res}, after its arrival at the cell surface, yet before it is digested by lysosomal proteases [2,3]. PrP^{res} is somewhat variable and poorly defined structurally, but most commonly is multimeric [1,4,5] and higher in β-sheet content [6] than PrP^{sen} [7]. Despite the usual association of PrP^{res} with TSE infectivity (prions), the full molecular nature of the TSE agent remains unclear. This is largely due to difficulties in purifying infectivity to biochemical homogeneity, so that its essential components and structures can be determined unequivocally.

Our long-term interests have been to understand the molecular basis of prion-disease transmission and pathogenesis. Along the way we also have attempted to identify better therapeutic and diagnostic tools for coping with these diseases. In this article we summarize highlights of our efforts over the last decade or so.

31.2 PrP Conversion

Our early efforts to understand the molecular basis of TSE diseases revealed that PrP^{res} can cause PrP^{sen} to convert into a protease-resistant state like PrP^{res} itself [8]. This conversion occurs via what appears to be a selectively seeded or templated polymerization mechanism [8–12] that can be stimulated by sulfated glycans [13] and the attachment of PrP^{sen} and PrP^{res} to contiguous membranes [14–16]. Although PrP^{sen} contains a disulfide bond, this bond appears not to be broken or rearranged during conversion [17,18].

This *in vitro* conversion reaction occurred with strain- and species–specificity [19,20], giving the first indication that PrP-res had at least limited self-propagating activity that could account for fundamental aspects of prion-disease biology. The ability of different strain-associated conformers of PrP^{res} to impose their specific conformation attributes on newly converted PrP^{sen} suggested that prion strains could propagate via the faithful seeding of conformationally distinct PrP^{res} oligomers of the same amino-acid sequence [20].

Many more recent studies are consistent with this hypothesis. The species and sequence specificity of the basic cell-free conversion reaction similarly provided a likely molecular basis for the profound barriers that are often encountered when attempting to transmit prions from one species to another. We performed various cross-species cell-free conversion reactions to gain initial insights into the relative transmissibilties of BSE, CWD and sheep scrapie to other species, including humans [21–23].

Although our original cell-free PrP conversion reactions reflected important attributes of prion replication, the yield of protease-resistant PrP from these initial cell-free conversion reactions was limited, and, the newly made PrP-res was

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not proven to be infectious (Ref[2] and our own unpublished results). Thus, we could not conclude that PrP conformational conversion was all that was required for prion replication.

31.2.1 PMCA, rPrP-PMCA and QuIC Conversion Reactions

More recent advances by other laboratories led to the development of much more continuous and productive prion-seeded PrP conversion reactions—specifically the protein misfolding cyclic amplification (PMCA) reactions [24–26]. One striking feature of these PMCA reactions is their ability to propagate prion infectivity, largely disproving the need for a protein-encoding, nucleic-acid genome for prions. However, these experiments have required brain-derived PrP^{sen} and polyanionic cofactors, at a minimum, so the PrP-only nature of prion infectivity remains to be established. Another feature of PMCA reactions is their unprecedented sensitivity in detecting PrP^{res} (~1 ag) [27]. However, the existing PMCA reactions were far too impractical and time-consuming for routine prion detection in medicine and agriculture. In fact, two of the most daunting challenges that remain in handling prion diseases are the lack of practical antemortem diagnostic tests and sensitive assays for prion contamination of at-risk materials, such as blood products; transplanted tissues; agricultural products; biotechnology products; pharmaceuticals; medical devices, and environmental samples.

In attempts to identify more precisely the essential molecular constituents and structure of the prion-infectious unit and improve the practicality of the PMCA assays for prions, we developed a reaction called rPrP-PMCA, in which PrP^{res} seeds conversion of bacterially expressed recombinant PrP^{C} ($rPrP^{C}$) to specific protease-resistant fibrillar forms ($rPrP^{res}$). Although not quite as sensitive as the conventional PMCA, rPrP-PMCA is much faster at detecting PrP^{res} at levels ≥ ~50 ag. PMCA uses intermittent sonication to break up PrP aggregates. To overcome difficulties controlling sonication, we developed another $rPrP^{C}$–based assay, called QuIC, for quaking-induced conversion, which uses automated tube shaking rather than sonication [28]. Within one day, the QuIC assay can detect sub-femptogram amounts of PrP^{res} (less than one lethal dose, intracerebrally) in hamster-brain homogenates and can discriminate scrapie-infected and normal hamsters using 2-μl samples of cerebral spinal fluid (CSF).

Recently, we have adapted the QuIC assay to species of greater medical and economic importance: humans and sheep [64]. These assays allow the detection of femtogram levels of PrP^{res} in brain homogenates from vCJD-infected humans and scrapie-infected sheep. Our findings demonstrate that QuIC assays can amplify sub-infectious levels of PrP^{res} in both human and sheep brain tissues to easily detectable levels, giving the assays potential applicability in the screening of biological samples (i.e. blood, lymphoid tissues, and nasal fluids) [65].

Importantly, prion-seeded products of the rPrP-PMCA and QuIC reactions are infectious when inoculated into rodents [66]. Moreover, the ability to derive specific prion-seeded conversion products from bacterially expressed recombinant PrP^{sen} that is easily mutated and labeled should facilitate biophysical studies of the 3D structure of these prion-like PrP assemblies. Indeed, one of the greatest hurdles in understanding PrP^{res} is the lack of biophysical techniques suitable for determining the high-resolution structures of ultrastructurally diverse and often fibrillar protein assemblies. *In silico* model building based on low-resolution electron crystallography, molecular dynamics simulations and docking procedures have suggested a couple of different molecular models for prion-fibril assembly [29–32]. We have collaborated with Valerie Daggett's group to compare these models, with respect to various types of low-resolution conformational analysis, and have concluded that DeMarco and Daggett's spiral model is most consistent with the available data so far [31].

31.2.2 Strain-Dependent Ultrastructures of PrP^{res} Fibrils

The preparations of PrP^{res} that are of the highest purity have most often contained amyloid-like fibrils, which have been characterized in terms of fibril dimensions [33–35] and secondary structure composition [7,36–38]. Visualization of the protein core of wild-type prion fibrils can be obscured by heavy glycosylation and GPI anchors. However, we have recently isolated plaques and fibrils containing mostly unglycosylated and anchorless PrP^{res} from scrapie-infected transgenic mice expressing only anchorless PrP^{C} (Fig. 31.1) [35,39].

These fibrils contained protofilaments 3.0–3.5 nm in width, as measured by negative-stain, transmission-electron microscopy (Fig. 31.2). The protofilament widths and twist periodicities varied significantly with scrapie strain, indicating strain-dependent fibril ultrastructures. These ultrastructural variations expand the list of strain-dependent features of $PrP^{res,}$ which includes secondary structures [37,38]; proteolytic sensitivities [40]; glycoform patterns [41–44]; stabilities [45] and conformational templating activities [20].

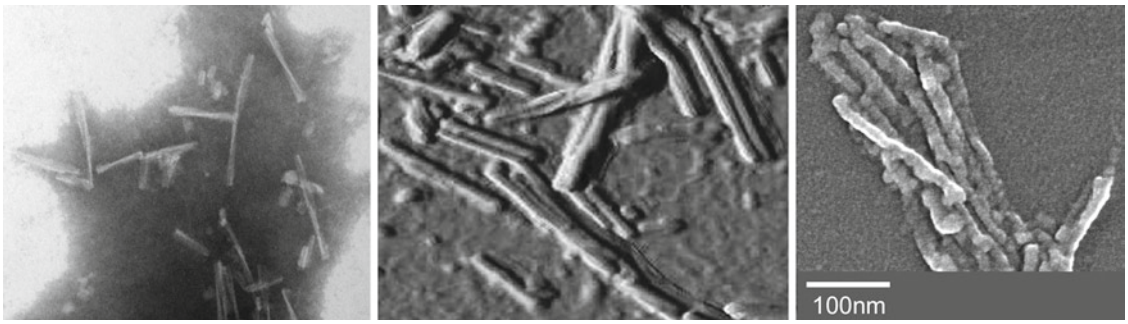

Fig. 31.1 Scrapie amyloid fibrils isolated from 22L scrapie-infected transgenic mice expressing only GPI-anchorless prion protein [as described in [35]]. From left to right: negative stained transmission electron micrograph, atomic force micrograph, scanning electron micrograph

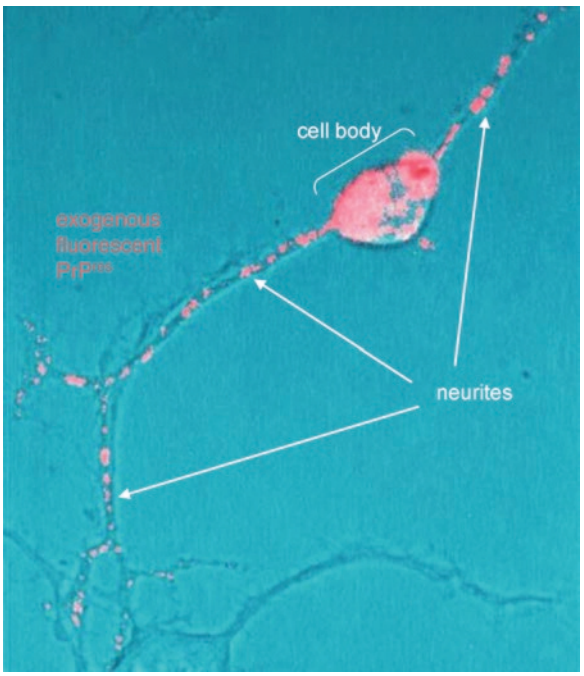

Fig. 31.2 Uptake and neuritic transport of exogenously added, fluorescently labeled scrapie PrP^{res} fibrils (pink; false-colored) by neurons in primary culture. The PrP^{res} initially contacted the cell body in the form of a single large aggregate. Over days, this aggregate was dissembled into smaller portions concurrent with endocytosis and transport via acidic intracellular vesicles along neuritic projections to points of contact with other cells as described in [63]

31.2.3 The Most Infectious Prion Protein Particles

Although PrP^{res} amyloid fibrils can occasionally be a prominent neuropathological feature in prion diseases, they may not be the form of PrP that is most relevant to TSE pathogenesis. Our assessments of the relative infectivity of PrP^{res} aggregates of various sizes indicated that the most infectious particle per unit protein is ~300–600 kDa, the mass equivalent of 14–24 PrP monomers, and the smallest oligomer with cell-free PrP^{sen} converting activity is larger than a PrP pentamer [46]. Light-scattering and electron-microscopic data indicated that these particles (a.k.a. "J-particles") are non-fibillar. Our results suggest that, as long as the infectious particles are above a certain critical size, it is the concentration of particles rather than the concentration of PrP^{res} molecules *per se* that correlates best with infectivity titer. Still, the multimeric nature and heterogeneity of infectious PrP oligomers, as well as uncertainties about the role of other potential prion constituents, greatly complicates more detailed analyses of prion structures by conventional techniques of structural biology.

31.2.4 Cellular Uptake and Neuritic Transport of PrP^{res}

One of the critical, but poorly understood, steps in the pathogenesis of prion diseases is the invasion of the nervous system and neuronal spread of infection. To characterize mechanisms for the uptake and intraneuronal trafficking of PrP^{res}, fluorescently labeled PrP^{res} was used to infect a neuronally derived cell line (SN56), and adult cortical neurons in primary culture. Coincident with the establishment of persistent scrapie infection, SN56 cells internalized PrP^{res} aggregates into vesicles positive for markers for late endosomes and/or lysosomes, but not synaptic, early endocytic or raft-derived vesicles. Internalized PrP^{res} was then transported along neurites to contacts with other cells. Similar trafficking was observed with Alzheimer's Aβ1–42 fibrils and non-infectious recombinant PrP fibrils, suggesting that PrP^{res} is internalized by a relatively non-specific pinocytosis or transcytosis mechanism. Primary cultured neurons were also capable of internalizing and disseminating exogenous PrP^{res}. Similar trafficking of exogenous PrP^{res} by cortical neurons cultured from the brains of PrP knockout mice showed that uptake and neuritic transport did not require the presence of endogenous cellular PrP. These experiments visualized and characterized the initial steps associated with prion infection and transport within neuronal cells.

31.2.5 The Search for Anti-Prion Compounds

No validated treatments exist for TSEs in humans or livestock. In the pursuit of anti-TSE drugs, we have focused primarily on blocking conversion of PrP^{sen} to PrP^{res}. Since identifying the first PrP^{res} inhibitor, Congo red, in 1992 [47], we have used scrapie-infected cell cultures to identify numerous classes of PrP^{res} inhibitors [for review, see [48]], some of which have strong *in vivo* anti-TSE activities, such as sulfated glycans [49], porphyrins, phthalocyanines [50,51] and phosphorthioated oligonucleotides [52]. A combined therapy with a sulfated glycan and porphyrin proved to have at least additive beneficial effects in prolonging the lives of scrapie-infected rodents, when administered directly to the brain [53]. The development of new prion-infected cell lines [14,54] and relatively high-throughput screens has hastened the identification of new PrP^{res} inhibitors [55,56] that may have therapeutic value. Our mechanistic studies have indicated that several different classes of TSE inhibitors share structural similarities, compete for the same site(s) on PrP^{C} in the amino-terminal flexible domain, and induce the clustering and internalization of PrP^{C} from the cell surface [48,57,58]. NMR studies indicate that such interactions of inhibitors with PrP can induce structure in the otherwise flexible amino-terminal third of the molecule [67]. Moreover, similar interactions with related, natural ligands such as hemin [59], sulfated glycosaminoglycans [60,62] and nucleic acids [52], might play a role in the as-yet enigmatic physiological function(s) of PrP^{sen} [1,58][1, 58].

In summary, to learn how better to cope with prion diseases or transmissible spongiform encephalopathies (TSEs), we have pursued biochemical, biophysical, and cell-biological studies of prion protein and its conversion to pathological forms. These studies have helped to characterize the fundamental infectious particles of these diseases (prions) and the mechanisms by which they invade cells, propagate themselves, and spread within the nervous system. We have developed a variety of cell-free, prion-protein conversion reactions that serve both as tools for probing the molecular pathogenesis of prion diseases, and rapid ultra-sensitive assays for prion detection. We have identified many inhibitors of prion-protein conversion, as part of efforts to develop effective anti-TSE drugs.

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Chapter 32
Neutrophils in the Resolution of Infection

Frank R. DeLeo

32.1 Introduction

The long-term objective of research in the Pathogen Host-Cell Biology Section (PHCBS) is to promote development of enhanced diagnostics, better prophylactic agents, and new treatments for emerging infectious pathogens, such as community-associated, methicillin-resistant *S. aureus* (CA-MRSA). To achieve that objective, the PHCBS (i) conducts a systematic molecular dissection of steps involved in the pathogen-host interaction, with specific emphasis on the interaction of bacterial pathogens with human neutrophils; (ii) investigates mechanisms mediating evasion of innate immunity by pathogens of special interest, such as *Staphylococcus aureus*; (iii) identifies new virulence genes involved in the pathogenesis of infections caused by pathogens of special interest, and (iv) utilizes appropriate *ex vivo* assays, animal models (including knock-out mice), and—if possible—human specimens, to test hypotheses developed from *in vitro* analyses. Major areas of research include neutrophil biology and function, neutrophil–bacteria interactions, with special emphasis on the interaction of methicillin-resistant *S. aureus* (MRSA) and human neutrophils, and *Staphylococcus aureus* virulence mechanisms. This article reviews progress made in the PHCBS toward a comprehensive understanding of the role of neutrophils in the resolution of the inflammatory response.

32.2 Neutrophils (PMNs) in the Innate Immune Response

Human polymorphonuclear leukocytes (PMNs or neutrophils) are essential to the innate-immune response against invading microorganisms. In contrast to the acquired-immune response, which requires time to develop and is dependent on previous interaction with specific pathogens, the ability of PMNs to kill infectious microorganisms is immediate, non-specific, and not dependent on previous pathogen exposure [1,2]. Inasmuch as PMNs produce toxic microbicidal components (Fig. 32.1) and are the predominant immune cell in most bacterial infections, moderation of infection-induced inflammation is critical for limiting host-tissue destruction. Studies published over the past several years suggest PMN apoptosis facilitates resolution of bacterial infections [3–6], an idea supported by the finding that some pathogens alter neutrophil apoptosis to survive [3,4,7–9]. However, few studies have focused on regulation of bacteria-induced apoptosis in PMNs, and relatively little is known about transcription networks that contribute to resolution of infection. An enhanced understanding of molecular signaling pathways induced in PMNs during phagocytosis of pathogens is critical for improving treatment and outcome of infectious diseases.

Although most bacteria are killed readily by PMNs, many pathogens have evolved mechanisms to circumvent destruction by neutrophils and thereby cause human infections. For example, strains of CA-MRSA cause disease in otherwise healthy individuals [10,11]. Although the molecular basis for the disease is not known, there is strong association between CA-MRSA infections and strains carrying *Panton-Valentine leukocidin (PVL)* [12], a toxin known to lyse neutrophils *in vitro*. *Streptococcus pyogenes* (Group A *Streptococcus*, GAS) successfully evades killing by PMNs to cause human infections, such as pharyngitis and necrotizing fasciitis (flesh-eating syndrome) [13]. Several extracellular molecules contribute to the ability of GAS to resist phagocytosis [14]. We hypothesize that the ability of *S. aureus* and GAS to cause disease is due to a combination of specific pathogen-derived factors and host susceptibility. Therefore, a better understanding of the pathogen-PMN interface, at both the cell and molecular levels, will provide information critical to our understanding, treatment, and control of diseases caused by these Gram-positive bacteria.

32.2.1 Global Changes in PMN Gene Expression During Phagocytosis

We performed studies to examine gene expression in PMNs, based on the hypotheses that (i) distinct PMN receptors

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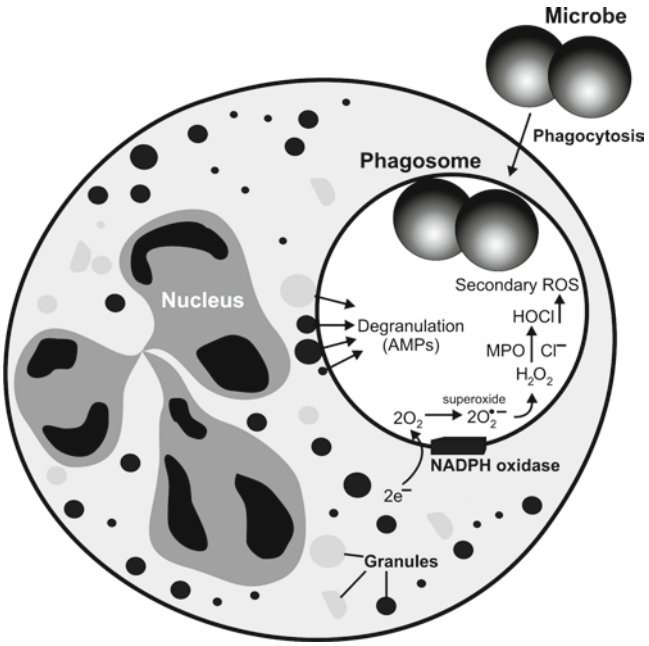

Fig. 32.1 PMN phagocytosis and activation. Phagocytosis activates PMNs to produce superoxide and secondarily derived ROS, including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Granules fuse with the phagosome, thereby enriching the lumen of the phagocytic vacuole with antimicrobial proteins and peptides (AMPs). MPO, myeloperoxidase; 2e, 2 electrons from cytosolic NADPH

would elicit unique cellular responses; (ii) receptor-specific responses are mediated at the level of gene expression, and (iii) gene expression in human PMNs mediates key innate immune and inflammatory responses**.**

To investigate phagocytosis mediated by antibody receptors (FcRs) and complement receptors (CRs), we used latex beads opsonized with antibody, serum complement, and antibody and serum complement to monitor receptor-mediated phagocytosis by human PMNs. We identified key differences in phagocytosis and in the production of reactive oxygen species (ROS) between FcR- and CR-activated PMNs [15]. Thus, our findings indicate events mediating activation of PMNs by FcRs or CRs proceed through distinct intracellular signal transduction pathways, consistent with previous studies in monocytes [16].

We used oligonucleotide microarrays to analyze global gene expression patterns in human PMNs during receptor-mediated phagocytosis [15]. Within six hours after phagocytosis, we identified differentially regulated, transcripts-encoding proteins involved in distinct apoptosis pathways [15]. Phagocytosis-induced apoptosis coincided precisely with the time at which specific apoptosis-related genes were differentially transcribed during phagocytosis. Actinomycin D and cyclohexamide, inhibitors of transcription and translation, respectively, blocked phagocytosis-induced apoptosis. These data demonstrate that PMN apoptosis is regulated, in part, at the level of gene transcription, a finding that has important implications for our understanding of host defense and the pathogenesis of inflammatory diseases.

32.2.2 PMNs Regulate Detoxification Pathways and Energy Metabolism During Apoptosis

In addition to predicting cell fate accuarately, gene-expression profiling identified complex biological pathways that accompany activation-induced apoptosis in human PMNs [17]. We used several biochemical approaches to confirm that these pathways, which included the glutathione system and γ-glutamyl cycle, were upregulated during the initial stages of neutrophil apoptosis [17]. The extent to which PMNs underwent apoptosis was reduced in the absence of glucose [17], indicating that glycolysis is required for activation-induced phagocytosis in human PMNs. These data suggest that neutrophil gene transcription facilitates energy metabolism and responses to oxidants during the induction of apoptosis.

32.2.3 Down-Regulation of Pro-Inflammatory Capacity Accompanies PMN Apoptosis

Using the FcR- and CR-phagocytosis model described above, we discovered that genes encoding pro-inflammatory receptors, signal-transduction mediators, and adhesion molecules were downregulated three to six hours after receptor-mediated phagocytosis in neutrophils [18,19]. There was a concomitant decrease in surface-protein expression for key PMN pro-inflammatory molecules, such as CXCR2, a high affinity receptor for interleukin-8 [18]. Decreased surface expression of pro-inflammatory molecules correlated with increased staining for annexin-V, following phagocytosis and FAS-ligation, confirming the receptors are downregulated during PMN apoptosis. Taken together, these data suggest that key components of PMN pro-inflammatory and bactericidal capacities are downregulated during the early stages of apoptosis.

32.2.4 Apoptosis In Activated PMNs From XCGD Patients

Leukocytes from patients with X-linked, chronic-granulomatous disease (XCGD) are defective in their ability to produce

ROS, due to abnormal or absent gp91*phox* [20]. Although the absence of ROS logically explains the increase in frequency and severity of pyogenic infections in patients with XCGD, the molecular basis for formation of granulomas in these individuals is unknown. Therefore, we studied gene expression in PMNs from XCGD patients to test the hypothesis that altered neutrophil apoptosis and the resultant unresolved inflammation contribute to formation of granulomas in XCGD patients.

To identify constitutive differences in gene expression that underlie chronic inflammation in XCGD patients, we first compared transcript levels in unstimulated PMNs from XCGD patients and healthy control individuals [21]. Our results suggest that pro-inflammatory capacity in XCGD patients is upregulated, either due to the absence of gp91*phox*- or ROS-dependent regulatory mechanisms, and/or the long-term influence of recurrent infection.

We next compared changes in gene expression in activated PMNs (those stimulated by receptor-mediated phagocytosis) from healthy control individuals and XCGD patients. Consistent with the data from unstimulated XCGD patient cells, genes encoding molecules that mediate host defense or the inflammatory response were upregulated significantly in activated neutrophils from XCGD patients. The observation that these transcripts are upregulated supports the idea that they potentiate chronic inflammation in individuals with XCGD. Notably, the gene encoding Bcl2-associated X-protein (BAX) was significantly upregulated in normal PMNs, but not in cells from patients with XCGD. These findings were confirmed at the protein level. Compared with cells from healthy control donors, apoptosis in PMNs from XCGD patients was reduced significantly following phagocytosis. Furthermore, there was no significant difference in apoptosis between unstimulated and stimulated PMNs from XCGD patients. These findings indicate that ROS produced during phagocytosis by normal PMNs accelerate apoptosis. Taken together, the data provide support to the idea that (i) ROS play a key role, either directly or indirectly, in regulating BAX in human PMNs, and (ii) altered gene expression in PMNs from XCGD patients underlies defective apoptosis.

32.2.5 Spontaneous Neutrophil Apoptosis

Neutrophils undergo constitutive (spontaneous) apoptosis as a mechanism to facilitate normal cell turnover and immune-system homeostasis. On the other hand, several proinflammatory molecules, including granulocyte-macrophage, colony-stimulating factor (GM-CSF), prolong neutrophil survival. The molecular mechanisms that regulate PMN apoptosis or survival remain incompletely defined. To test the hypothesis that normal turnover of PMNs or prolonged survival of cells exposed to GM-CSF is regulated in part at the level of transcription, we compared global-gene expression in human neutrophils during spontaneous apoptosis with that in cells cultured with human GM-CSF [22]. Genes encoding proteins that inhibit apoptosis, such as serum/glucocorticoid-regulated kinase (SGK), were downregulated coincident with neutrophil apoptosis. In contrast, those encoding inhibitors of apoptosis were upregulated in PMNs cultured with GM-CSF. Correspondingly, GM-CSF delayed PMN apoptosis, increased cell viability and prolonged neutrophil phagocytic capacity. Prolonged functional capacity was paralleled by upregulation of proinflammatory genes and proteins, including CD14, CD24, CD66, and HLA-DR. In addition, expression of SGK protein diminished during PMN apoptosis, but was restored by culture with GM-CSF, suggesting SGK is involved in leukocyte survival [22]. These studies provide a global view of the molecular events that regulate neutrophil survival and apoptosis.

32.2.6 Conclusions—Section B

Based on our discovery that cell fate is regulated at the level of gene expression [15,17,18,21,22], we propose that global changes in gene expression following phagocytosis comprise an apoptosis-differentiation program in human PMNs, a final stage of transcription-regulated PMN maturation or hematopoietic differentiation (reviewed in refs. 1,23]. Taken together, our studies indicate that the apoptosis-differentiation program regulates multiple processes in human neutrophils, including programmed cell death, pro-inflammatory activity, and responses to oxidants. Importantly, PMN-derived oxidants contribute to the resolution of inflammation in humans by facilitating the normal apoptosis-differentiation program. We propose that these processes are critical for the resolution of bacterial infections in humans.

32.3 Bacterial Pathogens and PMN Apoptosis

32.3.1 Bacterial Pathogens Alter the PMN Transcriptome

Our goal with the initial studies of gene expression in human PMNs [15,17,18,21] was to gain broad insight into cellular processes accompanying PMN phagocytosis. Having established that transcription and gene regulation are critical for modulating post-phagocytosis sequelae in neutrophils, we next examined global gene expression in human PMNs

following phagocytosis of bacterial pathogens [4]. Based on our preliminary studies of receptor-mediated phagocytosis in PMNs, we hypothesized that (i) PMNs differentially express a common set of genes during phagocytosis of bacterial pathogens, and (ii) induction or repression of a number of gene-regulated pathways involved in the innate immune and/or inflammatory responses is pathogen-specific.

The ability of PMNs to kill a diverse array of bacterial pathogens is essential for human innate-host defense. Therefore, we investigated phagocytosis of *Borrelia hermsii*; *Listeria monocytogenes;*, *Burkholderia cepacia*; *S. aureus*, and GAS by human neutrophils. Compared with the other human pathogens, GAS was significantly more resistant to phagocytosis and killing by PMNs [4,24]. We used oligonucleotide microarrays to analyze global changes in the PMN transcriptome following ingestion of these pathogens. Consistent with the data from the studies of receptor-mediated phagocytosis, our findings suggested that phagocytosis of bacterial pathogens induces an apoptosis-differentiation program in human PMNs.

There were few pathogen-specific changes in neutrophil-gene expression following phagocytosis of *Borrelia hermsii*; *L. monocytogenes*; *Burkholderia cepacia*, and *S. aureus* (strain COL), within the time-frame of the experiments (six hours). By comparison, phagocytosis of GAS resulted in differential expression of 393 genes that remained unchanged in PMNs stimulated with other bacteria [4]. Notably, phagocytic interaction of GAS with PMNs altered the expression of 71 cell fate-related genes, and the vast majority of GAS-specific changes in neutrophil-gene expression was mediated by live, rather than heat-killed bacteria [4]. These results indicate phagocytosis of GAS alters the normal apoptosis-differentiation program elicited by all other bacterial pathogens [4]. In accordance with the microarray data, phagocytosis of each of the pathogens accelerated PMN apoptosis. Unexpectedly, GAS induced rapid apoptosis/cell death in neutrophils, which was significantly greater in magnitude than that elicited by the other pathogens. The finding that heat-killed GAS had limited capacity for inducing PMN apoptosis is consistent with the idea that live GAS produces factors that alter apoptosis in human PMNs. Moreover, live GAS induced significant PMN lysis not observed with phagocytosis of heat-killed organisms, and necrosis correlated well with the accelerated apoptosis [4].

32.3.2 Anaplasma Phagocytophilum

Anaplasma phagocytophilum, the agent of human granulocytic anaplasmosis, survives within PMNs in part by altering normal host-cell processes, such as production of ROS and apoptosis. Thus, we hypothesized that an enhanced understanding of the molecular mechanisms used by *A. phagocytophilum* to alter neutrophil function would provide fundamental insight into pathogen survival mechanisms and general mechanisms of PMN apoptosis. To investigate the molecular basis of these phenomena, we used oligonucleotide microarrays to measure global changes in human PMN gene expression following infection with *A. phagocytophilum* [25]. Ingestion of *A. phagocytophilum* failed to trigger production of ROS and the neutrophil apoptosis differentiation program that typically follows phagocytosis. In addition, *A. phagocytophilum* blocked spontaneous PMN apoptosis and cell death induced by anti-Fas antibody (extrinsic pathway) [25]. The findings indicate that two separate anti-apoptotic processes work concomitantly to promote bacterial survival, (i) uptake of *A. phagocytophilum* fails to trigger the apoptosis-differentiation program typically induced by bacteria, and (ii) a protein or molecule on the pathogen surface can mediate an early delay in spontaneous neutrophil apoptosis.

32.3.3 Conclusions—Section C

We discovered that bacterial pathogens induce an apoptosis-differentiation program in PMNs that is essential for resolution of infection (reviewed in refs. 1,23]. Phagocytosis of bacteria, production of ROS, and killing of microorganisms were followed by global changes in PMN gene expression common to a broad range of bacterial pathogens. Neutrophil genes encoding pro-apoptosis factors were upregulated and genes encoding anti-apoptosis proteins were downregulated following phagocytosis of all pathogens. Consistent with this observation, phagocytosis of bacteria induced PMN apoptosis, which was accompanied by downregulation of pro-inflammatory molecules. Notably, some bacterial pathogens altered the apoptosis program in human neutrophils to survive. By integrating the findings from our genome-wide analyses with human neutrophil-function assays, animal infection models, and studies from other groups, we propose a model of host cell-pathogen interaction that provides fundamental insight into the resolution of infection (Fig. 32.2) [3].

Based upon our studies, there are two potential outcomes for the interaction of neutrophils with bacteria. On one hand, ingestion and killing of bacteria by neutrophils is followed by apoptosis, whereby infection can be resolved. This pathway predominates for most bacteria-neutrophil interactions and is healthy for the host. Alternatively, pathogens are ingested but not killed and neutrophils are destroyed rapidly or have delayed turnover that promotes

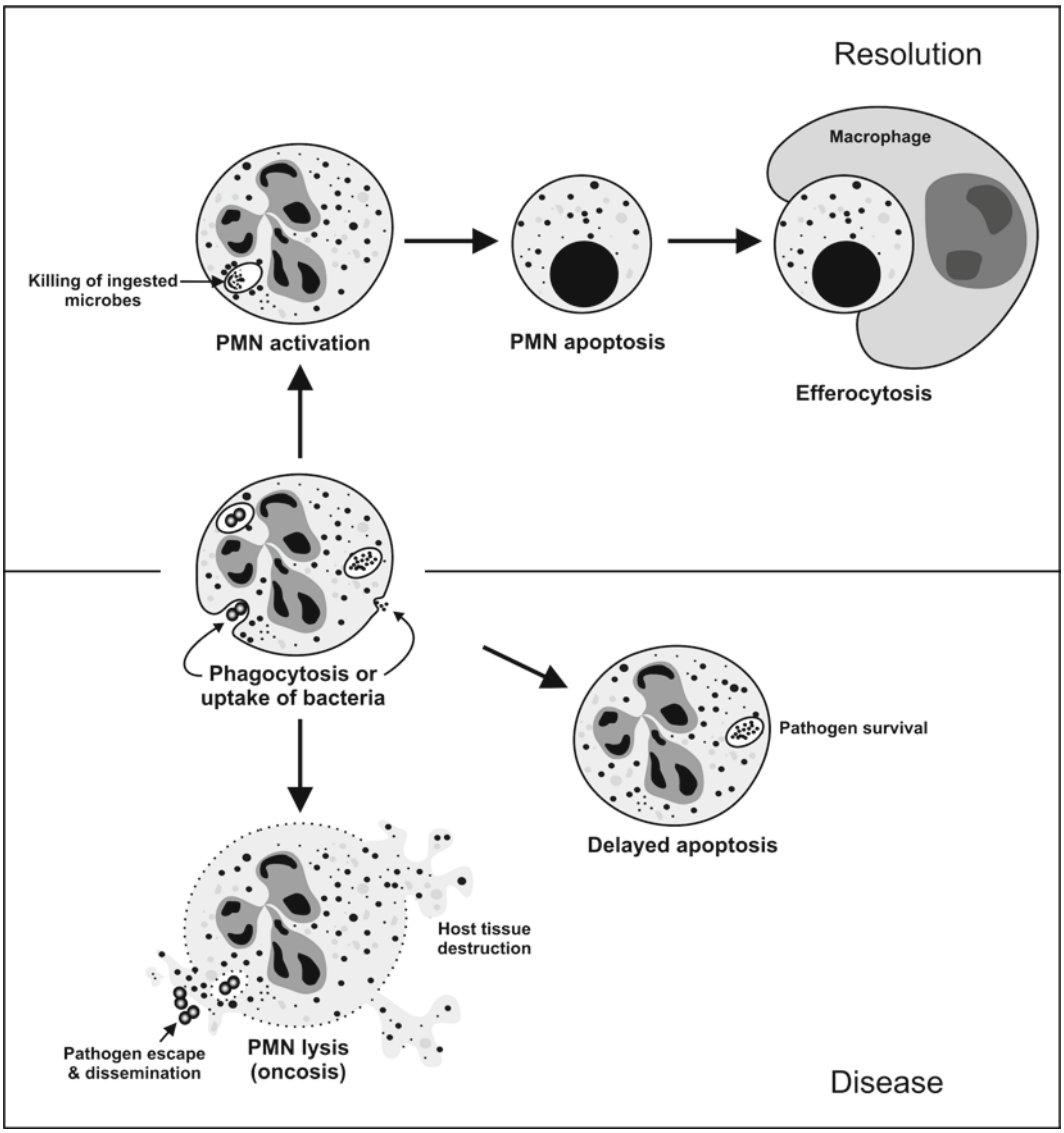

Fig. 32.2 A paradigm for the resolution of infection. There are two potential outcomes for the interaction of bacteria with human neutrophils. See text for details

pathogen replication (Fig. 32.2). These latter outcomes are counterproductive for the resolution of infection and may ultimately result in disease.

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Chapter 33
Reactive Oxidant-Dependent Innate Immune Defenses of the Airway Epithelium: The Dual Oxidase-Lactoperoxidase-Thiocyanate System

Thomas L. Leto and Balázs Rada

Abbreviations

ALI	air-liquid interface
ASL	airway surface layer
CaCC	calcium-activated chloride channel
CGD	chronic granulomatous disease
CF	cystic fibrosis
CFTR	cystic fibrosis trans-membrane conductance regulator
DPI	diphenylene iodonium
Duox	Dual oxidase
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide
LPO	lactoperoxidase
MPO	myeloperoxidase
O_2^-	superoxide free radical
$OSCN^-$	hypothiocyanite ion
SCN^-	thiocyanate ion
SOD	superoxide dismutase

33.1 Introduction

Neutrophils and other circulating phagocytes generate substantial amounts of reactive oxygen species (ROS) in response to infectious or inflammatory stimuli, in a process known as the respiratory burst [1]. The response is attributed to the activity of NADPH oxidase, an enzyme that uses NADPH and oxygen to produce superoxide, a precursor of other potent microbicidal ROS. Patients with chronic granulomatous disease (CGD) suffer from NADPH oxidase deficiencies, resulting in enhanced susceptibility to microbial infections and aberrant inflammatory responses [2] (see the article of Zarember and colleagues for a detailed discussion of CGD). Our program has explored the cellular mechanisms regulating the respiratory burst oxidase in phagocytes, and is characterizing related oxidant-generating NADPH oxidases expressed in non-phagocytic cells, now known as the "Nox family" of NADPH oxidases [3–5]. Genes for seven oxidase isozymes are now recognized in man: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2, where Nox2 is the catalytic core component of the prototypical phagocytic enzyme formerly designated as $gp91^{phox}$[3,4]. The non-phagocytic oxidases exhibit diverse, tissue-specific expression patterns, which have been related to a variety of functions requiring deliberate reactive oxidant production, including cell-proliferation responses; vascular regulation; oxygen sensing, and biosynthetic processes such as thyroid hormone synthesis and extracellular-matrix protein cross-linking [5]. Low-level ROS production by these enzymes can provide intracellular redox signals that affect gene-expression patterns during differentiation, oxygen sensing, or responses to infection, growth factors, cytokines, or hormones. Based on the effects of spontaneous or targeted Nox gene mutations in man and mouse, ROS generation by several of these novel oxidases has already been recognized as essential for a variety of functions. Nox1 defects cause blunted vascular hypertensive responses to angiotensin II [6,7]; the Nox3 system has roles in the formation of otoconial structures of the inner ear, as defects in this system affect balance and gravity perception [8–10], and mutations in Duox2 or its maturation factor (DuoxA2) cause hypothyroidism [11–13].

A particular emphasis of our program is focused on potential roles of these novel oxidases in host defense and inflammatory processes. The evidence supporting such functions has been summarized in recent reviews [3,14,15]: (i) several oxidases are induced by inflammatory signals; (ii) several are responsive to host-cell recognition of microbial factors; (iii) the highest expression of several of oxidases is on epithelial (mucosal) surfaces, and in several cases these ROS generators are aimed outward from the apical cell surface, and (iv) the Duox isozymes appear to function as hydrogen peroxide (H_2O_2) sources in support of lactoperoxidase, a well known antimicrobial enzyme in exocrine secretions. Duox expressed in the gut of *Drosophila melanogaster* was shown to be critical in responding to and controlling microbial infection [16], and several plant NADPH oxidases are known to serve roles in disease resistance [7]. Thus, it has become

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clear that the Nox family oxidases have ancient origins, and that a deliberate oxidative response against infection by pathogens is a primitive innate defensive function common to both plant and animal kingdoms.

33.2 Structure and Function of Nox and Duox Isozymes as ROS Generators

The importance of ROS generation in phagocyte microbial killing has been recognized for decades, as is evident from the enhanced susceptibility to microbial infections seen in CGD, but only within the last decade have we appreciated that the phagocytic NADPH oxidase is but one member of an entire family of seven related Nox oxidases in man. These proteins exhibit a range from 26–54% identical sequence, showing their highest homology within sequences considered critical for NADPH, FAD, and heme binding in the Nox2 prototype [3,4]. Fig. 33.1 depicts structural models of Nox and Duox isozymes, comparing common and unique structural features relevant to their functions as ROS generators. Nox1 through Nox4 are similar in size, containing six helical membrane-spanning segments encompassing the heme domain, followed by a *C*-terminal, cytoplasmic flavodomain. Nox5 is similar but has additional *N*-terminal sequence encoding four Ca-binding EF-hands. The Duox enzymes also have two EF hands, another transmembrane segment, and an extended amino-terminal ectodomain (~ 600 amino acids), showing significant homology with several hemoperoxidases. Hence, they were designated as "dual oxidases" for having both Nox and peroxidase related structures [18]. The responsiveness of Nox5 and Duox activities to increases in intracellular calcium levels is attributed to direct affects of calcium binding to the EF hands [19]. Duox1 and Duox2 activities are also stimulated by protein kinase A and protein kinase C, respectively [20]. Nox4 functions as a constitutively active enzyme, whereas Nox1, Nox2 and Nox3 are subject to control by cytosolic regulators, including Rac, Nox activators (Noxa1 or p67^{phox}) and Nox organizers (Noxo1 or p47^{phox}) [21,22].

Most information on the catalytic mechanism for superoxide formation by Nox enzymes, involving the donation of a single electron to molecular oxygen, derives from studies of the phagocytic (Nox2) system, which are reviewed in detail elsewhere [23]. Topologically speaking, all the oxidases function as trans-membrane electron carriers that accept electrons from cytosolic NADPH and eventually donate them to oxygen in an extracytoplasmic compartment, such as the phagosome or the extracellular medium. Two electrons derived from each NADPH molecule are transferred stepwise from FAD in the reductase domain, to two heme moieties bound within the membrane, and then to oxygen,

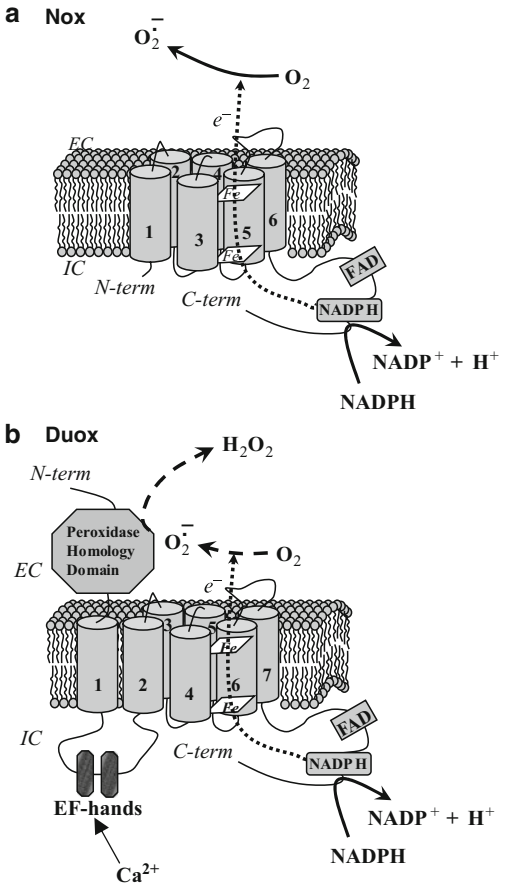

Fig. 33.1 Generation of reactive oxygen species by Nox- and Duox-based NADPH oxidases. Nox family NADPH oxidases function as electrogenic enzymes that transport electrons from a cytosolic source (NADPH), ultimately donating them to molecular oxygen in some extracytosolic compartment. The Nox enzymes produce superoxide as the primary product. However, Duox isozymes appear to produce H_2O_2 directly and no detectable superoxide, suggesting that the "peroxidase-like" ectodomains function in H_2O_2 generation (from superoxide) rather than H_2O_2 utilization. Several Duox isozymes accumulate in the plasma membrane and function in partnership with other extracellular peroxidases

thereby producing two molecules of superoxide from one NADPH molecule. Thus, all Nox family oxidases are electrogenic enzymes, passing electrons through the membrane as they generate protons in the cytoplasm from NADPH utilization:

$$NADPH \rightarrow NADP^+ + H^+ + 2e^-$$

All the Nox-type isozymes appear to produce superoxide as the primary product, although in the case of Nox4, H_2O_2 is the principle product released by most reconstituted cell models [24]. This can be explained by findings showing that Nox4 accumulates within intracellular sites, where superoxide can rapidly dismutate to H_2O_2, which is more stable and membrane-permeable. In contrast, the Duox isozymes appear to produce H_2O_2 *directly* [25–27]. This important distinction

from other Nox isozymes has become most evident recently following the development of efficient methods for cell-surface expression of recombinant Duox [25,26]. Surface-expressed Duox1 or Duox2, along with the corresponding maturation factor, produces only H_2O_2 and no detectable superoxide. Because the active surface-exposed proteins exhibit no detectable peroxidase activities, and their peroxidase homology domains lack the most conserved heme-binding residues common to all other hemoperoxidases, we hypothesized that the Duox ectodomains function in the *formation* of H_2O_2 from superoxide (produced by the Nox-like portions), rather than directly utilizing H_2O_2.

33.3 The Functional Partnership of Duox with Extracellular Hemoperoxidases

The significance of Duox isozymes serving as direct H_2O_2 generators has relevance to their proposed roles in acting cooperatively with other extracellular peroxidases. The dual oxidases were originally described as thyroid-specific sources of hydrogen peroxide that function in supporting iodination of thyroglobulin by thyroperoxidase during thyroid-hormone biosynthesis [28,29]. Direct evidence supporting this role soon followed when human Duox2 mutations were linked to severe hypothyroidism [11]. A Duox2 mutant mouse strain was also identified with a similar congenital hypothyroidism phenotype [13]. Duox1 is presumed to serve some non-redundant function in thyroid tissue, although no Doux1 mutations have been identified or linked with disease, to date. Duox in thyrocytes accumulates in the apical plasma membrane of thyroid follicles [30], allowing efficient thyroperoxidase (TPO)-mediated utilization of the Duox-derived H_2O_2 for iodination or cross-linking of tyrosine adducts on thyroglobulin within the thyroid follicular lumen. One study suggests that Duox and TPO can interact [31].

Duox isoforms expressed in non-thyroid tissues and in other organisms also appear to be functionally linked with other dedicated extracellular peroxidases (Table 33.1). Early work identified a nematode (*Caenorhabditis elegans*) Duox essential for oxidative cross-linking of extracellular matrix proteins in the developing worm cuticle [18]. Worms in which Duox expression was suppressed displayed defects in cuticle formation and decreased dityrosine formation in cuticle matrix proteins. A similar cuticle-blistering phenotype with collagen cross-linking defects was observed recently by RNA interference targeting of the peroxidase MoLT-7 [32]. Together, these findings suggest the cuticle dual oxidase and peroxidase proteins cooperate in collagen cross-linking to stabilize the cuticle extracellular matrix. The envelope proteins of sea urchin eggs are also oxidatively cross-linked during fertilization, and this appears to involve cooperativity of Duox (Udx) with a secreted ovoperoxidase [33]. Finally, our group surveyed expression patterns of Duox histologically in non-thyroid tissues and observed close correlations with lactoperoxidase expressed in exocrine glands, the gastrointestinal tract, and major airways [34]. The antimicrobial function of lactoperoxidase in exocrine secretions such as milk and saliva had been long appreciated [35–39], although the source of hydrogen peroxide supporting its activity had been unclear. These findings provided the basis for the hypothesis that Duox isozymes are major sources of H_2O_2 supporting lactoperoxidase antimicrobial activity in exocrine secretions, currently being investigation in several laboratories [40–42].

33.4 Oxidative Microbial Killing in Exocrine Secretions by the Duox-Lactoperoxidase System

Lactoperoxidase (LPO), present in milk, tears, saliva, and other secretions, has been recognized since the 1960s to function as a broadly effective antimicrobial enzyme capable of killing Gram-negative and Gram-positive bacteria, both in milk and in saliva [35–39,43,44]. LPO was later shown to exhibit antiviral and antifungal activities [45–48]. Renewed interest in LPO as an antimicrobial enzyme in the lung developed when significant concentrations of LPO were noted in major airway secretions [49,50]. Functional similarities between thyroperoxidase and lactoperoxidase activities prompted us to explore roles of Duox1 and Duox2 as potential

Table 33.1 Functional Partnership of Duox with Extracellular Peroxidases

Species	Tissue	Duox	Peroxidase	Function
C. Elegans	Dermal	Ce Duox	MoLT-7	ECM/ Collagen cross-linking [18,32]
S. purpuratus	Oocyte	Udx	Ovoperoxidase	Fertilization envelope cross-linking [33]
S. variegates				
H. sapiens	Thyroid	Duox1, 2	Thyroperoxidase	Thyroid hormone biosynthesis [28,29]
M. musculus				
H. sapiens	Airways Exocrine, Mucosal	Duox1, 2	Lactoperoxidase	Host defense [34]

H_2O_2 donors for lactoperoxidase. We examined expression of Duox isoforms in relation to LPO in several extra-thyroid tissues, and discovered their presence and activity in salivary glands, the gastrointestinal tract, and tracheal and bronchial surfaces [34]. Expressed sequence-tag databases (dbEST) also confirm high Duox transcript levels in most exocrine tissues. *In situ* hybridization revealed that Duox expression is limited to surface epithelial layers in all exocrine and mucosal tissues [34]. Duox2 is selectively expressed in terminal portions of the salivary duct system. In contrast, LPO mRNA was detected deep within secretory acini of salivary glands, whereas the sodium-iodide symporter mRNA was detected in intercalated ducts. These segregated expression patterns suggest that LPO and its stable substrate (mainly thiocyanate) is supplied in earlier stages of saliva formation, whereas H_2O_2 release by Duox completes the system only during the final stages of saliva formation, thereby enabling delivery of unstable ROS into the oral cavity and preventing oxidant-induced glandular tissue damage. These observations were the first indications of non-thyroid functions of Duox and the sodium iodide symporter related to host defense. Similar segregated expression patterns were observed in human airways, where the LPO mRNA was detected in submucosal glands, while Duox1 mRNA was detected at highest levels along the airway surface epithelial layer (Fig. 33.2). Anti-sense probes directed at Duox1 suppressed H_2O_2 release from primary human bronchial epithelial cells [34]. Conner and colleagues confirmed and extended these findings, showing that the LPO protein is synthesized in submucosal glands, but accumulates in the airway surface layer (ASL) [50]. In contrast, Duox protein accumulates within the apical plasma membrane of airway epithelium and releases H_2O_2 into the airway lumen from the apical plasma membrane [21,51]. Thus, airway mucosal Duox-LPO based oxidative defense is designed for extracellular microbial killing within the airway lumen ASL.

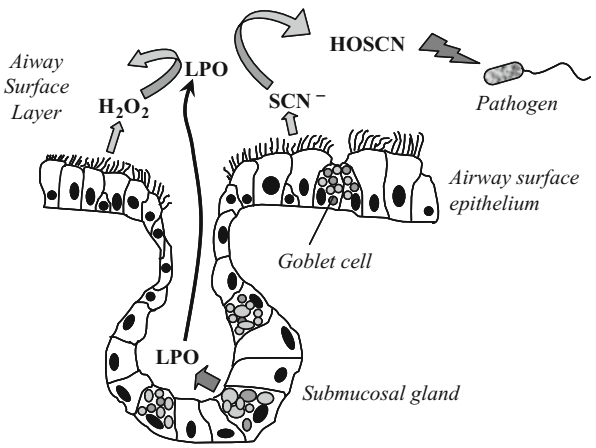

Fig. 33.2 Tissue expression pattern and function of the LPO-Duox-SCN^--based microbicidal system of major airways. LPO is synthesized deep within submucosal glands, but accumulates at high levels within the airway surface layer (ASL), whereas its substrates, H_2O_2 (from Duox) and SCN^- are released from epithelial cells of tracheal and bronchial surfaces. Inhaled pathogens are thereby subjected to oxidation by hypothiocyanite ($OSCN^-$) generated within the extracellular fluid phase of the ASL

Early work on LPO-based microbial killing in exocrine secretions, such as milk and saliva, revealed important differences from the peroxidases of phagocytes (myeloperoxidase and eosinophil peroxidase (MPO and EPO)), in that LPO does not use chloride or generate hypochlorous acid. Thiocyanate (SCN^-) is the favored LPO substrate, a pseudohalide that can reach concentrations approaching 0.5 millimolar in saliva and airway secretions [35–39,50,52–54]. Oxidation of thiocyanate by H_2O_2 and LPO produces hypothiocynite ion ($OSCN^-$), a broadly effective antimicrobial that is less harmful to host cells than hypochlorite.

$$\mathrm{SCN^- + H_2O_2 \xrightarrow{LPO} OSCN^- + H_2O}$$

The high concentrations of LPO detected in airway secretion were proposed to serve both anti-microbial and anti-oxidant functions [49,50], as airway H_2O_2 levels are maintained at low levels. Thus, Duox production of H_2O_2 may be the limiting component of this anti-microbial system. We suggested that the susceptibility of cystic fibrosis (CF) patients to microbial infection could reflect oxidative microbial killing defects involving the Duox-LPO-SCN^- system [34], as several of the pathogens that affect CF patients also affect CGD patients (*Staphylococcus aureus*, *Burkholderia cepacia*, and *Haemophilus influenzae*). Furthermore, we noted that the cystic fibrosis transmembrane conductance regulator (CFTR), commonly recognized as a chloride transporter, is also an efficient transporter of SCN^- [55,56]. Two later groups explored this hypothesis using cultured, polarized, human bronchial-epithelial cells and concluded that defects in SCN^- transport observed in CF airway cells were sufficient to compromise Duox and LPO-dependent microbial killing, which could be restored by expression of functional CFTR [40,41,54]. Other recent reports identified two other SCN^- carriers in the apical plasma membrane of airway epithelial cells, pendrin, and calcium-activated chloride channels (CaCC) [57,58], but further study is needed to establish the contributions of the three SCN^- transporters to Duox- and LPO-mediated microbicidal function.

As indicated in Fig. 33.3, the oxidants produced in phagosomes are distinct from those generated in Duox- and LPO-mediated killing in the extracellular medium, the latter (low H_2O_2 and high $OSCN^-$) being milder and less toxic to host cells. Mechanisms responsible for targeting the Nox2 and Duox isozymes to phagosomal and plasma membranes, respectively, were reviewed recently [21]. Phagocyte superoxide generation is prevented in resting cells, but is rapidly

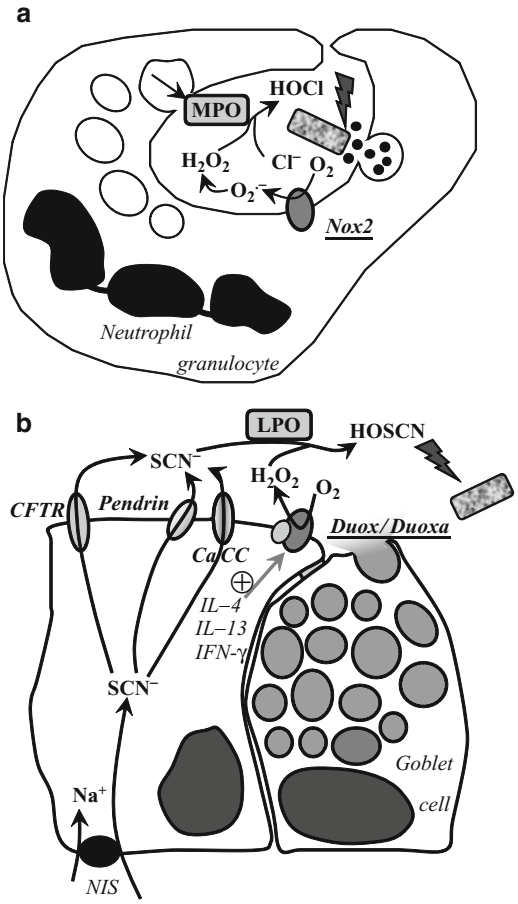

Fig. 33.3 Comparison of intracellular (phagocytic) and extracellular (mucosal epithelial) ROS-based microbicidal systems. (a) The Nox2-based system releases superoxide, H_2O_2, and hypochlorous acid (HOCl, produced by myeloperoxidase (MPO)) within the confines of phagosomes to kill ingested microbes. **(b)** Extracellular killing on apical epithelial cell surfaces produces milder oxidants (H_2O_2 and HOSCN) to avoid host cell damage. Lactoperoxidase (LPO) does not use chloride or generate HOCl

stimulated in newly forming phagosomes, as the Nox2-based oxidase assembles from its dissociated membrane and cytosolic components. The $p47^{phox}$ and $p40^{phox}$ components are directed to nascent phagosome membranes through PX and SH3 domain interactions, the Nox2-$p22^{phox}$ heterodimer is delivered from fusing granule membranes, and Rac translocates independently following GTP binding. Superoxide generation is accompanied by increases in phagosomal compartment ionic strength (mainly potassium), which were considered by some investigators to be even more important than ROS for microbicidal activity, as these changes affect the solubilization and activation of other granule-matrix microbicidal enzymes [59]. Otherwise, the traditionally held view is that superoxide is rapidly dismutated by SOD into H_2O_2, and MPO uses this to form hypochlorous acid (HOCl), which is the most abundant and effective microbicidal oxidant produced in phagosomes (Fig. 33.3A) [60]. In contrast, Duox in airways is concentrated along the apical plasma membrane of polarized epithelial cells. Mature airway cells produce H_2O_2 constitutively, which is further enhanced by several calcium-mobilizing agonists (ATP, ionomycin, thapsigargin) [34,40–42,51]. Constitutive levels of extracellular H_2O_2 are kept low by abundant LPO and SCN^- in these secretions, and any HOCl formed by secreted MPO is buffered by SCN^- [61]. Recent reconstitution studies suggest Duox becomes active in some post-Golgi cell compartment following carbohydrate modifications that are promoted by formation of Duox-Duox activator complexes [25,26]. Two Duox maturation factors (DuoxA1-alpha and DuoxA2) are efficient in targeting Duox1 and Duox2, respectively, to the plasma membrane, whereas a longer variant (DuoxA1-gamma) targets Duox1 to intracellular sites [25]. DuoxA1-alpha is detected in airway epithelial cells. Together these findings may explain how the polarized airway epithelium is designed for delivery of active Duox to the apical plasma membrane for H_2O_2 secretion into the ASL. Since the SCN^- transporters CFTR, pendrin, and CaCC are likewise targeted to the apical plasma membrane, it is tempting to speculate that the activities of these transporters could be linked to the electrogenic activity of Duox.

Other recent data suggest that Duox in airway epithelial cells may function as a signaling enzyme during wound healing responses, promoting cell migration and proliferation [62]. Other roles for airway Duox were proposed, related to host defense, including extracellular or intracellular signaling involved in mucin or IL-8 secretion [63,64]. The proposed role of Duox in proton secretion and acidification of the ASL [65] to support higher LPO activity seems unlikely, based on considerations of Duox functioning as a direct H_2O_2 generator [25]. Regardless of the mechanism involved in H_2O_2 formation, any protons produced in the cytosol through NADPH oxidation would be consumed in the ASL to support conversion of superoxide to H_2O_2.

33.5 Reconstitution of the Duox-Lactoperoxidase System on Polarized Airway Epithelium

Much of the initial evidence suggesting roles for Duox in microbial killing on epithelial surfaces has been circumstantial. The question of whether airway epithelial cells produce enough H_2O_2 to be effective in microbial killing has been addressed in recent studies using primary human bronchial cells that are redifferentiated into a mature ciliated phenotype in air-liquid interface (ALI) transwell culture-model systems [40–42]. We showed that Duox expression is dramatically induced in mature cells, coincident with the appearance of

cilia [21,42]. Such cultures are capable of killing several airway pathogens including *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Staphylococcus aureus* [40,42]. Fig. 33.4 shows that killing on the apical surface of ALI cultures depends on Duox stimulators (ATP or ionomycin) and supplementation with physiological concentrations of SCN^- and LPO. Microbial killing is also sensitive to diphenylene iodonium (DPI), a Duox inhibitor, and several antioxidant systems. Similar microbial killing processes were observed on the luminal surface of bovine tracheal explants [40]. Work by Harper and colleagues showed that human airway epithelial cell Duox1 levels are upregulated by T_H2 cytokines interleukin-4 and interleukin-13 [66], whereas Duox2 is responsive to interferon-gamma, rhinovirus, polyinosine-polycytidylic acid, or lipopolysaccharide [65,66], thus these oxidases can respond to inflammatory signals, as well as recognition of viral and bacterial pathogen-associated molecular patterns. We showed recently that the *P. aeruginosa* virulence factor pyocyanin is also capable of *suppressing* Duox activity and expression [42]. Interestingly, pyocyanin is itself a redox-active compound produced in overgrown cultures capable of entering host epithelial cells and utilizing NADPH to form superoxide in the cytoplasm, thereby inflicting oxidative stress on the host while competitively inhibiting

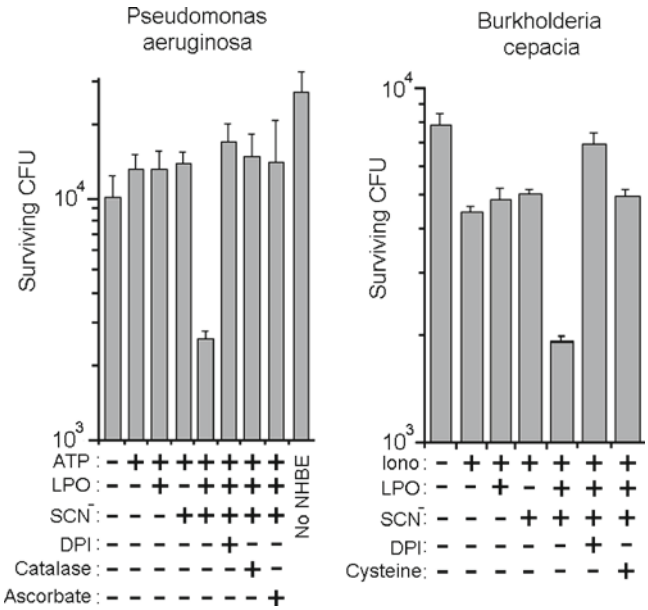

Fig. 33.4 Reconstitution of Duox/H_2O_2-based microbial killing on primary human airway epithelial cells. Cells grown on air-liquid interface transwell cultures were redifferentiated into a polarized, ciliated phenotype. The mature, differentiated cells release sufficient Duox-derived H_2O_2 to support killing of several airway pathogens on the apical cell surface. Optimum killing requires supplementation with physiological concentrations of LPO and SCN^-, as well as calcium-mobilizing agonists (ATP or ionomycin) to stimulate Duox activity. Killing is inhibited by the Duox/flavoprotein inhibitor diphenylene iodonium (DPI) or by oxidant scavenging systems (catalase, ascorbate, or cysteine). See Rada, et al. [42][42] for details

Duox activity. Such a mechanism may represent one means by which *P. aeruginosa* adapts to establish chronic infections in immunocompromised hosts such as CF patients. As long as sufficient H_2O_2 is available, an additional Duox-based defense mechanism exists, as both MPO and LPO were shown directly to detoxify pyocyanin and prevent its ROS-generating effects on host cells [42].

33.6 Conclusions

Several Nox/Duox family members appear to serve roles in host defense and inflammatory disease. The observations highlighted in this review show that high levels of airway Duox, together with lactoperoxidase, can provide an effective first line of defense against several respiratory infections. Studies in model air-liquid interface epithelial cell culture system indicate that Duox in the airway epithelium produces sufficient ROS to support the microbicidal activity of LPO; however, further studies are needed in whole animal models to establish how critical this system is in mucosal innate immunity. Recent observations regarding the inducibility of Duox1 or Duox2 by T_H2 and T_H1 cytokines, respectively, pose interesting possibilities for the roles of Duox in mucosal tissues. Strategies aimed at altering Duox expression or activity may have applications in treatment of a variety of infectious or inflammatory diseases of the oral, gastrointestinal, urogenital and respiratory tracts. Aside from the direct antimicrobial effects of high-level ROS production in exocrine secretions, lower-level ROS in many tissues provide intracellular "redox signals" that can regulate vascular tone, cell proliferation, inflammatory signaling, apoptosis, and oxygen sensing. It is thus plausible that low-level intracellular ROS generation by Duox may also affect processes such as wound healing, tissue remodeling or other profibrotic changes occurring in response to inflammatory stimuli or other chronic injuries. Therefore, future work should explore whether Duox genetic polymorphisms are determinants affecting susceptibility to infectious or inflammatory disease in conditions such as cystic fibrosis, asthma, and pulmonary fibrosis.

In summary, robust deliberate production of reactive oxygen species (ROS) by circulating phagocytic cells has long been considered an essential component of innate immune defenses against microbial infection. Recently, there is renewed interest in related oxidant-based anti-microbial defense systems on mucosal surfaces. In the last few years, following the identification of novel Nox family of NADPH oxidases related to the phagocytic system, growing evidence suggests that several of these novel enzymes are capable of responding to infectious and inflammatory stimuli, in some cases producing enough ROS for effective microbial killing in exocrine secretions. Mucosal surfaces of

airways represent a true first line of defense against exposure to the external environment. Microrbicidal ROS produced in the apical extracellular medium of the airway epithelium by the Dual oxidase-lactoperoxidase system are distinct from those generated within the confines of phagosomes. This chapter provides an overview of recent developments in our understanding of this oxidant-based anti-microbial system.

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Chapter 34
Chronic Granulomatous Disease: From Lethal Pediatric Mystery to Complex Chronic Disease

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34.1 History of Chronic Granulomatous Disease – A Remarkable Medical Detective Story

Chronic Granulomatous Disease (CGD) was first reported over 50 years ago by Heinz Berendes, Robert Bridges and Robert Good in *Minnesota Medicine* [1]. Four boys, starting with a one-year old who presented in 1950, were seen at the University of Minnesota Hospitals with a syndrome of chronic suppurative lymphadenitis, hepatosplenomegaly, pulmonary infiltrates, and eczematous dermatitis that did not match any then recognized diseases. Their short paper, published in 1957, defined the unique clinical entity of "Fatal Granulomatosus of Childhood" and outlined the clinical, laboratory, and pathologic findings in these patients, three of whom had died by the time of publication [1]. Later that year, Benjamin Landing and Harry Shirkey in Cincinnati described two more boys with a disease that closely matched this clinical picture [2].

While the cause of this disease was not yet known, one boy's maternal family history recorded the deaths of male infants from "boils and septicemia or scrofula" in three previous generations [1] possibly suggesting an X-linked inheritance. The families of the other patients were unremarkable. Landing and Shirkey noted the presence in some biopsied tissues of pigmented lipid histiocytes reminiscent of those seen in Niemann-Pick disease, a genetic metabolic disorder [2] that also presents in infancy and frequently also causes hepatosplenomegaly and infectious complications. When vaccinated, these children developed normal antibody titers and they also exhibited normal delayed-type hypersensitivity [3]. Phagocytosis of bacteria by neutrophils was normal, as were *in vivo* inflammatory responses as measured by Rebuck skin windows. Efforts to isolate mycobacterial, fungal, viral or bacterial agents from granulomatous lesions failed [3] although staphylococci, enterobacteria and *Candida* were recovered from various organs during infectious episodes or autopsy. Based on some similarity to Hodgkin's disease and other cancers, focal radiotherapy of cervical nodes was attempted with no benefit [1,3]. Interestingly, despite administration of antibiotics and intravenous immunoglobulin, the disease progressed relentlessly with worsening infections and the systemic sequelae of chronic childhood disease. Ultimately the patients developed pulmonary infections that never fully remitted and often presaged death. Laboratory findings were surprisingly unrevealing, given the patients' extremely poor health. The only consistent finding was hypergammaglobulinemia that correlated with neither disease severity nor progression. During surgical procedures and post-mortem examinations, lesions were noted on the surface of both the liver and the lungs, and microscopic examination revealed granulomas with central necrosis, surrounded by mononuclear cells and multinucleated giant cells with a striking infiltration of plasma cells surrounding the granulomas. Other diseases with a granulomatous component were considered, including scrofula (mycobacterial infections with cervical lymphadenopathy), Wegener's granulomatosis, midline granuloma of the face, and sarcoidosis, however, other characteristic signs of each of these disorders were absent.

While the possibility of X-linked inheritance for CGD [1,3] was strengthened by the description in 1965 of an additional 13 boys in 5 families with this same syndrome [4], a somewhat similar disease occurring in three sisters had been described as early as 1962 [5] hinting at another pattern of inheritance. In 1968, separate papers from Quie and Baehner described three female patients ranging in age from 9 to 17 years with a similar disease of nearly the same severity as that seen in the previously reported male children [6,7]. The molecular defects in X-linked and autosomal CGD (A-CGD) are discussed in detail below as are conditions associated with the carriers of CGD.

By 1966, the syndrome began to be referred to as Chronic Granulomatous Disease of Childhood, although different names continued to be used even in the same paper [8]. More importantly, an *in vitro* phenotype could be demonstrated in assays of polymorphonuclear leukocyte (PMN) killing of *S. aureus* with CGD PMN showing significantly less activity than normal [7–15]. Once a phenotype could be seen in an abundant and easily purified cell type, efforts rapidly focused on the identification of the mechanistic basis of defective

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killing. Phagocytosis of bacteria was apparently normal [3,10,14] or enhanced [16] and limited analyses of the enzyme content of CGD PMN had been normal [14,17]. Electron microscopic studies by Quie and coworkers [14] confirmed a previous observation [10] that CGD phagocytic vacuoles failed to enlarge suggesting a possible defect at the level of vacuole maturation. Degranulation, the movement of specialized PMN vacuoles to either the plasma or phagocytic vacuolar membrane, was sometimes reported as defective [14] but more often found to be normal [18–21].

In 1967, Baehner and Nathan demonstrated the first direct evidence of defective oxidative metabolism in CGD PMN following ingestion of styrene beads [22]. Later that year, Holmes, Page and Good also showed that CGD PMN do not increase respiration during phagocytosis of bacteria [17]. These two studies connected CGD for the first time to a phenomenon described by Baldridge and Gerard in 1932 and referred to as "the extra respiration of phagocytosis [23]". The metabolic basis for the respiratory burst, later shown to be defective in CGD cells, had been under intense study by Manfred Karnovsky's laboratory during the 1950s [24]. In 1961, Evans and Karnovsky reported that the enzyme responsible for the respiratory burst was likely granule-associated, utilized reduced nicotinamide adenine dinucleotide (NADH), and might be "…involved in the 'disposal' of molecular fragments formed from ingested particles…[25]". Later that year, Iyer, Islam and Quastel showed that PMN were capable of producing hydrogen peroxide (H_2O_2) upon phagocytosis of bacteria and proposed that it might be one of the agents responsible for the antibacterial activity of PMN [26]. While still debating whether the enzyme utilized NADH or NADPH, Cagan and Karnovsky [27] were able to measure the activity of the FAD-containing enzyme using nitro blue tetrazolium (NBT) in the presence of NADH, an assay exploited later by Baehner and Nathan to differentiate normal from CGD PMN [6]. Baehner and Karnovsky ascribed the cause of CGD to deficiency of NADH oxidase [28]. Notably, Rossi and Zatti showed that the activity of an NADPH-oxidase associated with PMN granules increased significantly when the cells had phagocytosed bacteria [29]. The model that emerged from these studies was that H_2O_2, thought to be produced intracellularly by PMN at levels between 1–100 mM [26], killed pathogens in normal PMN. CGD PMN did not produce H_2O_2 [17] thus explaining their infectious disease susceptibility.

Further revisions of this model came from Seymour Klebanoff who showed that myeloperoxidase (MPO), another neutrophil protein, could utilize H_2O_2 and chloride to form the potent antimicrobial compound hypochlorous acid ($HOCl^-$), the active component of household bleach [30]. In stark contrast to the severe infectious phenotype in CGD patients, genetic deficiency in MPO is relatively common, occurring in approximately 1 in 2000 people, and may only slightly predispose to infection. Later, it was found that while H_2O_2 is clearly produced by PMN, it is not the direct product of the phagocyte NADPH oxidase. In 1973, Babior, Kipnes, and Curnutte employed the now-standard cytochrome c-reduction assay and found that leukocytes reduced cytochrome c during phagocytosis and that reduction could be inhibited by the addition of superoxide dismutase [31] which converts $O_2^{\cdot-}$ and H_2O into H_2O_2 demonstrating that the product of the NADPH oxidase is superoxide. Significant challenges to the long-held belief that reactive oxygen species play a direct role in killing microbes have been mounted from several sides and will be discussed later in this chapter.

34.2 The History of CGD at the NIH Clinical Center

Understanding of CGD has grown from its initial presentation in 1950 as an unknown, fatal disease of young boys to recognition of the genetic basis of CGD as both an autosomal recessive and X-linked defect of the NADPH oxidase affecting males and females of all ages. To our knowledge, the first CGD patient entered the doors of the NIH Clinical Center in 1972 in the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Disease. This patient and affected family members continue to visit the NIH and serve to exemplify the importance of long-term clinical continuity both in understanding the natural history of CGD and providing a reliable source of clinical material for scientific study. Subsequent to this first visit, a large number of scientific and clinical milestones in the understanding of CGD have been reported by NIH researchers (see Figure 34.1, Timeline of Selected NIH CGD research) and are discussed further in this review.

34.3 Genetic and Biochemical Causes of CGD

CGD occurs in 1/200,000–250,000 live births [32] and is caused by defective activity of the NADPH oxidase of phagocytes. The catalytic center of the NADPH oxidase (see Figure 34.2A) resides within the 91 kDa glycoprotein subunit of the **ph**agocyte **ox**idase ($gp91^{PHOX}$), the component mutated in X-linked CGD (X-CGD). This enzyme catalyzes the single electron reduction of molecular oxygen to generate superoxide anion ($O_2^{\cdot-}$) using the reduced form of nicotinamine adenine dinucleotide (NADPH). As shown in Figure 34.2A, NADPH transfers electrons to an FAD moiety of $gp91^{PHOX}$ that then transfers electrons to two heme prosthetic groups situated among the 6 transmembrane domains of

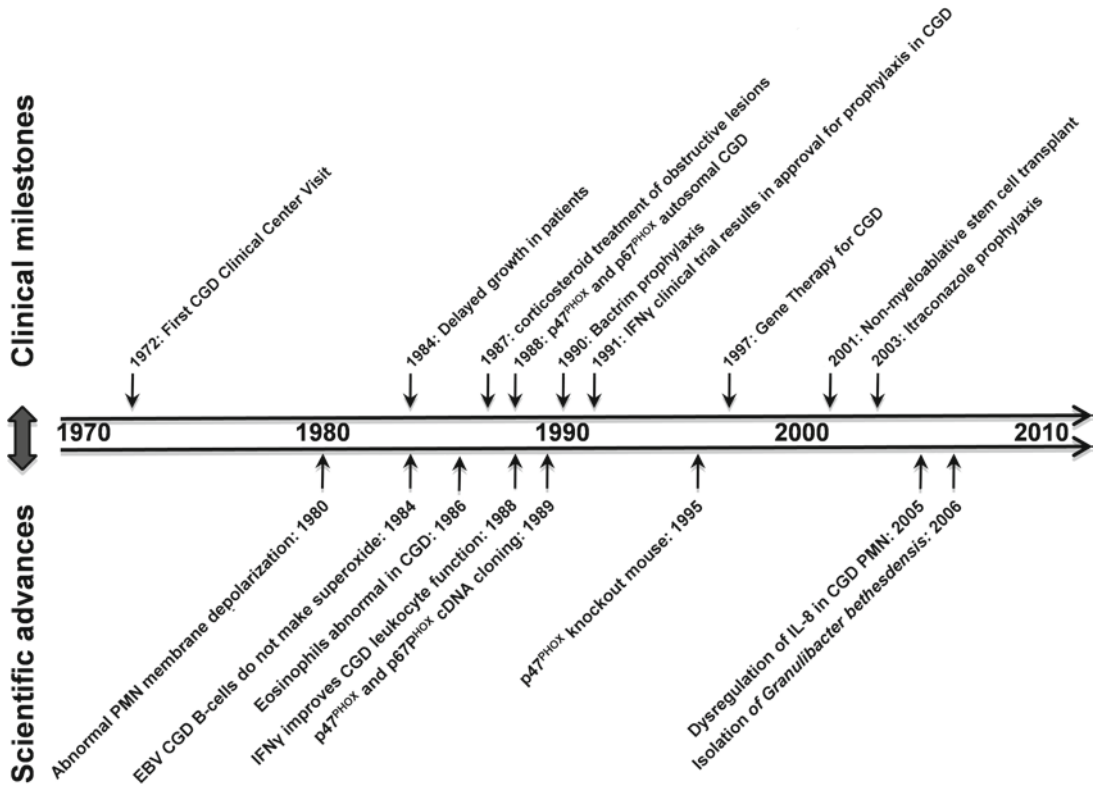

Fig. 34.1 Timeline of selected NIH CGD research

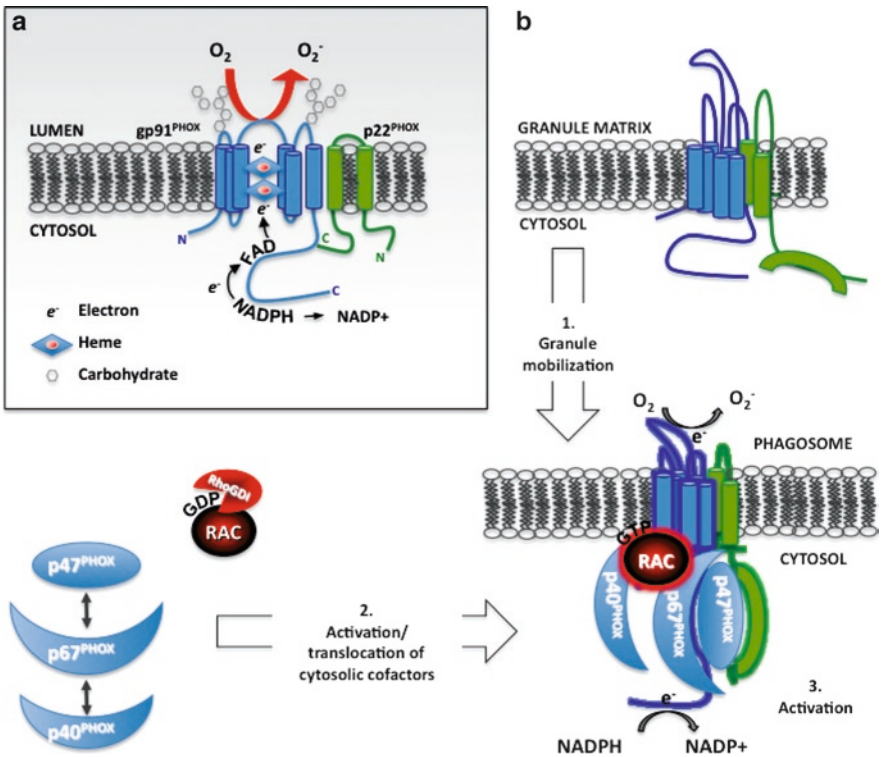

Fig. 34.2 Enzymatic activity of gp91^{PHOX}

gp91^{PHOX}. The luminal heme group then reduces molecular oxygen to form O_2^- either outside the cell or within the phagosomal membrane.

Maximal stability of gp91^{PHOX} requires another integral membrane protein called p22^{PHOX} and activation of the enzyme requires recruitment of the cytosolic components p47^{PHOX} and p67^{PHOX} to the heterodimeric membrane complex of gp91^{PHOX} and p22^{PHOX} as outlined in Figure 34.2B. Mutations in the genes encoding p22^{PHOX}, p47^{PHOX}, and p67^{PHOX} lead to the autosomal recessive forms of CGD (A-CGD). Numerous additional factors regulate NADPH oxidase activity *in vivo* and where relevant to CGD, these will be discussed. For a more complete review on the phylogeny, ontogeny, assembly and activation of the NAPDH oxidase, please see the excellent reviews by Nauseef [33–35], Segal [36,37], Leto [38] and others [39]. In addition to the mutations in NADPH oxidase components that cause CGD, several other diseases result in either positive or negative dysregulation of the neutrophil oxidase (NOX) and share some overlapping symptoms with CGD (Table 34.1).

Molecular cloning of the gene responsible for X-CGD without any primary sequence information was an impressive accomplishment and was the first reported success of positional cloning [41]. The protein, called gp91^{PHOX} and later Neutrophil oxidase 2 (Nox2), occurs in the membrane of PMN secondary granules as a heterodimer with p22^{PHOX}. Each protein seems to require the other for stability or targeting as the absence of either one tends to eliminate the other [42]. Deficiency in p22^{PHOX} causes CGD and complementation studies using PMN and monocyte fusions from patients with different forms of CGD indicated a possible requirement for cotranslational stabilization the p22^{PHOX} and gp91^{PHOX} complex [43].

The molecular complex required for maximal superoxide (O_2^-) production by PMN is still being elucidated and is being informed by the discovery of homologous systems in other cell types. In simplified terms, activation of the NADPH oxidase (Figure 34.2B) occurs in at least three distinct steps. First, a large number of signals induce mobilization of PMN granules, the specialized and heterogeneous storage vesicles of PMN, to either the plasma membrane or the phagosome. Of the three main classes of PMN granules, the membrane-bound complex of p22^{PHOX}/gp91^{PHOX} occurs in the so-called secondary or 'specific' granules [44]. The three granule populations are mobilized differentially with tertiary (gelatinase) granules mobilized most readily, followed by the secondary granules, and lastly the primary granules [45]. Concurrent with granule mobilization, the cytosolic components are activated by several phosphorylation events that enhance their affinity to the gp91/p22^{PHOX} complex. These components are also attracted by modifications of membrane lipids by phosphoinositol kinases (e.g., PI3K). The final step of Nox2 activation is still not certain nor are the processes ending its activation.

Many of the biochemical studies performed to build this model rely on the assumption that PMN remain inactive during purification and isolation, however PMN are extraordinarily sensitive to microbial products, such as lipopolysaccharide (LPS), that are ubiquitous contaminants of laboratory media and supplies. Our laboratory reported the first patient with IRAK4-deficiency, which manifested as a profound insensitivity to LPS both systemically and in PMN [46]. IRAK4-deficient PMN produce significantly less O_2^- in response to formyl-Met-Leu-Phe (fMLP) and fail to prime with LPS compared to normal donors [46]. The lower basal activation of the NADPH oxidase in this patient suggests that normal cells are either activated during purification or that the low levels of LPS to which humans are chronically exposed acts as a 'priming' agent to enhance responsiveness of PMN to additional stimuli. Further complicating the construction of a

Table 34.1 Recognized genetic defects resulting in abnormal NADPH oxidase activation

Gene involved	Other names	OMIM*	Relative frequency [32,40]
p22^{PHOX}, cytochrome b_{558} α subunit, cytochrome b_{558} light chain	Autosomal recessive, cytochrome b-negative CGD	#233690	2%-5%
p47^{PHOX}, NCF1	CGD, autosomal recessive cytochrome b-positive, Type I	#233700	12%-20%
67^{PHOX}, NCF2	CGD, autosomal recessive cytochrome b-positive, Type II	#233710	3%-5%
gp91^{PHOX}, cytochrome b_{-245}, Nox2, cytochrome b_{558} β subunit, cytochrome b_{558} heavy chain	CGD, x-linked, cytochrome b-negative Type I	#306400	70%-80%
Rac2	Rac2-deficiency, neutrophil immunodeficiency syndrome	#608203	rare
Glucose 6-phosphase-β (G6pc3)	Glucose 6-phosphase-β (G6pc3) deficiency, severe congenital neutropenia	#612541	rare
Glucose-6-phosphate dehydrogenase	G6PDH-deficiency		rare

* Online Mendelian Inheritance in Man ID# (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim)

comprehensive model of NADPH-oxidase activation, many studies have used different stimuli, animal species, and assays (intact cell, broken cell, cell-free). For these reasons, more questions remain about the assembly of the NADPH oxidase than can be summarized here.

34.3.1 gp91^{PHOX}: the Enzymatic Center of the NADPH Oxidase

Translocation of NOX to the plasma membrane in response to a calcium ionophore was noted by Borregaard and coworkers [44]. Studies at the NIH exploring different stimuli (Ca^{2+} ionophore, phorbol myristate acetate (PMA), and fMLP) demonstrated that translocation of Nox2 did not correlate directly with the amount of O_2^- generated by PMN suggesting additional levels of regulation [47]. Electron microscopy studies also demonstrated that localization to either the plasma membrane or phagolysosome membranes depends on the stimulus used [48] and that H_2O_2 could be detected within the phagolysosome itself [49]. The cytoplasmic domain of gp91^{PHOX} provides a docking site for p47^{PHOX} that can be blocked with antibodies or small peptides and appears to be required for maximal p47^{PHOX} phosphorylation [50]. PMN with a mechanistically revealing P415H mutation in gp91^{PHOX} demonstrated normal gp91^{PHOX} and p22^{PHOX} protein levels [51], normal recruitment of p47^{PHOX} and p67^{PHOX} to PMN membranes [52] but no NOX activity. Cytosolic factors do associate with phagosome membranes in the absence of gp91^{PHOX} however the interaction is far more transient [53]. Previous studies had shown that p47^{PHOX} recruitment to the membrane is deficient in X-CGD however the absence of gp91^{PHOX} typically also results in lower levels of p22^{PHOX} so that it remained possible that the docking site was on p22^{PHOX}. An additional regulatory step in the interaction of gp91^{PHOX} with cytosolic factors involves phosphorylation, apparently by protein kinase C [54]. The complex containing p22^{PHOX} and gp91^{PHOX} also contains several small GTPases including RapIA [55] that may regulate these processes.

Mice deficient in gp91^{PHOX} recapitulate many of the phenotypes seen in CGD patients including decreased killing of *A. fumigatus* and *S.aureus* [56], and have been extensively used as model for human CGD.

34.3.2 p22^{PHOX} CGD

CGD caused by mutations in p22^{PHOX} results in two cellular phenotypes; one in which gp91^{PHOX} is present but subnormally active, and another in which gp91^{PHOX} is absent. Cell hybridization (fusion) studies of p22^{PHOX}-deficient cells demonstrated complementation of X-CGD cells in a protein-synthesis dependent manner [57]. A particularly interesting CGD patient had a substitution mutation in the cytoplasmic tail of p22^{PHOX} resulting in defective translocation of cytosolic components despite normal gp91^{PHOX} levels [58]. A biochemical assay of phospholipid-activated O_2 consumption by membrane fractions of PMN from this patient demonstrated normal enzymatic activity thus proving that the enzymatic activity of the NADPH oxidase does not require the cytosolic factors. Studies have shown that p22^{PHOX} provides a docking site for p47^{PHOX} proximal to gp91^{PHOX}, and that p22^{PHOX} stabilizes gp91^{PHOX}, probably through a cotranslational mechanism, but does not contribute to the catalytic activity of gp91 ^{PHOX}. Studies have also shown that p22^{PHOX} is a heme protein [59] but it is not yet clear whether this heme plays a role in NADPH oxidase activity.

The mRNA for p22^{PHOX}, unlike other PMN oxidase genes, is widely expressed but the protein is apparently only detectable in myeloid cells. A mouse strain with a naturally occurring mutation in a p22^{PHOX} transmembrane domain demonstrates defective O_2^- production and increased sensitivity to the CGD pathogen *B. cepacia* [60]. These p22^{PHOX} mutant mice have difficulty with balance due to defective formation of gravity-sensing organs (otoconia) [60]. This phenotype has not been reported in p22^{PHOX}-CGD in humans, although patients with nearly complete p22^{PHOX} deletions are known [61]. Several single nucleotide polymorphisms (SNP) with clinical phenotypes have been reported in p22^{PHOX}. For example, the C242T (H72Y) SNP seems to increase O_2^- production and is associated with aggressive periodontitis [62].

34.3.3 CGD Caused by Mutation of Cytosolic Cofactors: p47^{PHOX} and p67^{PHOX}

Early cellular fractionation studies of patients with CGD [63], demonstrated deficient NADPH oxidase activity in the membrane fraction in all but one donor (a female who presumably had A-CGD). Further biochemical evidence for at least two complementation groups for CGD came from somatic cell hybrids which demonstrated NBT reduction when monocytes from X-CGD were fused to those from A-CGD indicating that each form of CGD lacked a necessary component [64]. Cell fractionation of PMN suggested that the cytosolic components form a stable ~240–250 kDa complex [65,66]. Curnutte developed a 'cell-free' system of NOX that could be activated by arachidonic acid and used this method to demonstrate that cytosol from X-CGD patients was normal [67]. The cell-free system showed that while cytosol from A-CGD patients failed to activate the membrane component, cytosol from siblings and parents typically

had about 50% of normal NOX activating ability, a result expected from heterozygous individuals [68]. A ~48 kDa protein typically phosphorylated in normal PMN during NOX activation was found to be absent in A-CGD PMN, the first hint that a factor is deficient in A-CGD [69]. In two back-to-back papers published in Science in 1988, a group at the NIH [70] and one at the University of Iowa [71] reported that two cytosolic components, eventually known as $p47^{PHOX}$ and $p67^{PHOX}$, were absent in different patients with A-CGD. This result was confirmed by studies showing that different A-CGD patients fell into two complementation groups in cytosol reconstitution assays [72]. Both $p47^{PHOX}$ and $p67^{PHOX}$ were present in the ~240 kDa complex [66] and other work suggested a relatively loose association of components [73]. It should be stressed that due to activation of PMN during purification, the true resting state of these components may not yet be known. While $p47^{PHOX}$ and $p67^{PHOX}$ translocate to membranes normally when $gp91^{PHOX}$ is present (as demonstrated in the P415H cytochrome b_{558}+ mutant mentioned above), translocation in A-CGD revealed important mechanistic insights into NOX assembly. In A-CGD PMN lacking $p67^{PHOX}$, normal translocation of $p47^{PHOX}$ was observed whereas $p67^{PHOX}$ did not translocate in $p47^{PHOX}$ –deficient CGD PMN [52]. Furthermore, $p47^{PHOX}$ is phosphorylated normally in $p67^{PHOX}$ deficiency [74]. This led to the view that $p47^{PHOX}$ is an organizer of the complex while $p67^{PHOX}$ is the activator of NOX. The mechanism of $p67^{PHOX}$ activation of the NADPH oxidase may relate to its ability to regulate the reduction of FAD by NADPH [75].

The first cDNA sequence of a cytosolic NADPH oxidase cofactor, $p47^{PHOX}$, was published by NIH researchers in 1989 [76] and was shown to complement $p47^{PHOX}$-deficient PMN cytosol. Mice deficient in $p47^{PHOX}$ were generated at the NIH [77] and displayed a CGD phenotype including significantly increased risk of spontaneous infections due to *Staphylococcus xylosus* and fungi. Haploinsufficiency is apparent in PMN but not monocytes from $p47^{PHOX}$ mice suggesting that this protein is a limiting factor in the activation of Nox2 [78].

Shortly after the observation that a 67 kDa protein was absent in some cases of A- CGD, the NIH and Iowa groups published the cDNA of $p67^{PHOX}$ and demonstrated functional complementation of $p67^{PHOX}$- deficient CGD cytosols using a recombinant expressed form [79].

An additional cytoplasmic component, $p40^{PHOX}$ has been reported as both a positive and negative regulator of the NADPH oxidase. To date, no CGD patients have been reported with defects in $p40^{PHOX}$ but mice lacking $p40^{PHOX}$ display reduced clearance of *S. aureus in vivo* and their PMN are less able to kill this organism *in vitro* [80]. $O_2^{\cdot-}$ production and $p67^{PHOX}$ expression were also lower in PMN from $p40^{PHOX}$ -deficient mice [80] suggesting that $p40^{PHOX}$ may stabilize $p67^{PHOX}$.

34.3.4 Other Defects in Oxidant Production by Leukocytes

Rac2-deficiency is a rare immunodeficiency associated with decreased $O_2^{\cdot-}$ production [81]. Cells from these patients also display a severe chemotactic defect and fail to release their primary granules although secondary granule release is normal [81]. As depicted in Figure 34.2B, Rac plays an important role in NOX activation.

Deficiency in Mo1 (complement protein 3 bi receptor) results in decreased $O_2^{\cdot-}$ production in response to particulate stimuli (*e.g.,* zymosan) that depend on opsonization for internalization whereas $O_2^{\cdot-}$ production in response to fMLP is normal [82].

Glucose-6-phosphate dehydrogenase deficiency: Cooper et al. described an adult patient with leukocyte glucose 6-phosphate dehydrogenase (G6PD) deficiency that resulted in multiple infections with *E. coli* and *Klebsiella* [83]. This patient's leukocytes were unable to generate H_2O_2 upon phagocytosis and demonstrated deficient killing of catalase positive but not negative bacteria. In cells with <5% of normal levels of G6PDH activity, the pools of reduced pyridine nucleotides (*e.g.,* NADPH) are also nearly absent due to the requirement for G6PDH to initiate the hexose monophosphate pathway/pentose shunt.

Glucose 6-phosphase-β (G6pc3) deficiency: The laboratory of Janice Chou (NIH/NICHD) noted neutrophil dysfunction in several glycogen storage diseases. They generated a knockout mouse lacking G6pc3 and noted increased apoptosis, decreased $O_2^{\cdot-}$ production and increased susceptibility to bacterial infection [84]. A human disease resulting from deficient activity of G6pc3 was reported in 2009 and results in neutropenia and severe infections [85] however $O_2^{\cdot-}$ production and microbial killing activity of cells from these patients is not yet reported.

Myeloperoxidase (MPO) deficiency is particularly interesting because in contrast to CGD patients, MPO-deficient patients are usually clinically well. MPO is thought to utilize H_2O_2 and Cl^- to generate hypochlorous acid (bleach, $HOCl^-$) and to be required for maximal antimicrobial activity of the PMN. Susceptibility to *Candida* infection in MPO-deficiency has been reported but is low (~5% of patients) and seems to be correlated with diabetes mellitus [86]. *In vitro* studies show that MPO-deficient PMN do not kill *Candida*, *Staphylococcus* or *Serratia* as well as normal [87]. MPO-deficient mice are resistant to smaller inocula of *Candida albicans* that are lethal to $gp91^{PHOX}$-deficient CGD mice but are equally sensitive to larger inocula [88]. Interestingly, MPO-deficient PMN produce significantly more $O_2^{\cdot-}$ than normal PMN [89,90]. Furthermore, MPO-deficient PMN display increased phagocytosis [90] possibly due to ability of MPO to damage opsonizing epitopes on C3b and IgG [91]. A recent

clinical study found that MPO-deficient patients have significantly more infections (including by bacteria) than normal controls but suffer from fewer cardiovascular pathologies [92]. Interestingly, increased levels of MPO predicted adverse cardiac events [93] suggesting a role for MPO and ROS in cardiovascular health.

IRAK4- and NEMO-deficiency are primary immune deficiencies that result in frequent infections and dysregulated inflammatory signaling [46,94–96]. Our laboratory has studied NOX activation in PMN from both types of patients and found that their cells respond normally to fMLP or phorbol ester (PMA) induction of O_2^- production, but show defective O_2^- production during an LPS-primed fMLP-stimulated respiratory burst [96a]. A role for NF-κB in NOX activation has been suggested by cell culture models employing either NF-κB repressors or NEMO-mutant EBV cells [97]. An attractive hypothesis derived from our studies of primary human cells is that defective signaling through TLR and/or NF-κB pathways results in subnormal NADPH oxidase activation thereby contributing to the increased susceptibility of these patients to microbial infection.

Table 34.2 Microbes causing disease in CGD patients

Microbes	Relative frequency	References
Gram-positive		
Staphylococcus aureus	Extremely common	3,32,99–103
Nocardia spp.	Common	32,99,102
Mycobacterium species	Rare	99,104
Gram-negative		
Klebsiella sp.	Common	32,101–103
Burkholderia cepacia	Common	32,100,105
Serratia marcescens	Common	100,101,103
Escherichia coli	Previously common (currently sporadic)	101 3, 101
Chromobacterium violaceum	Common	99
Granulibacter bethesdensis	Rare	106
Salmonella typhimurium	Common	99
Fungal		
Aspergillus fumigatus	Common	32,101,103,107
A. nidulans	Rare	108
Pneumocystis carinii	Rare	109
Candida species	Common	101,107
Pseudallescheria boydii	Rare	110
Paecilomyces	Rare	32

34.4 Clinical Manifestations of CGD

The classical presentation of CGD during infancy is well known. Late onset "variant" CGD may have been reported as early as 1962 [5] but was first named as such in 1977 by Dilworth and Mandell [98]. Variant CGD may lack certain features in some patients and in some cases inflammatory rather than infectious disease conditions may predominate due to the intermediate killing defect sometimes reported [98]. Studies segregating CGD patients into groups according to their genotype will likely reveal a more consistent genotype/phenotype correlation than has been possible thus far. In this section we describe the major clinical features of CGD as well as variant forms and the carrier state.

34.4.1 Infectious diseases in CGD

Patients with CGD develop frequent infections due to a large variety of opportunistic bacterial and fungal pathogens (Table 34.2). Infections in practically every organ system have been reported [32,99]. Coagulase-positive Staphylococci, most likely *S. aureus*, was recognized in the initial case reports [3] and *S. aureus* remains one of the most commonly isolated infectious agents in CGD [32]. Therapeutic developments, differences in environmental exposure, and changes in microbiologic culture techniques have caused the incidence of infection due to specific organisms to evolve. The rank order of frequency also depends on the site of infection [32] and is beyond the scope of this review. It is notable that the causative agent of a particular infectious episode often cannot be identified. It is possible that a large number of uncultivatable pathogens in CGD have yet to be described as attested by the recent discovery of a new pathogen that requires specific media for efficient isolation (see below). Another explanation for the failure to isolate an organism may also be that the microbe is sterilized by host defenses or therapy but continues to produce the exaggerated inflammatory response typical of CGD patients described below. Studies at the NIH indicate that when organisms can be identified, bacterial infections in CGD patients are usually due to genetically distinct strains causing recurrent infection, rather than persistence of the same strain [100]. A child infected with *Chromobacterium violaceum*, *Serratia marcescens*, *Granulibacter bethesdensis*, *Aspergillus* species or *Paecilomyces* should alert the care provider to the possibility of CGD.

The early clinical observation that CGD patients are infected by microbes that fail to secrete H_2O_2 remains generally true. Failure to secrete H_2O_2 correlates with production of catalase, an enzyme that degrades $2H_2O_2$ into $2H_2O$ and O_2. Mandell and Hook showed that catalase producing organisms were resistant to killing by CGD PMN whereas organisms that did not produce catalase (and therefore accumulated

H_2O_2) were killed normally by CGD PMN [111]. This microbially-derived H_2O_2 was thought to supplement the deficient PMN myeloperoxidase/halide system causing production of microbicidal hypohalous acids (*e.g.*, bleach) and microbial death. Support for this long-held view came from experiments in non-CGD mice that showed that following intraperitoneal infection, the virulence of *Staphylococcus aureus* was positively associated with the level of catalase produced and that addition of exogenous catalase tended to increase virulence [112]. The ability of exogenous H_2O_2 to fully reconstitute ROS-dependent microbicidal activities of the PMN has been shown by *in vitro* mixing experiments using PMN from patients with CGD and MPO-deficiency [113]. These studies showed that the presence of as little as 1 MPO-deficient PMN (able to produce $O_2^{\cdot-}$ and H_2O_2 but unable to make bleach) among 15 CGD PMN (unable to make $O_2^{\cdot-}$ or H_2O_2 but able to make bleach) resulted in normal levels of fungicidal activity against *Aspergillus* [113].

Evidence against the role of catalase during pathogenesis comes from several sources. June Kwon-Chung's group (LCID/ NIAID) showed that deletion of the two main catalase genes in *Aspergillus nidulans* did not alter its virulence in $p47^{PHOX-}/_{-}$ or wild type mice [114]. Other labs have shown that catalase-deficient *Staphylococcus aureus* mutants were fully able to establish infection in CGD mice [115]. In fact, while catalase-deficient *S.aureus* were more sensitive *in vitro* to H_2O_2, they appeared to survive better than wild type in CGD mice. MPO-catalyzed iodination (dependent upon H_2O_2) was not detected in either catalase producing or deficient *S. aureus* internalized by CGD PMN but could be readily measured in normal cells indicating that H_2O_2 production by this organism is unlikely to be sufficient to induce its killing [115]. These observations challenge the long-held view that expression of microbial catalases are key virulence determinants during infection in CGD, however further studies are clearly required to elucidate the relatively complicated roles H_2O_2 might play during infection.

Long term sequelae of frequent infections and abscesses can be seen in most organ systems of CGD patients and are discussed below. Hepatic abnormalities similar to cirrhosis have been reported and it has been suggested that baseline scans should be performed to facilitate detection of active lesions [116]. Infectious episodes in CGD patients generally require aggressive antimicrobial and supportive care.

Two pathogens are currently under intense study in our laboratory, *Aspergillus fumigatus* and *Granulibacter bethesdensis*. *A. fumigatus*, a ubiquitous environmental mold, is the leading infectious cause of death in patients with CGD [32,117]. Work at the NIH has shown that treatment of CGD patients with IFN-γ augments the ability of their PMN to damage *Aspergillus* hyphae (mycelia) *in vitro* [118]. As described below, the NIH has also found that antifungal prophylaxis using itraconazole can drastically reduce the frequency of aspergillosis in CGD patients [119]. These frequent infections often occur after handling common materials including hay and mulch that are replete with *Aspergillus* conidia (spores). Serious pulmonary aspergillosis has been the presenting complaint of several previously undiagnosed CGD patients [120]. Interestingly, not all CGD patients get aspergillosis suggesting either that infection requires a very large dose of conidia or that additional host defense mechanisms and/or predisposing factors are at work. Our laboratory recently found that normal and CGD PMN can equally arrest growth of *Aspergillus* conidia through the action of the iron sequestering PMN protein, lactoferrin [21]. Further studies in our laboratory have found that several small-molecule iron chelators similarly act against *Aspergillus* and can synergize with classical antifungal drugs thus possibly representing a novel and potentially useful therapeutic target [96b].

In 2006, a new pathogen of CGD patients was isolated and characterized at the NIH [106,121] and has since been isolated from another CGD patient in Spain [122]. *Granulibacter bethesdensis*, is a Gram-negative catalase-positive non-motile member of the acetic acid bacteria family. In contrast to other CGD pathogens, such as *B. cepacia, S. aureus*, and *Serratia marcescens* that appear to re-infect patients rather than persist [100], virtually identical isolates have been recovered from a single patient over several years during a waxing and waning clinical course [123]. Collaborative studies between the Gallin laboratory and the laboratory of S. Holland (Laboratory of Clinical Infectious Diseases/NIAID) have indicated that despite rapid internalization by PMN, *Granulibacter* fails to activate a respiratory burst in neutrophils and is a relatively poor stimulator of cytokines compared to other microbes [abstract [124]].

For the microbe, there are essentially three options to subvert NOX-dependent host defenses during pathogenesis: (i) detoxify ROS or their products; (ii) inhibit NOX activation; or (iii) avoid activation of cells. Examples of each of these strategies have been observed in CGD pathogens and may represent possible targets for antimicrobial therapeutics. Importantly, given the number of host defenses that are triggered by ROS, microbial inhibition of NOX activation may do far more than protect the microbe from oxidative damage.

In addition to catalase, many microbes also produce peroxidases and superoxide dismutase (SOD). SOD production by *S. aureus* was not associated with virulence in wild-type mice [112] and addition of SOD did not increase virulence. However, deletion of SOD from *Candida albicans* does increase sensitivity to macrophages [125]. The variable impact of ROS-detoxifying enzymes on microbial pathogenesis has prevented a simple model of the role of ROS in host defenses from emerging. Importantly, pathogens dynamically regulate genes involved in resistance to oxidative stress. Our laboratory, in collaboration with the LCID, showed that

A. fumigatus exposed to primary human PMN from normal donors upregulated catalase and SOD while those exposed to CGD PMN did not [126] indicating that oxidative stress from host-derived ROS induces a dynamic microbial response.

A striking example of microbial inhibition of NOX activation exists in the secondary metabolite, gliotoxin, of *A. fumigatus*. Gliotoxin inhibits several steps in NADPH oxidase activation including $p47^{PHOX}$ phosphorylation and membrane translocation of cytosolic components [127] and is present at bioactive levels in human biopsy specimens [128]. Gene deletion studies performed by the Kwon-Chung's group showed that removal of either a transcriptional regulator (LaeA) or a non-ribosomal peptide synthase (GliP) involved in gliotoxin synthesis resulted in significantly attenuated virulence *in vivo* and diminished or absent production of gliotoxin [129,130]. In collaboration, our laboratory demonstrated that while culture supernatants from wild type or gene-complemented *A. fumigatus* inhibited O_2^- production by PMN, similar supernatants from GliP- [129] or LaeA-deficient [130] fungi did not.

Cellular activation by microbes is a complex phenomenon involving both microbial factors (e.g., LPS, fMLP) and host factors (complement, cytokines). Our laboratory's studies of *G. bethesdensis* (see above) provide an example of a microbe that is 10–100 times less stimulatory of NOX activity than *E. coli* [published abstract [124]]. Studies are underway to identify how this bacterium eludes host recognition. Other pathogens, for example *Neisseria gonorrhoeae,* are known to suppress NOX activation in a contact-dependent manner that is still being elucidated [131] and several pathogens with relatively non-stimulatory LPS have been described, for example *Francisella tularensis* [132].

34.4.2 Inflammatory Disorders in CGD

CGD is clearly more than simply an increased susceptibility to certain infections. Early case reports noted the granulomatous inflammatory process with a predilection for the liver and lungs sometimes leading to organ failure and death [1–4]. Prior to the advent of biochemical and genetic testing, the characteristic granuloma formation in children with frequent infections was thought to be diagnostic for CGD. However, it is not always clear whether inflammatory disorders in CGD are a consequence of infection, or occur in the absence of infection. Granulomata are a common pathologic finding in many diseases ranging from mycobacterial infection to autoimmune diseases such as Wegener's granulomatosis and sarcoidosis. Granuloma formation in mycobacterial infection is presumed to be the consequence the persistence of intracellular organisms resulting in chronic local tissue damage and accumulation of PMN, macrophages and other cells that attempt to isolate the offending microbe. As stated above, efforts to identify microbes in granulomatous lesions from CGD patients are often unsuccessful [133,134]. Furthermore, formation and persistence of granulomata does not appear to be altered by either prophylactic or high dose antibiotic regimens suggesting that viable microbes are not required. Finally, corticosteroids are efficacious in treating CGD granulomata, a finding that would not be expected if the lesions were due solely to infectious processes [133,135,136].

As was recognized in the initial pathologic descriptions, the granulomas of CGD are distinguished by their increased numbers of plasma cells and pigmented histiocytes [1–3]. Pigmented lipid histiocytes were seen in other diseases, but their association with granulomas and various staining properties was unique. Since other patients with recurrent infections (e.g., cystic fibrosis, Hurler's disease, aplastic anemia and hypogammaglobulinemia) do not demonstrate this lipid histiocytosis, it was proposed that the histiocytosis was the cause of the susceptibility to infection. It is now known that lipid-laden histiocytes are common in CGD, but uncommon in other granulomatous diseases [134,137,138]. Histochemical staining and electron microscopy suggest that the pigment is likely the product of oxidation of bacterial lipid residues that forms conglomerated lipofuscin within the lysosomes and accumulates as a result of impaired clearance of intracellular organisms by CGD phagocytes [134,137,139].

Inflammation in CGD often has other features differentiating it from non-specific acute or chronic inflammation[134] including a relative paucity of PMN compared to acute inflammatory processes, prominent eosinophils, abundant nuclear debris and granulomata. These features are also useful to differentiate CGD from other inflammatory diseases such as Crohn's disease and tuberculosis [134]. The granulomata themselves are generally non-caseating and may contain large numbers of giant cells [134] although neutrophilic microabscess with pallisading histiocytes were described here at the NIH [140]. The inflammatory manifestations of CGD are the result of localized, often chronic damage to particular organs or organ systems rather than systemic inflammation. Granulomas may form large, macroscopic lesions resulting in mechanical disturbances, or may be microscopic as part of more diffuse organ inflammation. As with other forms of chronic inflammation, fibrosis may also be present.

34.4.2.1 Inflammation of the Gastrointestinal Tract

By far the most commonly recognized non-infectious manifestations of CGD occur in the GI system [141]. In fact, GI involvement was one of the earliest recognized manifestations of CGD [142]. In a series of 140 patients with CGD

followed at the NIH, more than 30% of patients have GI manifestations [136]. This finding was confirmed in an analysis of 368 patients reported in the national CGD registry, which includes the patients followed at the NIH, in which nearly 34% of patients had some GI manifestation [32]. A history of pyloric stenosis in a newborn should prompt inclusion of CGD in the differential diagnosis. Localized or diffuse GI inflammation can occur at any point in the GI tract causing symptoms clinically similar to autoimmune diseases such as Crohn's disease, or mechanical obstruction due to formation of large granulomas requiring surgical intervention. In the NIAID series, all of the patients with GI symptoms had abdominal pain, but other symptoms were also common (Table 34.3) [136]. Despite aggressive treatment including surgical intervention, corticosteroids, and anti-tumor necrosis factor (TNF) therapy with infliximab, nearly 11% of patients in this series had recurrent or unremitting GI symptoms. Although malabsorption resulting in hypoalbuminemia was common (70%) and often resulted in failure to thrive and growth restriction, most (>80%) had laboratory values including hemograms that were normal.

The most common site of GI obstruction is the distal stomach resulting in symptoms typical of gastric outlet obstruction in 15%-50% of patients [143] and which may be the presenting complaint for some CGD patients [32,144–147]. Obstructive lesions can also occur in the esophagus and duodenum [136] with an average age at onset of 44 months [144]. Diffuse inflammation of the GI tract (most commonly the distal small bowel and colon) is more frequent and more severe in X-CGD compared to A-CGD [32,136,148] and many patients are given a diagnosis of ulcerative colitis or Crohn's disease. Anatomic findings include friable mucosa with pseudopolyps, ulcerations, petechial hemorrhages, fissures and strictures [148]. Much like Crohn's disease, the lesions seen in CGD tend to be patchy in distribution but can become confluent in severe cases. Histologic examination reveals cryptitis and crypt abscess formation with infiltrates of eosinophils and macrophages that often contain lipid pigments. PMN are present but are typically less prevalent than in other inflammatory bowel diseases. Granulomas associated with Crohn's disease in CGD patients are well formed with dense histiocytes surrounded by a cuff of lymphocytes [137]. By comparison, in Crohn's disease in non-CGD patients the granulomas are more loosely formed and less prevalent, and eosinophilic infiltrates and pigmented histiocytes are not typically seen which allows for differentiation between the diseases by experienced pathologists [136,148]. GI manifestations such as acute obstructive lesions are very responsive to short bursts of high-dose corticosteroids and longer courses at lower doses have been used for maintainance [133,136,149,150]. Long-term toxicity and relapse in up to 70% of patients once off treatment, however, has lead to exploration of other immunomodulatory approaches such as TNF-α blockade and sulfasalazine [136,148]. As in other inflammatory bowel diseases, surgery is sometimes required.

Table 34.3 Prevalence of GI Symptoms in CGD Patients

Symptoms	% Reporting
Abdominal Pain	100
Diarrhea	39
Nausea/Vomiting	24
GI Tract Obstruction	35
Gastric Outlet	15
Esophagus	7
Duodenum	7
Other	7

* modified from Marciano et al. [136]

34.4.2.2 Inflammation of the Urogential Tract

Urogenital complications are common in CGD and include obstructive uropathy due to thickening of the bladder wall, granuloma-associated obstruction, or ulceration [141,151–154]. Chronic cystitis with hematuria, and even renal insufficiency resulting from chronic obstruction or infection is also relatively common [155,156]. Interestingly, an eosinophilic cystitis has also been described in children with CGD who are otherwise asymptomatic [151]. Whether urogenital lesions are the result of chronic or recurrent infections, urostasis, or other causes is unknown, however treatment with corticosteroids is frequently successful. *Pseudomonas* bladder infections and kidney abscesses have been reported in CGD patients [157] and antibiotics may be effective in some cases [158].

34.4.2.3 Inflammation of the Skin

Inflammatory disorders of the skin are extremely common in CGD. In addition to an increased risk of cutaneous infections including cellulitis and abscess formation, biopsies of apparently unaffected skin reveals granulomatous inflammatory changes containing the same pigmented histiocytes seen in inflamed GI tissues [159]. In 1983, this laboratory described a prolonged inflammatory response in skin window studies in CGD patients compared to normal volunteers [160]. Normal accumulation of PMN was observed in CGD patients during the first 5 hours. However, unlike the normal patients in whom the PMN are typically replaced by monocytes during the 8 to 24 hour period, PMN persisted alongside the monocytes in the CGD patients. These findings suggest there is a pathological maintainance of acute inflammation in CGD and that ROS-dependent processes normally modulate acute inflammation.

Table 34.4 Autoimmune Manifestations of CGD

Inflammatory Bowel Disease
Anti-phospholipid Syndrome
IgA nephropathy
Juvenile Rheumatoid Arthritis
Cutaneous and Systemic Lupus Erythematosus
Autoimmune Thrombocytopenia
Idiopathic Thrombocytopenic Purpura
Rheumatoid Arthritis
Eosinophilic Cystitis
Sarcoidosis
Celiac Disease with Hemosiderosis

Studies utilizing gp91^{PHOX}- [56] or p47^{PHOX}-knockout mice [77] demonstrated that intraperitoneal challenge with the sterile irritant thioglycollate induced a neutrophilic leukocytosis significantly greater than that observed in normal mice. This abnormally robust inflammatory response may explain, in part, why the cutaneous manifestations observed in patients with CGD are similar to autoimmune conditions. Indeed, cutaneous lesions resembling those seen in discoid lupus are among the most common manifestations in X-CGD carriers and some patients [141,145] and cannot be readily differentiated from discoid lupus by histologic examination [161]. It has also been shown that the occurrence of both lupus-like lesions and aphthous stomatis, another common finding in CGD patients, is closely related to the degree to which O_2^- formation is reduced [161]. In light of this finding, it is particularly interesting that carrier mothers of X-CGD appear to be even more susceptible to these manifestations than the patients.

34.4.2.4 Autoimmune Disease and CGD

As a result of a somewhat more inclusive definition of autoimmune disease, autoimmune conditions are being increasingly recognized as part of the disease complex in patients with many different immunodeficiencies. In CGD in particular, several autoimmune diseases are common. Recently, NIAID researchers reported on the increased incidence of autoimmune disease including patients with anti-phospholipid syndrome, IgA nephropathy, juvenile rheumatoid arthritis, cutaneous lupus or recurrent aphthous stomatitis [162]. In addition, up to 37% of CGD carriers also complain of joint pain that improves during corticosteroid treatment of lupus-like skin lesions [163], and there are isolated reports of autoimmune thrombocytopenia [164], idiopathic thrombocytopenic purpura [165], rheumatoid arthritis [5,98,166], eosinophilic cystitis [151], IgA nephropathy [167], sarcoidosis [168], and celiac disease with pulmonary hemosiderosis [169]. In contrast to the relatively low incidence of autoimmune conditions in the general population, more than 10% of the CGD patients followed at the NIH have an autoimmune complication other than inflammatory bowel disease [162]. The autoimmune complications in these patients are responsive to immunosuppresive therapy and corticosteroids or steroid-sparing agents such as methotrexate and anti-TNF-α agents should be considered despite the potential increased risk of infection.

The underlying etiology of autoimmune disease in CGD and most other primary immunodeficiencies remains unknown. Studies at the NCI examined seven genes involved in inflammation (MPO, mannose binding lectin (MBL), Fcγ receptors IIa, IIIa, and IIIb, TNF-α, and IL-1 receptor) in a cohort of 129 CGD patients [145]. Several MPO, FcγRIIIb, and FcγRIIa genotypes were associated with GI complications while variant MBL and FcγRIIa alleles associated with an increased risk of autoimmune or rheumatologic disorders. Since that time, Fcγ receptor polymorphisms have been associated with an increased risk of many infectious and inflammatory disorders [170], but have never been shown to be more common in patients with CGD.

34.4.2.5 Lung Involvement in CGD

In patients with CGD, a destructive process occurs in the lungs of some patients resulting in the loss of lung tissue and formation of large bullae with loss of the normal architecture. Early reports compared CGD to sarcoidosis due to the presence of granulomas in the lungs, but also noted that the wide distribution of granulomas and young age at onset were very atypical for sarcoidosis. On pathological examination, chronic inflammation and granuloma formation are often seen in lungs and pulmonary lymph nodes of CGD patients [134]. Due to frequent pulmonary infections in CGD, it remains unclear whether this inflammation is the result of chronic infection, or due to a separate inflammatory process like those described above. As discussed in greater detail elsewhere, some sterile irritants can induce granuloma formation in CGD mice [171] suggesting that deranged inflammation, rather than infection alone, may play a major role in lung pathogenesis. Finally, symptoms of atopy in CGD patients and their unaffected siblings were compared and only exercise-induced wheezing occurred more frequently in the CGD patients [172].

34.4.2.6 Nervous System Involvement in CGD

Although discrete non-infectious brain lesions are uncommon, inflammatory manifestations involving the central nervous system (CNS) are recognized and include granulomas and

lipid-laden histiocytes [173,174]. Cognitive deficits have been recognized in some CGD patients with 23% having an IQ of ≤70 [175]. Although chronic illness and frequent and prolonged hospitalization may contribute to this, over the past several years new roles for NADPH-oxidases (including NOX2) in normal brain function have been described [176]. Alterations in neurophysiology related to abnormal NADPH oxidase expression in microglia [177–179] and astrocytes [180] have been identified. Microglia require $p47^{PHOX}$ for normal O_2^- production [181] and work here and elsewhere has shown that both $p47^{PHOX}$- and $gp91^{PHOX}$-deficient mice display cognitive deficits [182]. It has also been shown that NADPH oxidase is highly expressed in the synapses of neurons suggesting this enzyme may generate the reactive oxygen species required for normal neuronal function [183,184].

34.4.2.7 Ocular Involvement in CGD

Chorioretinal lesions have been described in CGD since early reports of the disease [4]. In a more recent study, ¼ of X-CGD patients had similar lesions with a "punched out" appearance, retinal vessel bowing and pigmented margins [185]. Interestingly, several X-CGD carriers also exhibited the same lesions. It has been proposed that the retinal pigment epithelial cells are affected, but the pathophysiology of these lesions had not been fully elucidated. Blepharoconjunctivitis and corneal thinning have also been described in patients with PMN functional defects including CGD, Job's syndrome, Chediak-Higashi syndrome and others [186] and may be a consequence of abnormal regulation of eyelid flora.

34.4.2.8 Skeletal System Involvement in CGD

Infections of bone are a common occurrence in CGD patients and sometimes lack typical signs (pain, fever, local inflammation) [103]. Most infections occur through spread from adjacent loci (e.g., rib or vertebrae from pulmonary aspergillosis) however some cases of hematogenous spread to bone have been noted [187].

34.4.3 Atherosclerosis and Malignancy in CGD

Free radical stress is felt to play an important role in the development of both atherosclerosis and malignancy. In the pathogenesis of atherosclerosis, oxidation of lipids within atheromatous plaques may hasten progression of the lesions and lead to plaque rupture resulting in myocardial infarction. It has been suggested, therefore, that patients with CGD are protected from atherosclerosis. Although this has not been systematically studied in humans, experiments in mice suggest that this is not always the case. In a study examining the role of O_2^- in atherogenesis, mice overexpressing cytosolic SOD did not demonstrate an accelerated rate of atherosclerosis [188]. Several NADPH oxidases are present in vessel walls including $gp91^{PHOX}$ and Nox4 [189,190]. In one study, $gp91^{PHOX}$-deficient mice were crossed with mice lacking apolipoprotein E (ApoE) which are highly susceptible to developing atherosclerosis [191]. ApoE/$gp91^{PHOX}$ double knockout mice developed had elevated lipid levels but developed atherosclerotic lesions at the same rate as non-CGD ApoE-/- mice. In a subsequent study using the same murine model, $gp91^{PHOX}$-deficient vascular smooth muscle cells were actually shown to proliferate somewhat faster than wild type cells [192]. In that study, however, $p47^{PHOX}$ knockout mice were also crossed with ApoE knockouts and demonstrated decreased smooth muscle cell proliferation, less vascular ROS production, and significantly fewer atherosclerotic lesions. It is possible that other free radical producing enzymes present in vessel wall cells produce sufficient O_2^- to promote atherosclerosis. It has been suggested, for example, that the oxidase mox1 can supplement O_2^- production in $gp91^{PHOX}$-deficient smooth muscle cells [192]. However, a murine model in which $p47^{PHOX}$ -/- monocytes were transplanted into ApoE-/- mice demonstrated that monocyte-derived ROS is important for development of atherosclerotic lesions [193]. The study of atherosclerosis in humans is made difficult by the rarity of CGD and the small number of patients who live to an age at which they would normally develop cardiovascular disease.

As with cardiovascular disease, the natural history of most malignancies prevents them from being easily studied in patients with CGD. Since O_2^- and other free radicals can damage DNA, it has been hypothesized that CGD patients would be somewhat protected from developing cancer. On the other hand, CGD is disease of chronic inflammation that may be expect to predispose to transformation. For example, *Mycobacterium tuberculosis* infections have, in some cases, been associated with carcinoma [194]. It is well recognized that the risk of malignancy is increased in AIDS, but studies have also demonstrated an increased risk in primary immunodeficiencies including CVID and WHIM syndrome [195,196]. A 1996 study from the Netherlands reported a patient with $p47^{PHOX}$-deficient CGD who developed retinoblastoma at age 1, another with $p47^{PHOX}$-deficient CGD who developed melanoma at age 26, and an X-CGD patient who developed rhabdomyosarcoma of the liver at age seven [197]. Based on this, the authors calculated that the relative risk of developing any malignancy was 13.8 compared to the general Dutch population, and 93.4 for the specific malignancies described. Acute lymphoblastic leukemia in an

X-CGD patient has also been reported as the first occurrence of malignancy in the Israeli cohort of 23 CGD patients [198]. Both papers suggest that CGD patients may be predisposed to malignancy due to impairment of antibody-dependent cellular cytotoxicity, however there are conflicting reports of whether extracellular killing by this mechanism is affected in CGD [199–201]. An analysis of large patient cohorts will be required to determine the true incidence of malignancy in patients with CGD.

34.4.4 Function of Lymphocytes in CGD

While the role of NADPH oxidase in myeloid cells is well described, altered T- and B-cell number and function has also been reported. Although antibody responses to specific antigens in CGD patients appear to be normal [3,4] hypergammaglobulinemia was not correlated with disease severity [1,4] and was one of the early and still unexplained clinical observations. Recently, workers at the NIH have reported that CGD patients and carriers display a reduced memory B-cell population although other aspects of B-cell function (e.g., somatic mutation) are normal [202]. In CGD patients less than 3 yrs of age, increased numbers of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes were observed compared to age-matched controls [203], although another study found decreased $CD3^+$ lymphocytes in CGD patients in this age group [204]. In both studies, the differences in $CD3^+$ lymphocytes diminished as the patients aged. $CD4^+CD29^+$ memory T-cells and $CD8^+CD11b^+$ suppressor T-cells have been reported to be diminished in CGD in patients ≤19 yrs [204]. Diminished PHA-driven proliferation of PBMC and decreased CD4+ CD40 ligand levels have been observed in CGD patient cells [205]. A role of the NADPH oxidase in the regulation of T-cell apoptosis [206] has been explored by Sharon Jackson's group (LHD/NIAID) and is presented elsewhere in this volume.

34.5 The X-CGD Carrier State

While CGD was originally described as an X-linked disease that occurred in young boys, a disease with many characteristics of CGD was described in three young girls in 1962 [5]. Quie and Baehner later described three female children with the characteristic phenotype of CGD and nearly the same severity as that seen in the previously reported male children [6,7]. Several hypotheses were suggested for the occurrence of the disease in females including the presence of a carrier phenotype and an alternate inheritance pattern causing a similar disease. It has subsequently been shown that both are true.

The early presumption that CGD was an X-linked disorder was supported by the description of an intermediate phenotype of abnormal NBT reduction and bactericidal activity in mothers of affected boys [15,207]. However, abnormal bactericidal activity and NBT reduction of intermediate severity were subsequently demonstrated in both parents of two brothers with CGD [208]. Since the father could not be a silent carrier of an X-linked disorder, this lent support for an autosomal mode of inheritance. A subsequent analysis of 8 families of children with CGD revealed bactericidal and NBT reduction abnormalities in many of the fathers, all of whom were presumably clinically unaffected[9]. Ultimately, it was determined that CGD could be either an X-linked or autosomal recessive disease confirming both hypotheses. While this explained some of the variability in the severity of the disease, other characteristics of carriers were also recognized and are contrasted with the clinical phenotype of CGD patients (both X- and A-CGD) in Figure 34.3.

Carriers of X-CGD are usually free of severe infectious complications despite significantly subnormal production of O_2^- in response to particulate stimulus [209]. Due to the fact that there is no selective advantage for cells that make O_2^- or those that do not, carriers have a mixed population of competent and incompetent PMN [210]. The risk of infection is felt to be determined by the percentage of functional PMN present. While most carriers produce sufficient normal PMN to avoid infection, several have been described who exhibit significant skewing of X-chromosome inactivation resulting in the production of a large proportion of defective PMN [211–214]. One X-CGD carrier contracted aspergillosis following a progressive, apparently age-related decrease from 40% normal at 21 years of age to 6% normal PMN at age 45 [212]. Although carriers are often asymptomatic, they do sometimes exhibit manifestations that are similar to patients with CGD and some that are unique to the carrier state (Figure 34.3). A lupus-like autoimmune condition can occur in X-CGD patients, but is even more common in carriers [141,145]. In one study, half of the carrier mothers reported photosensitive skin rash, recurrent aphthous ulcers, or extreme fatigue, and 1/3 reported joint pain [163]. Interestingly, in this and other studies reviewed by those authors, antinuclear antibody testing was positive in only a small fraction of the carriers. A lupus-like disease is present in patients with A-CGD as well, but is not present in autosomal heterozygous carriers [32].

Patients with so-called variant CGD have also been reported [98]. These patients were older and presented with a phenotype of mild infections but with polyarthritis, pulmonary fibrosis, and glomerulonephritis. As this description predated genetic testing for CGD, it is not clear what defect caused the disease in these patients. In light of the documented defects in NBT reduction, H_2O_2 production, and oxygen consumption, it is likely that these and other

reported variant CGD phenotypes are actually attributable to A-CGD. Traditionally, carriers of A-CGD are considered to be asymptomatic. It remains possible, however that the wide variability in both severity and age may be in part due to manifestations of CGD in some A-CGD carriers. Importantly, carrier states have been variably reported and further efforts utilizing modern methods, such as the DHR assay and gene sequencing, will be required to explicitly determine whether heterozygous autosomal individuals have a clinically relevant phenotype.

In summary, the clinical manifestations of CGD include many inflammatory complications and autoimmune conditions involving most organ systems in the body (Figure 34.3). While infection remains the most common cause of mortality, these non-infectious manifestations can be lethal and are the source of significant morbidity in these patients. Immunomodulatory therapy (e.g., corticosteroids, TNF-α blocking agents) is usually beneficial in the treatment of inflammatory bowel disease and may promote resolution of complications due to granuloma formation and possibly other autoimmune conditions [133,136,162,215,216], however it must be carefully considered and monitored in patients with underlying immunodeficiency. X-CGD carriers can benefit from active clinical management and should be monitored closely for autoimmune and even infectious problems.

34.6 Clinical Management of CGD Patients

Initial attempts to treat CGD were hampered by ignorance of its cause. Antibiotic therapy was attempted in all of the early patients with little or no benefit [1,2] which called into question the underlying etiology, however neither long courses of antibiotics nor prophylactic regimens were typically used. Ultimately, increased choice of antibiotics and the aggressiveness with which they were used in conjunction with surgical drainage of suppurative lesions [217] improved survival.

34.6.1 Antibiotic Therapy and Prophylaxis

Recurrent, often life-threatening infections are the most recognized consequence of CGD. In the first several clinical reports, relatively short courses of antibiotics were used without apparent benefit [1–3]. Longer courses of antibiotics were tried with some success [4,12]. By 1968, it was recognized that early use

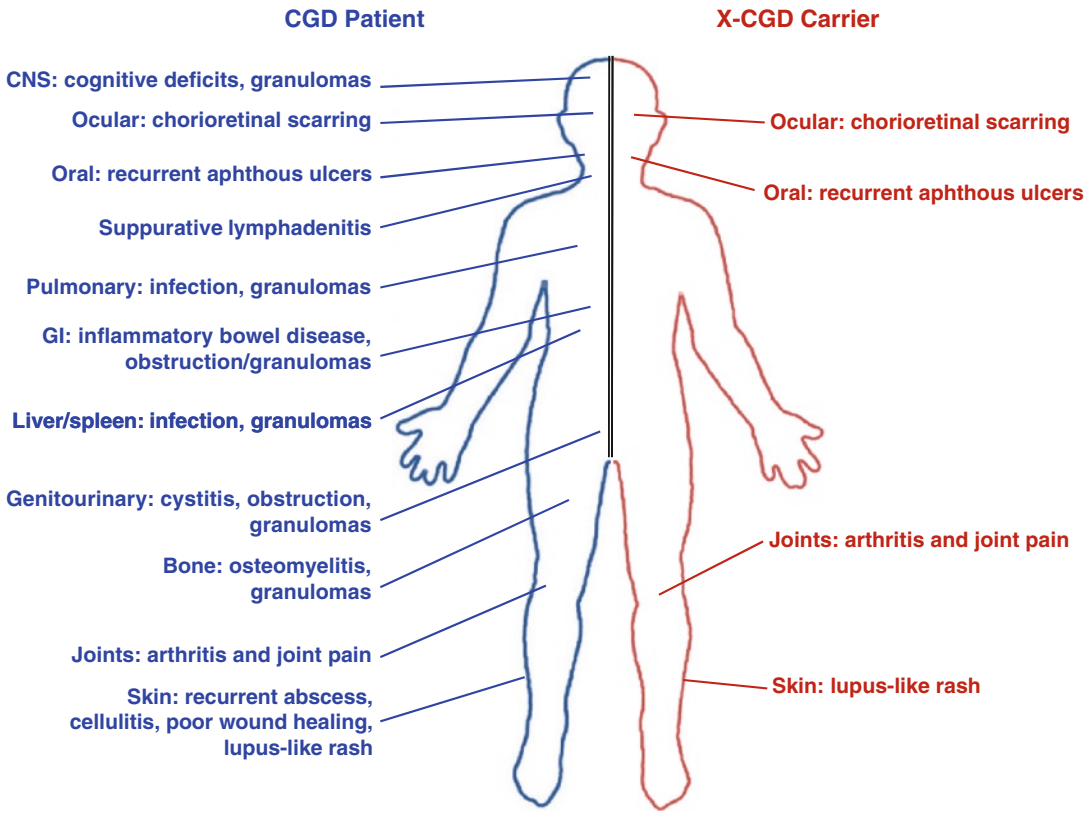

Fig. 34.3 Clinical manifestations of CGD in patients and carriers

of prolonged courses of antibiotics could reduce the frequency of serious infection; however the efficacy and safety of prophylactic antibiotics to prevent infection remained in doubt [218]. The use of continuous nafcillin therapy was reported in 1972 to decrease both total days of hospitalization/yr and number of surgical interventions in a group of six CGD patients [219]. It is recognized that patients with CGD frequently do not have the typical signs and symptoms of infection [99,103]. In a series of CGD patients with aspergillosis followed at the NIH, fully 1/3 were asymptomatic at diagnosis, and only 20% were febrile [220]. As a result, clinicians must be vigilant in not only searching out, but also treating and preventing infection in patients with CGD. As previously stated, patients with CGD are susceptible to certain organisms (see Section 34.4.1). It is now clear, from work done at the NIH and elsewhere, that prophylaxis with trimethoprim-sulfamethoxazole can significantly reduce the number and severity of infections in these patients as it is effective against most of the commonly encountered bacteria in CGD [99,221,222].

Although bacterial infections were recognized early, and candidiasis was reported in early papers, it was not until a decade later that the role of pathogenic fungal infection was fully appreciated [223]. *Aspergillus* species are frequent fungal pathogens in CGD but other organisms also occur (see Table 34.2). Use of antifungal drugs, such as amphotericin, itraconazole and others has been the focus of a great deal of study at the NIH. While amphotericin is clearly effective and was initially the only antifungal agent available, toxicity is often dose limiting or precludes its use. Itraconazole was found to be efficacious in treating *Aspergillus* [224–226] and a 10-year trial conducted at the NIH demonstrated that prophylactic itraconazole significantly reduced fungal infections in CGD patients without serious adverse consequences [119]. The efficacy and safety of posaconazole, voriconazole [227,228] and caspofungin [229] have been demonstrated in other conditions predisposing to fungal infections and are being actively used to treat patients with aspergillosis.

34.6.2 Interferon-γ (IFN-γ) Prophylaxis

In PMN from many CGD patients, especially those with the autosomal recessive mutations, $O_2^{\cdot-}$ production still occurs, albeit at levels 10–100 times lower than in normal PMN. As described above, the catalytic center of the NOX complex resides in $gp91^{PHOX}$ and the cytosolic components are regulators of activity rather than obligate coenzymes. This may contribute to the somewhat better prognosis of CGD patients with defects in cytosolic factors and may provide a basis for IFN-γ therapy.

As early as 1983, it was recognized that IFN-γ could regulate macrophage oxidative and antimicrobial activity [230]. Furthermore, IFN-γ increased the respiratory burst in normal PMN by a mechanism requiring protein synthesis [231]. *Ex vivo* IFN-γ treatment of PMN and monocytes from normal and CGD patients resulted in increased $O_2^{\cdot-}$ production by all cells except those that failed to produce any $O_2^{\cdot-}$ at baseline [232]. Independently, investigators from the Laboratory of Clinical Investigation (NIAID) cultured CGD monocytes with IFN-γ and found significantly greater production of $O_2^{\cdot-}$ by cells from nearly all of the A-CGD patients and a significant minority of the cytochrome b_{558}-negative patients [233]. Three patients whose cells responded to IFN-γ *in vitro* were selected for a limited *in vivo* trial of subcutaneous IFN-γ that resulted in increased $O_2^{\cdot-}$ production for up to one week after IFN-γ administration [233]. Similar results were reported by Ezekowitz and coworkers [234]. In both studies, increased *S. aureus* killing did not correlate with $O_2^{\cdot-}$ production suggesting that the effects of IFN-γ may also occur by mechanisms independent of NOX activity. These positive results lead to a multi-center, double-blind, randomized, controlled trial of IFN-γ therapy in 128 patients with CGD followed for 10 months [235]. Significant reductions were found both in the number of patients experiencing infections and the number of infections per patient in the IFNγ group. Furthermore, the use of IFN-γ was beneficial regardless of the age of the patient, use of prophylactic antibiotics, or the mode of inheritance of the disease. Based on the later study, IFN-γ was licensed by the FDA for prophylactic use in CGD.

Over the next several years, additional studies reported similar results and safety profiles for IFN-γ therapy [236,237]. Long-term safety of IFN-γ was studied in 76 CGD patients followed at the NIH during treatment for up to 9 years [238]. The rate of serious bacterial or fungal infection was 0.3 per year compared to 1.1 per year in patients not on IFN-γ therapy [235]. Furthermore, although 47% of patients reported mild adverse effects of IFN-γ therapy, this was usually limited to fevers and myalgias that responded to acetaminophen and did not require discontinuation of therapy. There was no increase in the incidence of inflammatory complications. Thus, IFN-γ treatment in CGD appears efficacious and generally well-tolerated over long periods. Its use is far from universally accepted because some patients do not appear to respond clinically. Analysis of the association of certain types of mutations in CGD with IFN-γ responsiveness is currently underway in our laboratory and will be presented elsewhere (Kuhns et al. in preparation).

34.6.3 Leukocyte Transfusion

Even before the biochemical abnormality in CGD was identified, efforts were made to provide normal leukocytes to CGD patients in the hope of treating life-threatening infections.

In an early study of CGD patients, the function of circulating leukocytes collected 4 hours after transfusion of normal cells was no different than prior to the transfusion [217]. The first success occurred in the salvage therapy of a pediatric CGD patient with advanced pulmonary aspergillosis who was treated with $5x10^{10}$ granulocytes isolated from his father [239]. Despite a minor HLA mismatch, a rapid and significant clinical improvement was observed. Moreover, leukocytes isolated 5 minutes after transfer demonstrated near normal killing activity that returned to the patient's baseline poor activity by 18 hours after the transfusion. Interestingly, there was only a very mild improvement in O_2^- production, even 5 minutes after transfusion. By the end of the 1970s, two additional reports of granulocyte transfusion in CGD patients were published [240, 241]. These studies demonstrated significantly better donor PMN survival due to more careful HLA matching. In some cases, NBT positive leukocytes were present for up to 42 hours following transfusion [242].

Therapeutic leukocyte transfusion was developed for CGD throughout the next decade both at the NIH and at other centers [243], although generally only for patients who failed to respond to aggressive conventional therapy. Over the next several years, leukocyte transfusions were given to patients to treat specific infections, such as *Aspergillus* and *Pneumocystis carinii* that were resistant to treatment [109]. Intralesional granulocyte instillation was combined with IFN-γ therapy in the treatment of a hepatic abscess in one patient followed at the NIH [244]. More recently, leukocyte transfusion was given prior to bone marrow transplant to a CGD patient with aspergillosis [245] and it is still used as adjunctive therapy by some centers for patients undergoing bone marrow transplant.

34.6.4 Bone Marrow/ Hematopoetic Stem Cell Transplant

In addition to therapies aimed at ameliorating symptoms by treating infections or reducing inflammation, there is also significant work being done to develop potential cures for CGD. Among these are bone marrow transplant, hematopoetic stem cell transplant, and gene therapy.

The first reported bone marrow transplant for CGD was performed in 1973 at the Westminster Hospital (London, UK) [246]. The 2 year old patient had nearly continuous infections requiring antibiotics and no O_2^- production by patient neutrophils was detected. An HLA-matched unrelated female donor was found and confirmed to be non-reactive by mixed lymphocyte cultures. Cyclophosphamide was employed prior to transplant to provide receptive niches for transplanted donor cells (marrow pre-conditioning). The patient received $5.5x10^9$ nucleated donor cells and within 12 days, circulating NBT positive donor PMN were detected. Three months post-transplant, 12% of peripheral PMN were NBT positive and significant clinical improvement of infection was observed. The patient did well clinically after the transplant and it was learned that a very small population of normal PMN, estimated to be between $1\text{-}5x10^8$/L, can be protective against infection.

Further bone marrow transplant efforts in CGD were met with complications of graft rejection and graft versus host disease [247,248]. Based on their experience with bone marrow transplant in patients with thalassemia, osteopetrosis, and Wiskott-Aldrich syndrome, Kamani and colleagues added busulfan to the preconditioning regimen [249]. This CGD recipient achieved 23% donor engraftment and remained free from infections for six years after the transplant.

Between 1985 and 1999, eight more CGD patients underwent bone marrow transplant and nearly all had 100% engraftment and marked clinical improvement without graft rejection [250–257]. Two patients developed veno-occulsive disease [252,254] and one developed graft versus host disease that was successfully managed with standard therapy [251].

In treating these initial patients, a preconditioning regimen of busulfan and cyclophosphamide was found to be effective in suppressing both graft versus host disease and graft rejection in patients with CGD. What remained unclear, however, was the optimal time to transplant these patients to minimize the risks of transplant but also reduce the accumulation of irreversible tissue damage due to infection and granuloma formation. A review of the 27 reported patients given a bone marrow transplant between 1985 and 2000 confirmed that a myeloablative regimen with busulfan followed by HLA-matched transplant was effective in treating CGD [258]. Myelosuppresive regimens were found to be less effective. Graft versus host disease occurred in 11 patients, of whom one died. At a median follow up of two years, 23 of the 27 patients survived and were doing well, and 22 had been cured of their CGD. An important fact gleaned from this set of patients is that survival was 100% for those transplanted without infection at the time of the procedure.

Despite the successes of bone marrow transplant, high dose chemotherapy carries the risk of infection and organ toxicity. Consequently, methods were sought to reduce the need for myeloablation in favor of myelosuppression. In a trial conducted at the NIH, a myelosuppressive regimen of anti-thymocyte globulin, cyclophosphamide and fludarabine produced excellent results with 60% develop-

ing full engraftment [259]. Three patients died from infection and although most of the adult patients had either graft rejection or acute graft versus host disease none died from graft-related complications. It was recognized as early as 1964 that transplantation of hematopoietic stem cells could be performed [260] and was an alternative to bone marrow transplant for the treatment of malignant and nonmalignant hematologic diseases [261]. Hematopoietic stem cell transplant was tested in a 6 year old boy with CGD using hematopoietic stem cells from an HLA-matched sister and a myelosuppressive preconditioning regimen [262]. Three months post transplant, the patient underwent donor lymphocyte infusion (DLI) to ablate the remaining abnormal PMN. This resulted in 100% donor cell engraftment and normal O_2^- production with no graft versus host disease two years post transplant. Hematopoietic stem cell transplant in CGD using umbilical cord blood as a source of hematopoietic stem cells was reported [263]. Preconditioning consisted of busulfan and cyclophosphamide and the patient achieved 87% engraftment by day 33 that continued to increase at one year post-transplant and no graft versus host disease.

Although these techniques have been widely utilized, concerns remain regarding the optimum preconditioning regimen and age for transplant. Transplant procedures are associated with significant risks for both graft versus host disease and graft rejection and improvements are needed and deserve further exploration.

34.6.5 Gene Therapy

The most exciting, and perhaps the most promising, treatment being developed for CGD is gene therapy. While the desirability of gene therapy in general was first discussed as early as 1947 by Keeler [264], it was not until the 1960's that proof-of-principle *in vitro* experiments demonstrated the transfer and expression of corrected genes into deficient cells [265,266]. The first successful gene therapy approach in a human occurred at the NIH Clinical Center in 1990 with the correction of Severe Combined Immunodeficiency due to adenosine deaminase deficiency in a four year old girl [267,268].

The first report of gene therapy attempted for human CGD was published by Harry Malech et al. (Laboratory of Host Defenses/NIAID) in 1997 [269]. Prior experiments demonstrated that viral transduction of a cDNA encoding $p47^{PHOX}$ could restore $p47^{PHOX}$ protein production in $p47^{PHOX}$-deficient CGD EBV B-cells [270].

Vectors with $gp91^{PHOX}$ and $p22^{PHOX}$ cDNAs [271] and $p67^{PHOX}$ cDNA [272] were also developed and could correct $CD34^+$ hematopoetic stem cells in culture. Experiments in murine CGD models firmly established the potential efficacy of *ex vivo* gene therapy by restoring phagocyte oxidant-dependent host defense in $p47^{PHOX}$-deficient mice [273]. As the gene therapy approach consumes large numbers of hematopoietic stem cells from pediatric CGD patients, optimization of GM-CSF dosing and kinetics for mobilization of $CD34^+$ hematopoietic stem cells was required [274]. Finally, in 1997 five patients with $p47^{PHOX}$-deficient CGD were given transfusions of autologous $CD34^+$ hematopoietic stem cells that had been transduced *ex vivo* with a recombinant retrovirus encoding a normal $p47^{PHOX}$ protein [269]. Unlike bone marrow transplant or hematopoietic stem cell transplant performed previously, these patients did not receive preconditioning. Despite this, corrected hematopoietic stem cells populated the bone marrow of patients as demonstrated by the appearance within 3–6 weeks of between 0.004 and 0.05% of NADPH-oxidase positive granulocytes in the peripheral circulation.

Although corrected PMN were detected, the net production of such corrected PMN was insufficient to confer long-term clinical benefit. In a murine model using these transfected cells, it was determined that up to 2.5% of PMN arising *in vivo* could express $gp91^{PHOX}$ using the original vector [275]. An HIV-based lentiviral vector achieved higher yields of $gp91^{PHOX}$-expressing PMN differentiating *in vivo*. Modifications made to the original vector and transduction technique resulted in a significant increase in $gp91^{PHOX}$-expressing PMN to 14%-22% in the murine model [276]. Concurrently, research using similar vectors to improve B- and T-lymphocyte abnormalities in patients with X-SCID that are also sometimes seen in CGD patients after bone marrow transplant was generating successful results [277,278].

Enthusiasm for gene therapy in general was dampened when three of the patients treated for X-linked SCID experienced leukemic T-cell expansions associated with retroviral insertions [279]. Although leukemia has not been seen in the CGD gene therapy trials, some patients have developed clones of cells bearing viral insertions in certain gene loci with some similarities to the treated SCID patients [280]. Research continues to develop next generation gene therapy approaches with safety features such as self-inactivating vectors and insulators to reduce the probability of untoward regulatory interference [275,276].

Although to date no patient has been "cured" of CGD with gene therapy, one patient treated at the NIH had circulating corrected PMN 2 years post-gene therapy with no evidence of oligoclonality or myelodysplasia and an apparent reduction in frequency of infections [281]. Another NIH patient achieved 5% reconstitution of corrected cells

in the peripheral blood but succumbed to infection after these cells were apparently cleared by a host immune mechanism. Despite these mixed results, the curative potential of gene therapy spurs continued research to develop safer and more effective techniques for CGD and many other diseases.

34.6.6 Other Experimental Treatment Concepts

34.6.6.1 Correction of Deficiency by Exogenous Superoxide Generating Systems

Some genetic disorders can be ameliorated by reconstitution with the deficient factor (e.g., hemophilia due to factor VIII deficiency). A similar approach was first considered for CGD in 1970 in experiments employing latex particles coated with glucose oxidase to deliver superoxide-generating capacity to CGD PMN [282,283]. Improvements in both H_2O_2 production and microbial killing were observed. Liposomes containing glucose oxidase were shown to fully reconstitute bleach production and *S. aureus* killing by CGD granulocytes [284]. While the efficacy of this experimental approach has yet to be demonstrated clinically in humans, the potential to circumvent the pitfalls of other approaches is attractive.

34.6.6.2 Enhancement of PMN Antimicrobial Activity by Agents that Increase Phagosomal pH

Several studies suggested that the phagosomal pH was dysregulated in CGD, specifically that the initial increase in pH is due to the NADPH oxidase and is absent in CGD PMN resulting in a lower phagosomal pH [285]. While this phenomenon has been disputed, regulation of phagosomal pH has been considered for therapeutic intervention in CGD. Amantadine is a weak base that increases phagosomal pH and has been FDA-approved for anti-influenza virus prophylaxis and is also used in treating Parkinson's disease [286]. This drug was used to treat a *Pseudomonas aeruginosa* infection in a CGD patient [287]. Two additional CGD patients have been reported to benefit clinically from the addition of amantadine to antibiotic regimens that had been ineffective [288]. In both cases, resolution of disseminated aspergillosis was reported and *in vitro* activity of patient PMN against *Candida* improved. In normal PMN, it has been shown that compounds that raise the phagosomal pH (e.g., chloroquine, NH_4Cl, methylamine and the antibiotic clindamycin) tend to inhibit the respiratory burst [289] however a transient increase in pH may be required for other PMN functions as discussed below.

34.7 Consequences of Aberrant Superoxide Production

Phagocyte production of $O_2^{\cdot-}$ by the NADPH oxidase is clearly deficient in CGD, however what little is known of the functions of $O_2^{\cdot-}$ in host defenses has come mostly from inferential and circumstantial evidence rather than direct proof. Indeed, there is a growing appreciation that $O_2^{\cdot-}$ and its derivatives exert a large number of regulatory effects, the absence of which may be more important in pathogenesis than the absence of the direct microbicidal effects of ROS.

34.7.1 Reactive Oxygen Species (ROS) Absent in CGD

Most biologic activities ascribed to $O_2^{\cdot-}$ have been studied *in vitro* in whole cells, with enzymes that produce $O_2^{\cdot-}$, or with compounds that transform it. Whole cell experiments (using CGD or NADPH oxidase inhibitors) suffer from the presence of myriad other factors that may modify the endpoint being examined. Enzyme studies, for example those employing xanthine oxidase and its substrate hypoxanthine, can produce superoxide *in vitro* and have been used to model direct effects of $O_2^{\cdot-}$ on a variety of processes including microbial killing [290]. Enzymatic production of $O_2^{\cdot-}$ *in vitro*, however, may not adequately mirror the dynamic aspects of natural $O_2^{\cdot-}$ production. Superoxide dismutase (SOD), which catalyzes the reaction $2\,O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$, has been used study the action of $O_2^{\cdot-}$ in certain processes, however because its products include other ROS, namely H_2O_2, it is difficult to draw conclusions about the direct activity of $O_2^{\cdot-}$ itself.

With these caveats in mind, it has been shown that addition of SOD to PMN antimicrobial assays decreases killing of *E. coli* and *S. aureus* [209] and that some SOD-deficient pathogens are less resistant to phagocytes [125]. $O_2^{\cdot-}$ is also likely to form products with other compounds such as NO to form peroxynitrite, thus SOD activity is required to favor production of H_2O_2. SOD activity can also be demonstrated in MPO [291] and, since $O_2^{\cdot-}$ is not likely to diffuse out of the phagosome and contact cytosolic SOD, the presence of this activity in MPO in the phagosome may be important.

H_2O_2 solutions are microbicidal and many organisms are sensitive to H_2O_2 at doses thought to be produced in the PMN phagosome. Moreover, degredation of H_2O_2 by catalase reduces leukocyte antimicrobial activity [209]. Early studies by Klebanoff and co-workers demonstrated that H_2O_2, in the presence of halide ions, is utilized by MPO to form hypochlorous acid (bleach) that can exert potent antimicrobial activity. However, as discussed previously, MPO

deficiency is common but has a far less obvious phenotype than CGD arguing against a major role for MPO-dependent processes *in vivo*.

Interestingly, MPO-deficient PMN display significantly increased O_2^- production [89] suggesting that the MPO pathway is a major consumer of PMN-derived O_2^- and H_2O_2. Compelling biochemical models suggest that O_2^- accumulates in normal phagosomes to levels of ~25μM while H_2O_2 occurs at concentrations of ~2 μM [292]. Removal of MPO from this model results in a 4-fold increase in O_2^- concentration while H_2O_2 remains in the low μM range.

Normal and MPO-deficient PMN, but not CGD PMN, produce the extremely reactive hydroxyl radical (·OH) [293,294]. Singlet oxygen ($^1O_2$) is also produced in PMN. Scavengers of ·OH and $^1O_2$ (mannitol and benzoate, respectively) were found to slightly inhibit phagocytic killing of bacteria [209] however the specificity of these compounds may not be absolute. Nitrogen radicals also play important roles in regulating immunity. NADPH oxidase activation results in consumption of nitric oxide (NO) by normal PMN, a process not seen in CGD PMN [295]. Following NOX- and MPO-dependent production, $HOCl^-$ contributes to the generation of chlorine gas (Cl_2) during phagocytosis [296]. It is expected, but still not shown, that CGD patients would also lack this potentially antimicrobial product. It has been estimated that about 1/3 of the oxygen consumed by PMN during NOX activation is transformed into reactive chlorine compounds [297] and both PMN and microbial proteins appear to be chlorinated during phagocytosis [298]. Another MPO-dependent process occurring in activated PMN is the production of reactive aldehydes from amino acids that contribute to production of advanced glycation end products [299].

Interestingly, the bactericidal activity of ROS was inhibited by PMN granule proteins including MPO at amounts thought to be present in the phagosome [300]. ROS generated by glucose and glucose oxidase failed to kill *Aspergillus* conidia but, when CGD monocytes were added, killing was observed suggesting that other cell-associated factors utilize O_2^- to generate cidal activity [301]. Coupled with the relative health of MPO-deficient individuals, these findings suggest that activities dependent upon O_2^- and other O_2^--dependent ROS may be more important than the direct antimicrobial activities of ROS in normal host defense.

34.7.2 Altered mRNA and Protein Production in CGD

Several groups have performed microarray analysis to study differences in mRNA expression between CGD and normal cells. NIAID researchers identified large numbers of genes that were differentially expressed in CGD and normal PMN both at baseline and following phagocytosis [302]. In particular, genes promoting inflammation (e.g., IL-8) were upregulated in CGD as were anti-apoptotic genes that may prolong PMN lifespan. Similar results were described in CGD monocytes that demonstrated elevated resting and TLR-stimulated levels of inflammatory gene mRNAs and proteins (e.g., IL-6, TNF)[110]. Several immune receptors on PMN were also shown to be dysregulated in CGD at both the protein and mRNA level (e.g., TLR5 and TLR9 were lower whereas TLRs-1, 2, 4 were normal) [303].

Using $p47^{PHOX}$ -/- mice that also expressed an NF-κB luciferase reporter allowing *in vivo* detection of activation, Sadikot and colleagues showed that while more *Pseudomonas* were viable in various tissues, the level of NF-κB activity in those tissues was lower compared to WT mice [304]. This *in vivo* finding supports a growing body of work suggesting that ROS regulates transcription [305–307], in some cases through NF-κB. The PMN chemoattractant IL-8 is regulated by ROS in several ways. IL-8 mRNA is induced by inflammatory stimuli such as bacterial LPS, and also by H_2O_2 [308]. Interestingly, CGD cells accumulate higher levels of IL-8 mRNA and seem to prolong transcription of this mRNA in response to fMLP [309]. Treatment of normal PMN with catalase mimicked the CGD finding suggesting either that H_2O_2 somehow downregulates transcription or, alternatively, it may decrease signaling potency of fMLP. In support of these results, CGD patients displayed higher serum levels of IL-8 compared to normals and their neutrophils produced more IL-8 basally and with stimulation *in vitro* [310].

34.7.3 pH Regulation in the Phagolysosome

In 1981, Segal and coworkers reported that activation of NOX upon ingestion of fluorescently labeled *S. aureus* in normal PMN results in a transient alkalinization of the phagolysosome followed by acidification [285] while CGD phagosomes failed to alkalinize and became significantly more acidic. The hypothesis was that alkaline pH is required for the action of antimicrobial granule factors and acid pH was required for optimal activity of the hydrolases present in PMN granules. This pattern of pH changes was confirmed in other studies using yeast as the stimulus [311], however other methods have called into doubt whether the transient alkalinization phase exists [312]. The hyperacidification of CGD PMN has been manipulated by addition of the weak base methylamine which increased vacuolar pH of CGD PMN and, interestingly, resulted in increased killing of *S. aureus* by CGD PMN [285]. As mentioned above, three CGD patients have been treated with amantadine

[287,288], a drug that increases the pH of the phagolysosome, and in each of these patients was associated with clinical improvement as well as improvement of *in vitro* neutrophil antimicrobial activity.

34.7.4 H^+ and K^+ Channels and the NADPH Oxidase

It was recently proposed that concurrent with O_2^- secretion into the phagosome, a large potassium flux occurs to compensate for the production of superoxide anion [313]. In this model, K^+ ions liberate granule components, such as proteases, from the granule matrix and allow their action on target microbes. Mice deficient in the proteases elastase and cathepsin G were as sensitive to infection by *S. aureus* or *C. albicans* as $p47^{PHOX}$ -/- mice. Protease deficient mice had normal ROS production suggesting that protection from infection is not due to the direct antimicrobial activity of ROS but rather that ROS are required to release antimicrobial proteases [313]. Candidate channels for the pumping of K^+ into the phagosome are the large-conductance Ca^{2+}-activated K^+ channel (also called BK_{Ca} channels). Pharmacologic blockade of BK_{Ca} using paxilline or iberiotoxin decreased bacterial killing and microbial digestion but had no effect on O_2 consumption [314]. Further study has shown that BK_{Ca} channels have no effect on NOX activity but do seem to increase TNF-α production [15]. There is still debate about the roles of BK_{Ca} channels as other groups have found that human PMN do not express this protein and that paxilline and iberiotoxin do not inhibit killing of bacteria [316].

Several laboratories have found that H^+ channels are required for antimicrobial activity. Activation of the Na^+/H^+ antiport and the H^+-ATPase was normal in CGD cells, however H^+ conductance was reportedly abnormal [317] and assembly of the NADPH oxidase seemed to be required but not the enzymatic activity [318]. CGD PMNs are also unable to depolarize their membranes in response to chemoattractant [319]. More recent results have suggested that NOX activity somehow regulates the H^+ channel but is not a part of the channel [320]. Finally, the cystic fibrosis transmembrane conductance regulator (CFTR) appears to play a role in PMN killing with PMN from cystic fibrosis patients showing slower bacterial killing than normal cells [321]. Taken together, these studies show that charge compensation is required to balance both the appearance of NADP+ in the cytosol and the extrusion of electrons through $gp91^{PHOX}$ to form O_2^- in the phagosome. The role of ion channels in controlling the composition and pH of both the PMN phagolysosome and the cytosol upon activation of NOX is reviewed elsewhere [322,323].

34.7.5 Defective Apoptosis in CGD

CGD PMN persist in inflammatory responses *in vivo; e.g.,* in skin windows in X-CGD patients [160] or peritoneal exudates in CGD mice [56,77,324]. In addition to the possibility of increased PMN recruitment in CGD, another explanation for these findings could be a delay in apoptosis in the typically short-lived PMN. The rate of spontaneous apoptosis *in vitro* due to aging was significantly lower in CGD PMN compared to normal PMN [325,326]. Furthermore, catalase inhibited induced apoptosis while H_2O_2 promoted apoptosis [325]. TNF-α-induced apoptosis of PMN was decreased under hypoxic conditions [327] which may mimic the environment within an abscess. Slowed apoptosis resulting in longer life may prolong the normally transient contribution of PMN to inflammation, particularly if during this longer life the CGD PMN continues to produce compounds such as IL-8 and LTB_4. Another possible explanation for this effect could be delayed removal by monocytes of PMN showing early signs of apoptosis [328].

34.7.6 Defective Neutrophil Extracellular Trap Formation in CGD

Under certain conditions, neutrophils undergo a poorly defined process of cell death [329] distinct from necrosis and apoptosis that leads to the extrusion of DNA and the formation of extracellular nucleic acid structures called NETs (Neutrophil Extracellular Traps) [330]. These structures are thought to bind cationic antimicrobial factors thereby preventing diffusion away from the site of neutrophil activation. The NETs also bind to microbes leading to their immobilization and exposure to higher concentrations of neutrophil products and possibly enhanced antimicrobial activity [330,331]. Eosinophils also release mitochondrial DNA to form extracellular traps in a ROS-dependent manner [332]. Although the physiologic stimuli that induce NET formation have yet to be identified, Fuchs et al. demonstrated that CGD neutrophils were unable to form NETs [329]. Addition of glucose oxidase, an enzymatic source of O_2^-, can restore the ability of CGD PMN to form NETs. NET formation *in vivo* has not been well established and it has been shown that NET formation is inhibited by as little as 2% serum [329]. Interestingly, however, many microbes secrete nucleases that may degrade NETs and deletion of an extracellular DNase from group A *Streptococcus* results in a less invasive phenotype [333] suggesting that escape from NETs may be important in pathogenesis. Whether defective NET formation in CGD contributes to disease remains to be established.

34.7.7 ROS Regulation of Inflammation

Complex lipids such as leukotriene B_4 (LTB_4) are important proinflammatory mediators acting as chemoattractants for leukocytes and regulating cell function. LTB_4 is made from arachadonic acid by 5-lipoxygenase, the target of the anti-asthma drug zileuton. CGD PMN both produce more LTB_4 and degrade less LTC_4 than normal or MPO-deficient PMN [334]. Increased levels of LTB_4 may contribute to the recovery of more leukocytes from $p47^{PHOX}$-deficient CGD mice following intraperitoneal thioglycolate or LTB_4 injection compared to similarly treated wild-type mice [324]. In normal and CGD mice, inhibition of 5-lipoxygenase significantly decreased peritonitis. Notably, recovery of LTB_4 from CGD mice was about 5-fold higher than wild-type confirming the earlier *in vitro* findings in human cells.

Another family of arachadonic acid-derived lipid mediators are the prostaglandins which are produced from arachadonate by cyclooxygenases, the targets of several non-steroidal anti-inflammatory agents. Some prostaglandins, for example PGD_2, have some anti-inflammatory properties and CGD PMN have been shown to produce less of this molecule [335].

While the mechanism of ROS or ROS-dependent regulation of arachadonate metabolism is still unclear, these findings have important implications for the control of inflammation. In addition to lipid mediators, ROS seems to be required for the control of several protein mediators of inflammation.

CGD mice ($p47^{PHOX\ -/-}$) spontaneously accumulate crystals of Ym1[336,337], an enzyme present in PMN and activated macrophages that might play a role in the degradation of microbial glucosamines. Subcutaneously injected crystallized Ym1 persisted in CGD but not wild-type mice. While such crystals have not yet been identified in humans, the failure of the host to degrade such products may well contribute to prolonged inflammation seen in CGD.

34.7.8 Lipid Metabolism in CGD Leukocytes

Pigmented lipid histiocytes (yellow-brown in color) were described in several of the initial case reports of CGD [2,4,102,338]. The molecular identity of these cells and the composition of their payload, whether undigested microbial or host materials, remains to be established. The lipid peroxidation product malonyldialdehyde (an antimicrobial lipid) is produced by normal leukocytes but inefficiently by those from CGD patients [339]. 4-hydroxynonenal, a bioactive lipid produced during inflammation, is also produced in the phagosome at lower levels by CGD PMN [340]. It is also known that CGD PMN fail to decrease membrane fluidity upon stimulation [341] however whether this is due to lipid modification or other cellular activation defects is unclear.

34.7.9 Degradation of Microbes in CGD

While there are a few exceptions, most studies have shown quite conclusively that CGD PMN are normal in assays of chemotaxis, phagocytosis and degranulation [19,20,342]. However, many workers have reported both morphologic and biochemical evidence that damage and degradation of phagocytosed microbes is markedly reduced in CGD PMN. ROS may play a role in the activation of the many enzymes that contribute to catabolic processing of microbes or may modify microbial substrates to make them more sensitive to enzymatic attack. Failure to degrade microbes (or damaged host structures or inflammatory mediators) may prolong the inflammatory response and may contribute to clinical phenotypes in CGD such as persistance of granulomas and abscesses, difficulty with wound healing, and altered antigen presentation. It is, of course, important to bear in mind that there is great variation among microbes in their susceptibility to degradation by PMN.

E. coli can cause significant disease in CGD patients and was more frequently isolated in the past [101] than it is now [32]. The microbicidal activity of CGD PMN against *E.coli* and *Salmonella* is reportedly normal and depends on O_2^- independent factors [343]. Interestingly, the degradation of *E. coli* protein by either CGD PMN or hypoxic normal PMN (under N_2 atmosphere) is reduced by 50% compared to normal PMN in room air [344]. Degradation of staphylococcal protein was also deficient in CGD PMN compared to normal [285]. Digestion of staphylococcal phospholipids by PMN in combination with group IIA phospholipase A_2 (PLA_2) also apparently requires products that are absent in CGD PMN [345].

The sensitivity of CGD patients to aspergillosis is discussed above. Interestingly, histopathology of CGD mouse lungs upon infection with this mold can be recapitulated following inoculation with autoclaved, pulverized and sonicated hyphae [171]. The same stimulus in normal mice resulted in a rapid inflammatory response in lungs that largely resolved within 72 hours. In contrast, an identical challenge of CGD mice causes extensive and prolonged cytokine production and histologic evidence of inflammation including microabscesses suggesting that the overly exuberant inflammatory response in CGD is largely due to dysregulated host responses rather than damage caused directly by pathogens. Interestingly, in CGD mice given normal or gene-corrected PMN at numbers sufficient to achieve ≥ 20% normal PMN in the circulation, there was resolution of many of the inflammatory signs in the murine sterile *Aspergillus* model [346].

34.7.10 Tryptophan Catabolism

Romani and coworkers recently reported that CGD mice do not produce kynurenine during aspergillosis infection and consequently suffer from excessive inflammation [347]. Kynurenine is produced from tryptophan by the IFN-γ-induced enzyme indoleamine 2,3-dioxygenase (IDO) and may stimulate the activity of γδ T-cells that may regulate inflammation [347]. They found that exogenous kynurenine, when co-administered with IFN-γ, could modulate the inflammatory response in mice. Until recently $O_2^{\cdot-}$ was thought to be a required cofactor for IDO, however a recent report implicates cytochrome b_5 as the major electron donor for this enzyme [348]. The possibility of multiple cofactors for IDO is important in reconciling the findings in the $p47^{PHOX}$ knockout CGD mouse mentioned above with the observation that human X-CGD patients actually secrete more kynurenine basally and after tryptophan loading [349]. Further work will be required to determine whether trypotophan catabolism is defective in human CGD patients.

In summary, *in vitro* observations comparing CGD with normal PMN have revealed a large number of potentially important findings that may help explain clinical phenotypes. For many of the findings shown in Table 34.5, the causes may be difficult to unravel. For example, the dysregulation of some immune receptors on CGD PMN could be due to differences in transcription seen in CGD or differences in the age of PMN with CGD PMN being older. All of these findings have been reported, however, and are fertile ground for further study into the aberrant mechanisms that result in CGD.

Table 34.5 Summary of abnormalities in CGD

PMN	$O_2^{\cdot-}$ [350]
	↓H_2O_2
	↓Hypochlorite
	↓tyrosinolation of protein [351]
	↓fail to decrease membrane fluidity upon stimulation [341]
	↓fail to depolarize membranes following stimulation [319]
	↓immune complex uptake [352]
	↓apoptosis [325,326]
	↓extracellular traps (NETs) [329]
	↓vacuole swelling [10,14,285]
↓consumption of NO [295]	
	↓phagosome pH
	↑IL-8 production [309]
	↑phagocytosis [16]
Monocytes	
	↓microbial killing [353]
Dendritic cells	
	↓phagosomal pH/↓antigen cross-presentation [354]
Lymphocytes	
	↓Memory T-cells [204]
	↓T-cell number [203]
	↓Memory B-cell compartment [202]
	↓CD40 on T-cells [205]
	↓T-cell apoptosis
Lipids	
	↓prostaglandin D_2 [335]
	↑leukotriene B_4 [334]
	↓Inactivation of Estrogen by PMN [355]
	↓lipid peroxidation by PMN [340]

34.8 Unanswered Questions

Among the most striking findings in patients with CGD is that the spectrum of common pathogens is strikingly narrow given the microbial diversity present in the environment and the apparent severity of the PMN functional defect. This clinical fact is best explained by the existence of large numbers of complementary host defenses which is not to say that they are redundant. More likely, each of these systems contributes to the multifactoral antimicrobial efficacy of the whole organism. The absence of any one factor tips the balance slightly in favor of the pathogen. Even in CGD, it is likely that other factors, such as transient neutropenia, may temporarily decrease the strength of these complementary mechanisms resulting in infection. A small change in the growth rate of the pathogen (e.g., depriving the organism of nutrients such as iron) and a small change in rate of dissemination (e.g., by NET or clot entrapment) may be just as important in controlling infection as direct microbicidal activity of the host.

The increased susceptibility to infection by catalase positive microorganisms in CGD has provided important insights about the role of the NADPH oxidase in innate immunity. Perhaps the most important unanswered question is why CGD patients are prone to granuloma formation. An intriguing possibility is that increased production of IL-8 is due to a lack of downregulation of PMN IL-8 mRNA transcription by H_2O_2 [309], although as mentioned in the previous section there are numerous other inflammatory processes that are deranged in CGD. Other observations that may relate to granuloma formation in CGD include inadequate degradation of antigen, persistence of inflammatory cells due to decreased apoptosis and persistence of inflammatory mediators. But the relationship of increased signals of acute inflammation in CGD and the transformation to the granulomatous event associated with delayed type hypersensitivity is totally unknown.

CGD patients may also provide important clues to the pathogenesis of some common diseases. Reactive oxygen species have been implicated in the pathogenesis of atherosclerosis [356–358], heart failure [359,360] malignant transformation [361,362], thyroiditis [363,364], and pancreatitis

[365,366]. These observations provide incentive to study whether CGD patients or mammalian models of CGD can provide *in vivo* evidence to support a role for ROS in these diseases, and may also help elucidate underlying mechanisms. Perhaps in the future, pharmacologic control of NADPH oxidase will prove an important approach for treating or preventing many common disorders of abnormally regulated inflammation.

34.9 Conclusions

Chronic granulomatous disease (CGD), caused by a genetic defect leading to defective production of the antimicrobial superoxide anion (O_2^-), was first recognized in the 1950s as a pediatric disease associated with frequent infections. Since the early 1970s, the NIH Clinical Center has become a major nidus for clinical and basic science research on this disease. The large CGD patient population followed at the NIH has accelerated clinical studies to develop treatment options such as recombinant interferon-γ therapy, antibiotic prophylaxis, bone marrow transplantation, and gene therapy. These patients have also participated in research that has advanced understanding of the molecular mechanisms of myeloid superoxide production including the identification of several components of the enzymatic machinery that produces superoxide and the mechanisms controlling activation of this system. In this review, we placed the contributions of the NIH into the context of global efforts to understand and treat CGD.

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Chapter 35
Pneumonia Virus of Mice (PVM): Exploring Novel Therapeutic Options In a Severe Respiratory Disease Model

Helene F. Rosenberg and Joseph B. Domachowske

35.1 Introduction

Respiratory syncytial virus (RSV) is the most important respiratory pathogen among infants and toddlers, with infections prevalent and nearly universal in this age group. Severe infections are more common among premature infants, those with cardiac and pulmonary anomalies, and the immunosuppressed. Effective prophylactic monoclonal antibody treatment is available for high-risk infants, but there is no effective vaccine. Mouse challenge models have been used for the study of the human RSV pathogen, but the most severe forms of RSV disease are not replicated by this approach. Pneumonia virus of mice (PVM; family *Paramyxoviridae*, genus Pneumovirus) is a mouse pathogen of the same family as human respiratory syncytial virus. PVM replicates efficiently in mouse-lung epithelial cells *in vivo* in response to a minimal virus inoculum, and replication is accompanied by local production of proinflammatory cytokines (MIP-1α, MIP-2, MCP-1, and IFN-γ) and granulocyte recruitment to the lung. PVM infection and the ensuing inflammatory response can lead to pulmonary edema and respiratory compromise. Our laboratories have pioneered the use of the PVM model for the study of human clinical disease, which has provided important insights into the role of the inflammatory response in the pathogenesis of severe respiratory virus infection. As part of this work, we have presented several immunomodulatory strategies that clearly reduce morbidity and mortality when administered to PVM infected, symptomatic mice, and thus hold promise as realistic therapeutic strategies for severe RSV infection in human subjects.

35.2 Human RSV Disease

Respiratory syncytial virus (RSV) infection is a near universal affliction of infancy and childhood, accounting for approximately 50% of all pneumonia and up to 90% of the reported cases of bronchiolitis in infancy. Of those infected during the first year of life, one-third develops lower respiratory tract disease, and 2.5% are hospitalized (more than 90,000 children in the United States every year). In many previously healthy infants, RSV disease is a mild and self-limited infection involving the upper and lower respiratory tract, with varying degrees of peribronchiolar and interstitial inflammation. In others, the disease progresses to severe bronchiolitis and pneumonia, including submucosal edema and bronchiolar obstruction requiring oxygen, and in the worst cases, mechanical ventilation. Infants at particularly high risk for severe disease include those born prematurely, infants and children with cardiac or pulmonary anomalies, and the immunocompromised. Prophylactic monoclonal antibody therapy is available for high-risk infants, but no vaccine is yet approved for use. RSV has also recently been recognized as an important pathogen in the institutionalized elderly. The clinical features and pathology of RSV disease have been reviewed extensively, and the reader is referred to these excellent sources of additional information [1–4].

35.3 Identifying an Appropriate Animal Model

There is no one animal model that can replicate all features of a human disease. This is particularly true when considering infectious pathogens, many of which have particular tropisms or specificities for a limited range of hosts, and in some cases present with completely different clinical illnesses in different species. While it would perhaps be ideal to study all human pathogens in natural, relevant human or higher primate hosts, this approach is of course completely limited and impractical. Despite clear and recognized differences between human and rodent immune and inflammatory responses, various factors (availability of characterized strains, ease of handling and breeding, availability of sophisticated experimental tools). together have provided a focus on inbred strains of mice as a centerpiece for human disease research. As such, it is critical that one understands what the unique features of each infectious disease model are and that

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the specific advantages and the individual limitations in each situation are clearly understood.

With these factors in mind, around 1997, we began our collaborative exploration of the pneumonia virus of mice (PVM) pathogen for the study of respiratory virus replication and the ensuing inflammatory response within a natural, evolutionarily relevant host-pathogen relationship. Further and more detailed consideration of our studies and those from other laboratories can be found in several of our recent reviews [5–8]; this article is largely excerpted from ref. 8.

35.4 The Pneumonia Virus of Mice Pathogen

PVM was originally discovered in 1939 by Frank Horsfall and Richard Hahn at the Rockefeller University as part of an attempt to identify pathogens from human clinical samples that would replicate in lung tissues of inbred mice [9]. Interestingly, PVM was isolated from lung tissue from the control (thought to be uninfected) mice, which yielded an infectious isolate after undergoing serial mouse-to-mouse passage. Choppin and colleagues [10] presented the first electron micrographs of these newly discovered virions (Fig. 35.1),

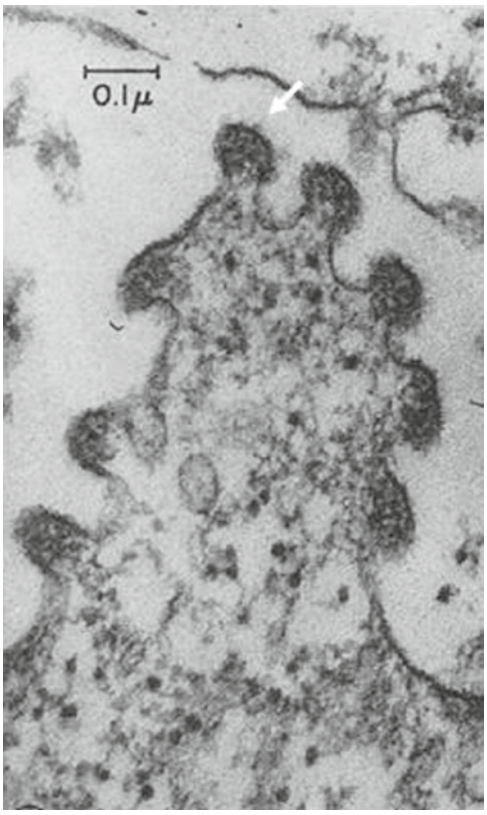

Fig. 35.1 Electron microscopic image of nascent PVM virions. Virions as shown budding (at arrow) from BHK-21 cells in culture. Reprinted with permission from Compans, R. W., et al. [10]

which they described as defining a new, third subgroup of myxoviruses. The polymorphic virions formed spheres of 80–120 millimicrons in diameter to filaments up to three microns in length, and replicated over a period of 24–30 hours in mouse lung tissue *in vivo*, with virus amplification proceeding at ~16-fold per cycle. Perhaps most interestingly, Ginsberg and Horsfall [11] recognized the potential of PVM for the exploration of acute respiratory virus infection in an evolutionarily relevant host. Among several studies, these authors were the first to relate the development of lung lesions to ongoing virus replication and to evaluate altered morbidity and mortality in response to rudimentary immumodulatory therapy, specifically in response to administration of bacterial capsular polysaccharide [11–13].

PVM has since been classified as a pneumovirus (Family Paramyxovirdae, genus Pneumovirus), together with the human and bovine respiratory syncytial virus pathogens. Viruses of this family are enveloped, and have non-segmented, negative-sense RNA genomes [14]. The molecular organization of the PVM genome has been elucidated primarily by Easton and colleagues [15–18], and has been the subject of several recent reviews [5,6]. The genomic organizations of PVM and RSV are shown in Fig. 35.2, which highlights the similarities in gene structure and gene order.

There are two major characterized strains of PVM in general use, although there is not complete clarity on all details related to their origin and maintenance. The original studies by Horsfall and colleagues [9,11–13] were performed on an isolate known as strain 15, which was at that time highly pathogenic in mice. Since that time, PVM strain 15 has reportedly undergone tissue-culture passage and lost some of its pathogenicity *in vivo*, although the extent to which this is so, and in which specific isolates, remains uncertain. A second strain, PVM strain J3666, also developed at the Rockefeller University, has been reportedly maintained in mice with minimal tissue-culture passage, and has recently been shown to be highly pathogenic in nearly all inbred strains of mice [19]. In our hands, PVM strain 15 from Dr. Andrew Easton's laboratory (PVM strain 15 Warwick) is highly attenuated and elicits

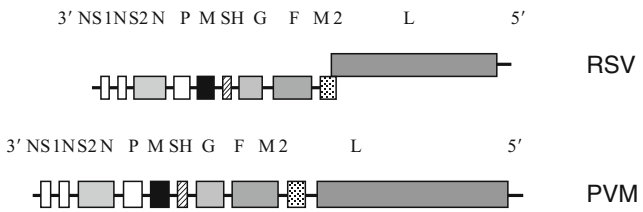

Fig. 35.2 Genomic organization of RSV and PVM. Shown are the 3′ to 5′ linear order of PVM and RSV protein encoding genes, including non-structural proteins (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix protein (M), surface hydrophobic protein (SH), attachment protein (G), fusion protein (F), the M2 genes, and polymerase protein (L). Reprinted with permission from Rosenberg, et al. [5]

a minimal inflammatory response in the highly susceptible BALB/c strain of mice [20]. In contrast, PVM strain 15 from the American Type Culture collection (PVM strain 15 ATCC) is pathogenic in BALB/c mice (unpublished findings), but results in little to no disease in the less susceptible C57BL/6 strain at similar inoculating doses [<100 pfu/mouse [21]]. Krempl and colleagues [22] likewise found PVM strain 15 (ATCC) to be highly pathogenic in the BALB/c strain. Complete sequence data are available for both PVM strain J3666 and PVM strain 15 [23,24]. The most remarkable differences are in selected regions of the G (attachment protein) and throughout the sequence of the SH small hydrophobic glycoprotein.

35.5 RSV and PVM: Inflammatory Responses and Disease Severity

We became interested in PVM in order to pursue studies of inflammatory responses to respiratory virus infections in a natural, evolutionarily relevant host. We initially recapitulated the aforementioned findings of Horsfall and colleagues and reported robust virus replication *in situ* (to titers $>10^8$ pfu/gm lung tissue), progressing to marked morbidity (hunching, fur ruffling), weight loss, and mortality, in our case in response to a minimal virus inoculum of the highly pathogenic strain PVM J3666 [25,26]. We have localized immunoreactive PVM to the bronchiolar epithelium [27], in a distribution similar to what has been observed for RSV in human post-mortem specimens [28] (Fig. 35.3). Microscopic examination of bronchoalveolar lavage fluid and lung tissue from morbid mice revealed profound inflammation, most notable for recruitment of granulocytes and for severe pulmonary edema consistent with the clinical findings characteristic of acute respiratory distress syndrome (ARDS; Fig. 35.4). Interestingly, severe inflammation, edema and recruitment of granulocytes have also been characterized in a recent series of RSV-diagnosed post-mortem samples evaluated by Welliver and colleagues [28]. PVM replication *in situ* results in local production of proinflammatory mediators, including MIP-1α, MIP-2, MCP-1 and IFN-γ [27]. A similar subset of proinflammatory mediators is produced in association with the more severe forms of RSV in human infants (reviewed in ref. 29].

35.5.1 MIP-1α (CCL3) Is a Crucial Component of the Virus-Induced Inflammatory Response

Similar to findings from the mouse model of influenza virus [30] we found that the chemokine, MIP-1α (CCL3), is crucial for granulocyte recruitment in response to PVM infection [26]. Specifically, MIP-1α gene-deleted mice are readily infected with PVM, although 10^5-fold fewer granulocytes are recruited to the lung tissue in response to the identical initial inoculum. Similar results were obtained upon infecting mice devoid of CCR1, the major receptor for MIP-1α on neutrophils and eosinophils. We have used this observation to design specific immunomodulatory strategies for the virus-induced inflammatory response and its associated pathology [31,32]

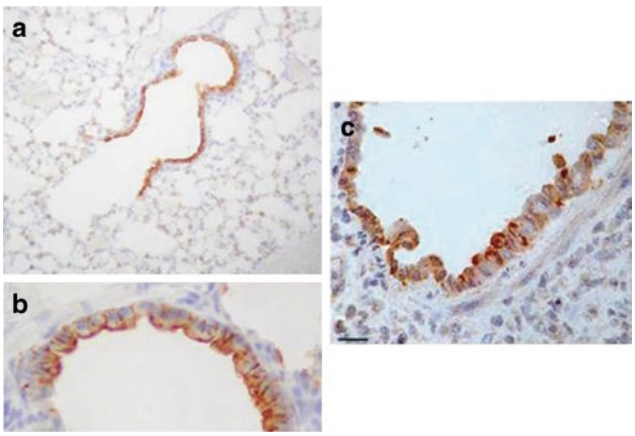

Fig. 35.3 Detection of immunoreactive PVM and RSV in bronchiolar epithelial cells. (**a**) Immunoreactive PVM is detected in infected mouse lung tissue by probing with convalescent mouse sera, original magnification 20X, (**b**) as in (**a**), original magnification 40X. (**c**) Detection of immunoreactive RSV in post-mortem human lung tissue, original magnification 40X. PVM images reprinted with permission from Bonville, et al. 27); RSV images reprinted with permission from Welliver, et al. [28]

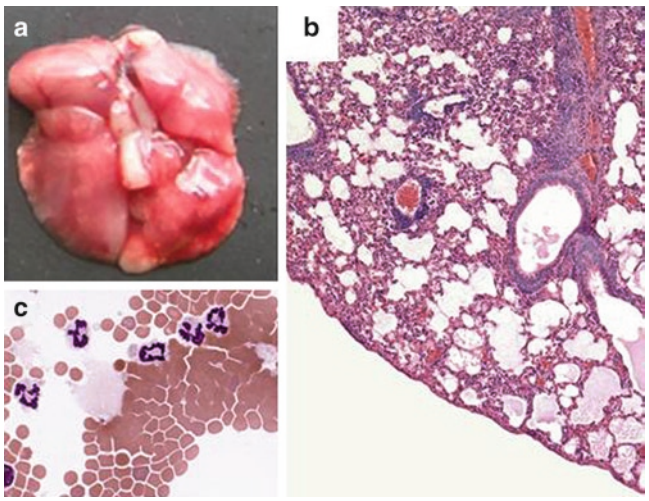

Fig. 35.4 Lung pathology. (**a**) Lung tissue from infected mice, with region of typical grey discoloration and multiple hemorrhagic foci; reprinted with permission from Ellis, et al. [21]; (**b**) Microscopic pathology, with evidence of profound cellular inflammatory response and developing pulmonary edema; original magnification, 20X, reprinted with permission from Garvey, et al. [36]; (**c**) Bronchoalveolar lavage fluid, with recruited neutrophils, original magnification, 63X

(discussed further below). Interestingly, although MIP-1α is clearly crucial for granulocyte recruitment in response to PVM infection, this chemokine, acting alone, even at high concentrations, does not induce ARDS, nor does it recruit granulocytes effectively in the absence of IFN-γ [33].

35.5.2 Inflammatory Responses In Aged Mice

As noted above, respiratory virus infection is also a growing problem in the aging population [34]. In a study performed using young adult (eight–12 weeks) through aged (up to 78 weeks), but otherwise immunologically naïve mice, we observed no change in the kinetics of PVM replication, but diminished local production of several proinflammatory mediators, including MIP-1α, MCP-1, and IFN-γ, along with diminished recruitment of granulocytes to the lung tissue [35]. The differences observed when comparing these results to those reported among elderly human subjects, who tend to have more severe, rather than less severe forms of RSV infection, may be related to virus re-exposure and its impact on the ensuing biochemical and cellular inflammatory responses. Interestingly, there is no published data on PVM replication and disease pathogenesis in neonatal mice, a critical target population given the prevalence of pneumovirus infection among human infants and toddlers.

35.5.3 Differential Responses In Type I IFN Receptor Gene-Deleted Mice

Pneumoviruses have unusual anti-interferon strategies, as they do not limit IFN production, nor do they interfere with receptor binding or signal transduction. Of particular recent interest are the ways in which IFNs and IFN-mediated signaling mechanisms interact with proinflammatory pathways and modulate the production of chemoattractant cytokines. As part of a larger exploration of the inflammatory responses to PVM, we examined the differential expression of cytokine genes in wild-type mice and mice devoid of the receptor for type I interferons (IFNαβR gene-deleted mice) [36]. As anticipated, PVM infection induces transcription of IFN antiviral response genes preferentially in lung tissue of wild-type over IFNαβR gene-deleted mice. However, we demonstrate that PVM infection also results in enhanced expression of eotaxin-2, TARC, and the proinflammatory RNase mouse eosinophil-associated RNase (mEar) 11, and we observe paradoxically prolonged survival among the IFNαβR gene-deleted mice.

35.6 Immunity to PVM Generated via Mucosal Inoculation

Using PVM strain 15 (ATCC), which, as noted above, is attenuated (replication-competent but does not induce a profound inflammatory response) in the C57BL/6 mouse strain, we explored the development of acquired immunity in response to a mucosal vaccination strategy. Neutralizing antibodies were detected at 14 days after inoculation in mice receiving live attenuated virus (but not heat-inactivated virus), which correlated with protection against subsequent challenge with the highly pathogenic strain J3666 [21]. Among the interesting questions, we are not certain of the duration of protective immunity. Interestingly, the responses of IFNγR gene-deleted mice were indistinguishable from the wild-type mice, indicating that any role for IFN-γ in generating acquired immunity in this setting is at least somewhat dispensable (Fig. 35.5). Among our other findings, PVM antigens, when prepared and administered in a manner analogous to the earlier hRSV lot 100 vaccine [37], will induce a Th2-mediated hypersensitivity response [38].

35.7 Therapeutic Strategies

Among the primary reasons to explore respiratory virus infection using the PVM model is to improve our understanding of the molecular basis of severe disease so as to design novel therapeutic strategies.

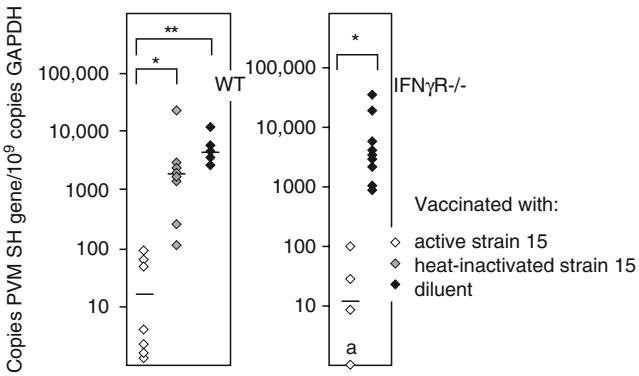

Fig. 35.5 Virus replication in lung tissue of vaccinated mice. Wild type and IFNγR gene-deleted mice were vaccinated with live-attenuated PVM strain 15 (ATCC), heat-inactivated strain 15, or diluent alone and challenged with virulent strain J3666. Replication was measured by a quantitative RT-PCR assay designed to detect the PVM SH gene. We observed no differences in the kinetics of virus replication, and no differences in responses to mucosal vaccination between the wild type and IFNγR gene-deleted mouse strains. Reprinted with permission from Ellis, et al. [21]

35.7.1 Antiviral Agents

The antiviral agent, ribavirin, is very effective at blocking replication of RSV in both tissue culture and in human subjects, yet the impact of ribavirin therapy alone on the course of actual clinical disease is insignificant [39]. The PVM model replicates this scenario, as ribavirin at concentrations of >10 μg/ml is effective at blocking virus replication in tissue culture, and at concentrations of 75 μg/kg/day, at blocking virus replication in mouse lung tissue *in vivo*. Yet, analogous to observations made in clinical studies, administration of effective doses of ribavirin alone to PVM-infected, symptomatic mice, has little impact on the ultimate outcome of disease, when measured in terms of morbidity and mortality [31,32]. In conjunction with this observation, we found that, although ribavirin was quite effective at blocking virus replication, it had no impact on the ongoing production of proinflammatory chemokines and associated recruitment of granulocytes to the lung tissue. Clearly, there is some disconnect between virus replication and the ensuing inflammatory response, in that inflammation is not necessarily controlled effectively at all given points in time by reducing or eliminating the primary stimulus.

35.7.2 Glucocorticoids

Glucocorticoids are in general use as broad-spectrum, anti-inflammatory agents, yet overall analysis suggests that they have only limited benefit for the treatment of severe hRSV-associated inflammation [40]. Although we have not evaluated specific combinations of ribavirin and glucocorticoids in PVM-infected mice, we have documented the effects of hydrocortisone alone on the inflammatory response. We have determined that hydrocortisone therapy has no effect on the production of MIP-1α or on the influx of neutrophils; PVM-infected mice responded to hydrocortisone with enhanced viral replication and slightly accelerated mortality [41]. These results suggest several mechanisms to explain why glucocorticoid therapy may be of limited benefit in the overall picture of pneumovirus infection. Interestingly, Thomas and colleagues [42] also determined that glucocorticoids had no impact on the virus-induced chemokine response in hRSV infection in human subjects.

35.7.3 Combination Therapy with Ribavirin and Specific Immunomodulatory Agents

Given our earlier observation on the crucial nature of the chemokine, MIP-1α, in promoting granulocyte recruitment in response to virus infection, we considered the possibility that blockade of this chemokine itself, or its signaling via its major receptor, CCR1, might provide appropriate immunomodulatory control in this setting. In a series of studies, we found significant improvements in long-term survival when ribavirin was administered to symptomatic mice in conjunction with anti-MIP-1α antibodies, or with small molecule blockade (met-RANTES) of the MIP-1α receptor, CCR1 [31,32] (Fig. 35.6).

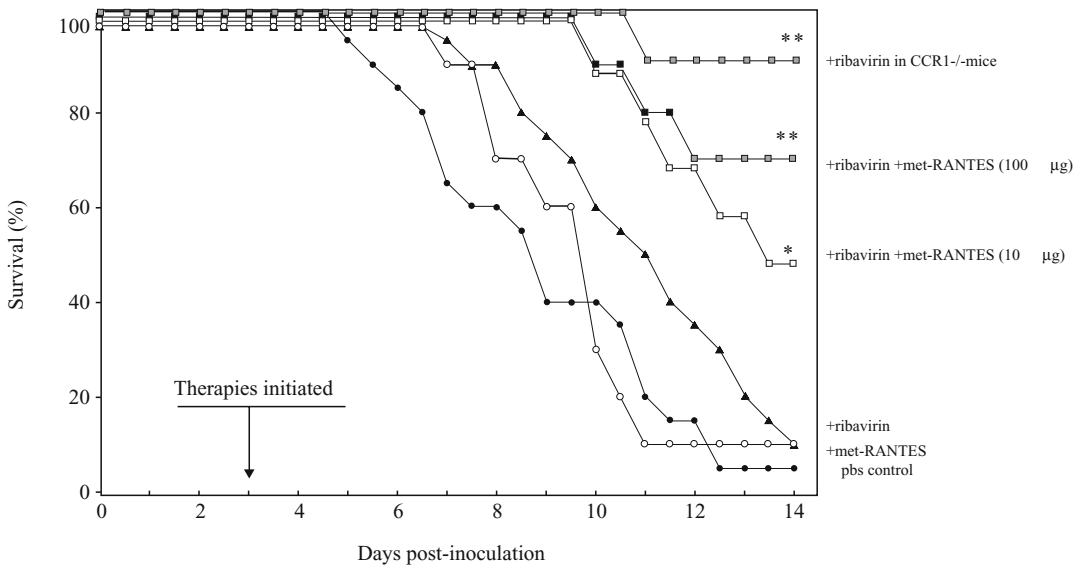

Fig. 35.6 Survival of PVM-infected mice treated with combined antiviral and immunomodulatory therapy. Improved survival resulted from treatment with ribavirin and met-RANTES (* $p<0.05$) compared individually to the groups treated with Met-RANTES alone, ribavirin alone, or PBS. Further improvement was observed in response to combination of ribavirin with the higher met-RANTES dose, 100 μg/day, over that observed in response to ribavirin-met-RANTES at 10 μg/day (** $p<0.05$), approaching that observed for CCR1 gene-deleted (CCR1 -/-) mice treated with ribavirin, representing theoretical complete receptor blockade. Reprinted with permission from Bonville, et al. [32]

A similar study documented the effectiveness of ribavirin in conjunction with the cysteinyl-leukotriene inhibitor montelukast [43]. Interestingly, although neither agent was effective at reducing morbidity or mortality as single-agent therapy, together, administered to infected, symptomatic mice, significant improvements in long-term survival were observed (50% vs. 10% for PBS control). Interestingly, montelukast had little impact overall on neutrophil recruitment, suggesting that the presence of neutrophils alone does not indicate inevitable progression to intractable disease.

35.8 Conclusions

The PVM model holds great promise for the elucidation of inflammatory mechanisms associated with virus infection and acute inflammatory responses in the lung. Studies carried out to date have provided an explanation for the lack of clinical efficacy of antiviral therapy, and have indicated that chemokine and/or chemokine-receptor blockade in conjunction with appropriate antiviral therapy might be more effective than antiviral therapy alone. Likewise, PVM is an excellent system in which to explore the molecular mechanisms through which natural immunity to pneumovirus infection develops, information which may assist in the development of novel vaccines and other prevention strategies.

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Chapter 36
The 1918 Influenza Pandemic: Pathology and Pathogenesis

John C. Kash and Jeffery K. Taubenberger

36.1 Introduction

Influenza viruses are among the most common causes of human respiratory infections [1], and among the most significant because they cause high morbidity and mortality. Influenza outbreaks have apparently occurred since at least the Middle Ages, if not since ancient times [2]. In the elderly, in infants, and in people with chronic diseases, influenza is associated with especially high mortality. In the United States, influenza results in approximately 200,000 hospitalizations and 36,000 deaths in a typical endemic season [3]. In addition to annual winter outbreaks, pandemic influenza viruses occasionally emerge [4,5], as they have every eight to 41 years, for at least several centuries. Up to 50% of the population can be infected in a single pandemic year, and the number of deaths caused by influenza can dramatically exceed what is normally expected [6].

In the past 120 years there were undoubted influenza pandemics in 1889; 1918; 1957; 1968, 1977, and 2009 [7]. Although most experts believe we will face another influenza pandemic, it is impossible to predict when it will appear, where it will originate, or how severe it will be [8]. Nor is there agreement about the subtype of influenza virus most likely to cause the next pandemic. The continuing spread of H5N1 highly pathogenic avian influenza viruses has heightened interest in pandemic prediction. Despite uncertainties in the historical record of the pre-virology era, study of previous pandemics may help guide future pandemic planning and lead to a better understanding of the complex ecobiology underlying the formation of pandemic strains of influenza A viruses. The most severe influenza pandemic on record occurred in 1918–1919. It is hoped that studying this past outbreak can help prepare for or mitigate the impact of a future pandemic [4].

The 1918–1919 "Spanish" influenza pandemic was responsible for an estimated 40 to 50 million deaths, worldwide, and approximately 675,000 deaths in the United States [9]. The genome of the 1918 (H1N1) influenza A virus was sequenced from viral RNA fragments retained in lung tissues collected from human fatalities during the pandemic [10,11]. Sequence analyses suggest that the 1918 pandemic virus arose after the adaptation and introduction of an avian-like H1N1 influenza A virus into humans [11,12]. The 1918 pandemic differed from other pandemics in several important clinical and epidemiologic aspects. Although the clinical course was usually self-limiting, a substantially higher percentage of cases developed severe pneumonic complications. As a result, the case mortality rate in the United States averaged 2.5%, over twenty-five times higher than the rate of other pandemics. Moreover, mortality during the 1918 pandemic was concentrated in an unusually young age group [4]. People under the age of 65 accounted for more than 99% of excess influenza-related deaths in 1918. In contrast, in the 1957 and 1968 pandemics, people less than 65 years old accounted for only 36% and 48% of excess deaths due to influenza, respectively [6]. The age group affected most severely by the 1918 pandemic was that between 20 and 40 years, and this group accounted for almost half of influenza deaths during the pandemic. The reasons for these unexpected patterns remain obscure [4,5].

In humans and other mammalian hosts, influenza viruses replicate in the epithelial cells throughout the respiratory tree. During acute infection, virus is recoverable from both the upper and lower respiratory tract of people naturally or experimentally infected [1]. Unfortunately, as histolopathologic changes in acute influenza infection are nonspecific, histologic analysis alone is insufficient to make a specific diagnosis (13]; diagnosis typically requires supporting diagnostic tests such as viral isolation, rapid diagnostic tests (including RT-PCR), serologic studies, or a biopsy or autopsy tissue section confirmed by *in situ* hybridization or immunohistochemical techniques [14]. Nonfatal influenza viral infections predominantly involve the upper respiratory tract and trachea, but fatal cases of influenza usually show evidence of pneumonia.

36.2 Why Did People Die during the 1918 Pandemic?

An important question related to pandemic preparedness remains unanswered: what killed people during the 1918

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–1919 pandemic and subsequent influenza pandemics? In recent work, re-cut tissue specimens obtained during autopsy from 58 influenza victims in 1918 –1919 were examined, and epidemiologic, pathologic, and microbiologic data from published reports for 8398 postmortem examinations bearing on this question were reviewed, as shown in Fig. 36.1. We have also reviewed relevant information, accumulated over nine decades, related to the circulation of descendants of the 1918 virus. With the recent reconstruction of the 1918 pandemic influenza virus, investigators have begun to examine why it was so highly fatal [15,16]. Based on contemporary and modern evidence, we conclude here that influenza A virus infection in conjunction with bacterial infection led to most of the deaths during the 1918 –1919 pandemic [17].

We believe that the weight of 90 years of evidence, including the exceptional, but largely forgotten, work of an earlier generation of pathologists, indicates that the vast majority of pulmonary deaths from pandemic influenza viruses have resulted from poorly understood interactions between the infecting virus and secondary infections due to bacteria that colonize the upper respiratory tract. These data are consistent with a natural history in which the virus, highly cytopathic to bronchial and bronchiolar epithelial cells, extends rapidly and diffusely down the respiratory tree, damages the epithelium sufficiently to break down the mucociliary barrier to bacterial spread, and if able to gain access to the distal respiratory tree—perhaps on the basis of receptor affinity [18]— creates both a direct pathway for secondary bacterial spread and an environment (cell necrosis and proteinaceous edema fluid) favorable to bacterial growth. It remains unresolved whether co-colonizing, non-pneumopathic upper respiratory–tract organisms such as *Haemophilus influenzae* play an ancillary role, or are merely innocent bystanders. It is uncertain why *Haemophilus influenzae* was much less prominent in 1957–1958 and thereafter, but this phenomenon may relate to antibiotic use and conceivably, in recent years, to *Haemophilus influenzae* b vaccination of children.

Even so, the extraordinary severity of the 1918 pandemic remains unexplained. That the causes of death included so many different bacteria, alone or in complex combinations, argues against specific virulent bacterial clones. The pathologic and bacteriologic data appear consistent with co-pathogenic properties of the virus itself, perhaps related to viral growth; facility of cell-to-cell spread; cell tropism, or interference with or induction of immune responses. Certain observers believed that co-transmission of the influenza agent and of pneumopathogenic bacteria was responsible for many severe and fatal cases, especially during the October–November 1918 peak of mortality and case-fatality rates [19]. We speculate that any influenza virus with an enhanced capacity to

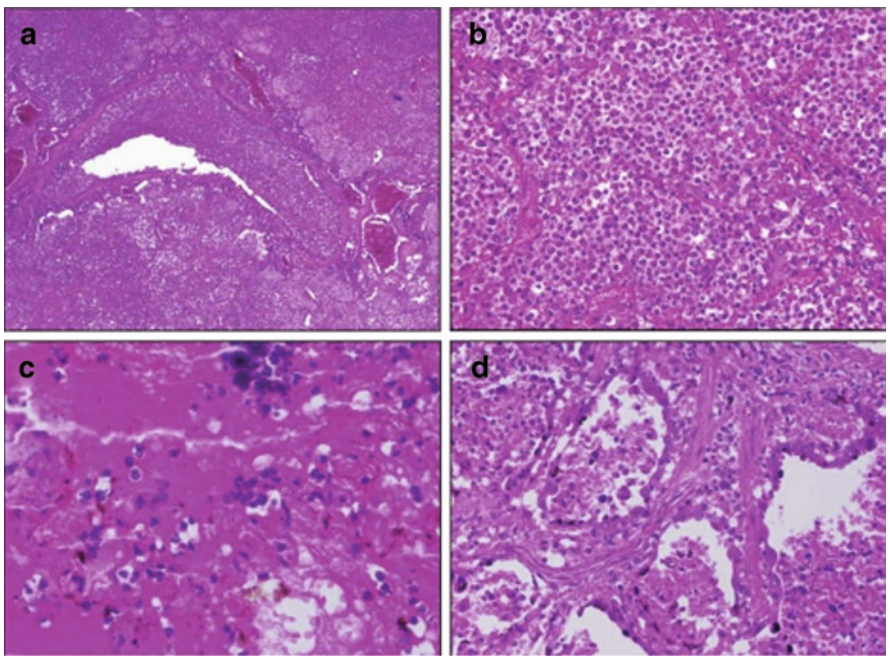

Fig. 36.1 Pathology of primary influenza and secondary bacterial pneumonias in humans during the 1918–19 pandemic. Examples of hematoxylin and eosin–stained postmortem lung sections from 4 victims of the 1918 –1919 influenza pandemic. **a**, Typical picture of severe, widespread bacterial bronchopneumonia with transmural infiltration of neutrophils in a bronchiole and with neutrophils filling the airspaces of surrounding alveoli (original magnification, 40x). **b**, Massive infiltration of neutrophils in the airspaces of alveoli associated with bacterial bronchopneumonia as in A (original magnification, 200x). **c**, Bronchopneumonia with intra-alveolar edema and hemorrhage. Numerous bacteria are visible both in the edema fluid and in the cytoplasm of macrophages (original magnification, 400x). **d**, Bronchopneumonia with evidence of pulmonary repair. The alveolar epithelium is hyperplastic; interstitial fibrosis is seen between alveoli (original magnification, 200x) [17]

spread to and damage bronchial and/or bronchiolar epithelial cells, even in the presence of an intact rapid reparative response, could precipitate the appearance of severe and potentially fatal bacterial pneumonia due to prevalent upper respiratory–tract bacteria.

In the modern era, the widespread use of antibiotics and the establishment of life-prolonging, intensive-care unit treatment make it more difficult than it was in 1918 to document the importance of bacterial lung infection for influenza-related mortality. Influenza-associated pneumonia patterns may now be influenced by the administration of pneumococcus, *Haemophilus influenzae* b, and meningococcus vaccines, and cases have tended to occur in elderly individuals, who rarely undergo autopsy. The 1968 influenza pandemic was mild, and autopsy studies were uncommon [4]. Fatal cases of influenza-associated viral pneumonia that are considered to be "primary" (i.e., with little or no bacterial growth) continue to be identified [20,21]; however, their incidence appears to be low, even in pandemic peaks. The issue of the pathogenesis of fatal influenza-associated pneumonia remains important; the fact that even severe, virus-induced tissue damage is normally followed by rapid and extensive repair [22,23] suggests that early and aggressive treatment, including antibiotics and intensive care, could save most patients [24,25] and also underscores the importance of prevention and prophylaxis.

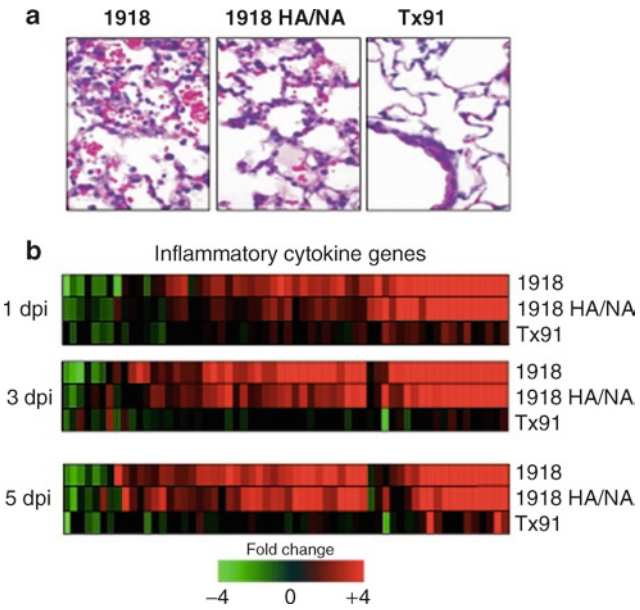

Fig. 36.2 Pathology and expression of inflammatory cytokine genes during 1918 virus infection in mice. a, hematoxylin and eosin–stained lung sections from mice infected with equivalent doses of the reconstructed 1918 influenza virus, A/Tx/36/91 virus expressing the 1918 HA and NA genes (1918 HA, NA) or parental A/Tx/36/91 (Tx91) virus at 3 dpi. **b**, Inflammatory cytokine related gene expression in lungs of 1918, 1918 HA, NA or Tx91 virus infected mice. For each infection point, the data presented are the error-weighted average expression changes calculated from four technical replicate micro arrays performed on three individual mice (n = 12 total). Genes shown in red were up-regulated and genes shown in green were down-regulated in infected relative to mock-infected mouse lung. Modified from (28)

36.3 What is the Molecular Basis for 1918 Pandemic Virus Pathogenicity?

Because influenza viruses were not known in 1918, no viral isolates were made, and thus the causative agent of the pandemic could not be directly studied. To study the virus, an archaevirologic approach was used in which archival influenza pneumonia autopsy materials from 1918 were screened, and from select cases, small influenza-viral RNA fragments could be amplified and sequenced. This procedure was used to determine the complete genomic structure of the causative influenza virus [10,11]. Advances in reverse genetics technologies for influenza A viruses [26] made it possible to construct infectious viruses containing some or all of the 1918 influenza virus gene-segments [16,27,28]. Utilizing this approach, experimental pathogenesis studies in animal models have attempted to identify virulence factors in the 1918 influenza virus that might correlate with the severity of the pandemic.

In a series of *in vivo* experiments, recombinant influenza viruses containing from one to eight gene segments of the 1918 virus have been produced [16,27,28]. As shown in Fig. 36.2, the reconstructed 1918 influenza virus and constructs bearing the 1918 HA and neuraminidase (NA) segment are all highly pathogenic in mice and caused severe necrotizing bronchiolitis, with accompanying moderate to severe alveolitis and alveolar edema. Analysis of the host immune-response to infection by gene-expression microarray performed on RNA isolated from whole lung tissue of mice infected with the 1918 and 1918 HA/NA recombinant showed dramatic upregulation of pro-inflammatory cytokine genes, in addition to genes involved antiviral responses, apoptosis, tissue injury, and oxidative damage, compared to a modern human influenza virus A/Tx/36/91 (Tx91) [28]. These findings were unexpected because the viruses with the 1918 genes had not been previously adapted to mice; control experiments in which mice were infected with modern human viruses, such as Tx91, showed little disease and limited viral replication. The lungs of animals infected with the 1918 HA, NA construct showed bronchial and alveolar epithelial necrosis and a marked inflammatory infiltrate, which suggests that the 1918 HA (and possibly the NA) contain virulence factors for mice, but the genotypic basis of this pathogenicity has not yet been fully mapped. Whether pathogenicity in mice effectively models pathogenicity in humans is unclear. Thus, the significance of these studies for human pathogenesis remains unclear. More recent work has examined the link between HA receptor-binding specificity and pathogenicity.

Influenza A virus infection requires binding of the hemagglutinin (HA) surface glycoprotein to sialic acid receptors on the host-cell surface. The HA receptor-binding site configuration is different for those influenza viruses adapted to infect birds and those adapted to infect humans. Influenza virus strains adapted to birds preferentially bind sialic acid receptors with α2–3 linked sugars. Human-adapted influenza viruses are thought to preferentially bind receptors with α2–6 linkages [29]. Previous studies have shown that the entire 1918 HA gene can act as a virulence factor in mouse studies, in that viruses bearing the 1918 HA gene can induce lethal infections [16,28]. However, the contributions that sequence differences in the receptor-binding domain make to virulence of the 1918 virus have not been previously examined. The presence of multiple positively charged amino acids at the cleavage domain is characteristically observed in highly pathogenic H5 or H7 subtype avian influenza viruses; however, this sequence does not appear in any 1918 HA [10]. Severe influenza viral pneumonias in humans, particularly those caused by highly pathogenic avian influenza (HPAI) viruses are often associated with significant lower respiratory track infections [30,31]. HPAI viruses such as H5N1 have a strong α2–3 binding specificity [32] and it has been hypothesized that mutations leading to increased α2–6 binding by HPAI viruses would facilitate human-to-human transmission and lead to a new pandemic. It has been hypothesized that, as part of the adaptation of an avian influenza virus to mammals, the receptor specificity would need to change from α2–3 to α2–6.

The HA gene sequence, containing the receptor binding domain, of the 1918 pandemic virus has been determined from five autopsy cases. When aligned to the sequences of H1 subtype HA genes from avian influenza isolates, the 1918 cases all have a shared change from avian H1's at residue 190. Interestingly, the 1918 cases differ at another receptor-binding residue at site 225. Three of the cases have a second mutation from avian sequences at this site, while two retain the avian amino acid [33]. The receptor-binding specificity of these variant 1918 HA proteins was evaluated using glycan arrays. Data generated in this manner demonstrated that the switch from the avian α2–3 receptor configuration for the H1 subtype HA required only the one amino acid change at residue 190, and as the HAs of all sequenced 1918 viruses have this change, it suggests that it could be a critical step in human host adaptation. Those HAs with this sequence had a mixed α2–3 and α2–6 specificity. The second change observed in the remaining three of five sequences greatly augmented virus binding to the α2–6 human receptor [18,34]. Thus, the 1918 pandemic influenza virus was apparently transmitted and caused severe disease with either α2–3/α2–6 or α2–6 binding activity. This observation suggests that 1918 HA likely encodes one or more additional unmapped virulence properties outside of the receptor-binding domain. It is intriguing to note that some recent H5N1 influenza isolates causing human infections in 2003 and 2004 showed mixed α2–3/α2–6 binding, but these viruses were not more easily transmissible in humans [35].

Given the differing receptor-binding specificities of influenza virus HA genes that were circulating during the 1918–19 pandemic, we need to revisit the hypothesis that avian-to-human influenza A virus host-switching and virulence is based on HA binding specificity to include important changes in HA outside the receptor binding domain. Factors allowing influenza A virus to be transmissible between humans are not well understood, but changes in the receptor binding domain are likely to be important factors in the process. It was recently demonstrated in a ferret transmission model with the 1918 influenza virus, that a virus with an α2–6 specificity was transmitted efficiently to contact animals, that the 1918 variant with the mixed α2–3 and α2–6 specificity transmitted less well, and that an 'avianized' form of the 1918 virus, in which the 190 position of the receptor binding domain was mutated back to the conserved avian glutamic acid at residue 190, did not transmit at all [36]. Moreover, this study showed that differences in sialic acid binding-specificity of 1918 HA proteins did not affect weight loss, viral replication, or pathogenesis in primary inoculated ferrets.

To examine the relationship between influenza virus receptor-binding specificity and virulence, we recently tested two hypotheses: (i) if changing α2–3 to α2–6 binding specificity of an avian-adapted HA would increase virulence, and (ii) if altering the α2–6 to α2–3 sialic acid specificity of the 1918 HA would abolish virulence in a mouse model [37]. The viruses used in this study contained identical internal genes and their replication in MDCK cells was efficient (titers between 10^6-10^7 PFU/mL), showing that neither replacement nor mutation of the HA genes blocked infection or replication. However, we observed dramatic differences in mouse virulence and pathogenesis. Given that the 1918 reassortant viruses were isogenic, apart from the surface glycoproteins, these properties can be attributed to the different HA and NA genes. Additionally, the 1918 and avian HA genes were isogenic outside single nucleotide changes in the codons of the receptor-binding domain. The parental human A/New York/312/2001 (H1N1) virus [NY312] showed limited lung replication at four days, post inoculation (dpi) and caused only slight weight loss, with minimal pathologic changes. Replacing the parental NY312 virus genes with wild-type avian HA and NA genes slightly increased weight loss but were still non-fatal. Changing avian HA α2–3 binding specificity to α2–6 led to prolonged viral replication of the avian mutant virus in mouse lung, but with minimal pathogenic changes and non-lethal outcome that was similar to the avian wild-type virus (data not shown).

In contrast, as shown in Fig. 36.3 panels A and B, replacement of the HA and NA genes of the parental NY312

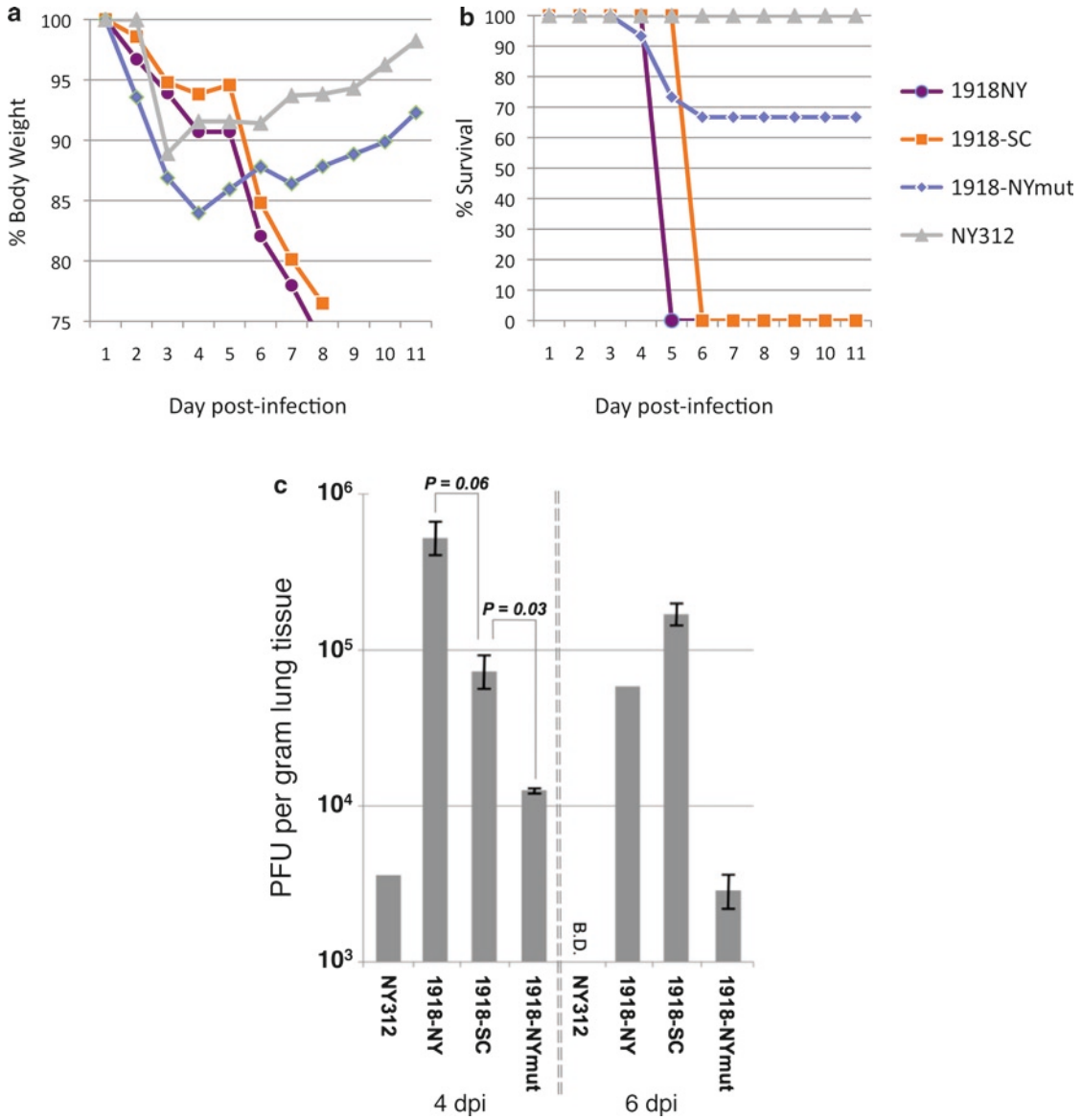

Fig. 36.3 1918 influenza virus HA gene contains virulence motifs outside of the receptor binding domain. Results of wild-type and mutant 1918 HA expressing influenza virus infection experiments in mice. (**a**) Weight loss of mice infected with rescued influenza viruses. 8-10 week old female BALB/c mice were intranasally inoculated with 2 x 10^5 PFU of indicated viruses and daily weights were measured. Only the 1918-SC and 1918-NY mice had uniformed significant weight loss leading to a lethal infection (n=5 mice per virus per group); while 1918 NYmut infected animals showed similar early weight loss with significantly lower mortality (n = 15 mice per virus per group). (**b**) Kaplan-Meier survival curve of 10–12 week old female BALB/c mice intranasally inoculated with 2 x 10^5 pfu of virus. The 1918-SC, and 1918-NY viruses caused uniformly lethal infections typically by 6 dpi; while mice infected with the 1918-NYmut virus had lethal outcomes in 33% of the infected animals (n=15). (**c**) Virus titers in the lungs of infected BALB/c mice (n = 2-3) described in plaque forming units (PFU) per gram of lung tissue as determined by plaque assay (B.D. indicates below detection threshold). Two-tailed P-values were calculated using an unpaired t-test [37]

virus with 1918 virus HA and NA genes dramatically increased mouse pathogenicity and lethality, confirming previous findings [38,39]. Both the 1918-SC and 1918-NY viruses were uniformly lethal at the highest dose, with significant lung replication that persisted until death and led to severe pathology, especially in the lower respiratory tree. The viral MLD_{50} and lung pathology observed for the 1918-SC virus was similar to previous studies evaluating similar viral constructs [16,28,39]. The increased pathogenicity of the viruses expressing the HA and NA genes representing the 1918 virus sequences was not solely due to the ability of these viruses to bind α2–6 sialic acid. Mutation of the 1918-NY HA gene (D190, G225) to a completely avian RBD consensus (E190, G225) did not completely attenuate the virus, which still caused lethal infection with bronchiolitis and alveolitis, albeit with less severity than 1918-SC or 1918-NY.

Intriguingly, as shown in Fig. 36.3, panel C, we observed a similar trend of the duration of viral replication in mouse lung between the avian and 1918 chimeric viruses. Both the Av-wt and 1918-NYmut viruses with α2–3-only binding activity were only detected at 4 dpi, but were either undetectable or significantly reduced, respectively, by 6 dpi. In contrast, the Av-mut, 1918-SC and 1918-NY viruses with α2–6 binding activity persisted in the lungs and were detected at both 4 and 6 dpi.

To determine how sialic acid receptor-binding specificity of the 1918 HA affected lung pathology and cell tropism of these chimeric viruses, we compared hematoxylin and eosin and viral-antigen-stained lung sections from mice infected with 1918-SC, 1918-NY or 1918-NYmut viruses. In contrast to the parental NY312, Av-wt and Av-mut virus-infected animals, lung sections from mice infected with the three 1918 HA, NA constructs all showed severe pathology and similar cell tropism. The lung sections from the α2–6 specific 1918-SC and the mixed α2–3/α2–6 1918-NY virus-infected mice had moderate-to-marked alveolitis and bronchiolitis with alveolar edema and/or hemorrhage (Fig. 36.3, panels A, B, D, E). Although the inflammatory infiltrate was mixed, the dominant cell type observed was neutrophils.

Immunohistochemistry for viral antigen showed widespread antigen in both bronchiolar epithelial cells and alveolar lining cells (Fig. 36.4, panels C, F). The α2–3 binding 1918-NYmut virus-infected mice showed a similar pathology with moderate alveolitis and bronchiolitis (Fig. 36.4, panels G, H) that was less extensive than observed in the 1918-SC- and the 1918-NY-infected mouse-lung sections. Viral antigen was also detected in alveolar lining cells (Fig. 36.4, panel I). These studies demonstrated that influenza viruses expressing 1918 hemagglutinin possessing either α2–6, α2–3 or α2–3/α2–6 sialic acid specificity were fatal in mice with similar pathology and cellular tropism. Moreover, changing the α2–3 binding specificity of an avian-adapted hemagglutinin to bind α2–6 did not increase lethality nor change tropism. Thus, the 1918 hemagglutinin contains important determinants outside the receptor-binding domain that contribute to enhanced virulence in mice and likely humans. Studies are underway to identify the responsible domains and amino acid changes associated with them.

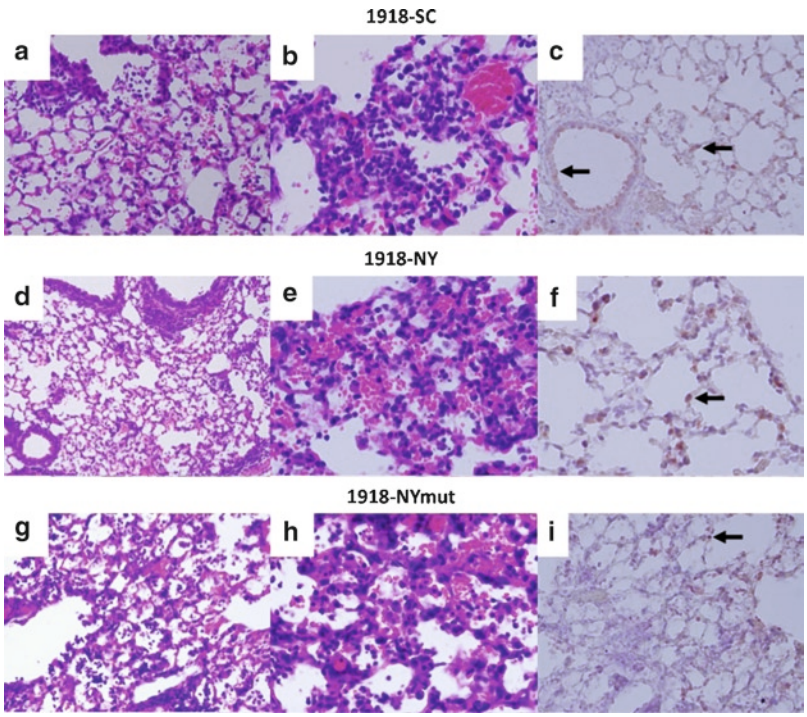

Fig. 36.4 Similar lung pathology in both α2–3 and α2–6 sialic acid binding 1918 HA expressing viruses in mice. Pathology and immunohistochemistry of 1918 HA expressing virus infected mouse lung. Photomicrographs of hematoxylin and eosin-stained tissue sections and immunohistochemically stained sections to detect influenza viral antigen from mice infected with different influenza virus constructs 6 dpi. Viral antigen is stained red-brown on a hematoxylin stained background. Arrows show example positive cells. (**a–c**) Sections from an animal infected with the 1918-SC virus. A moderate-to-marked acute alveolitis and bronchiolitis were seen (**a** orig. mag. 20x; orig. mag. 40x **b**). Viral antigen was observed in alveolar lining cells and in the epithelium of terminal bronchioles (**c** orig. mag. 20x). (**d–f**) Sections from an animal infected with the 1918-NY. A moderate-to-marked acute alveolitis and bronchiolitis was seen (**d** orig. mag. 20x; E orig. mag. 40x). Viral antigen was observed in alveolar lining cells (F orig. mag. 40x). (**g–i**) Sections from an animal infected with the 1918-NYmut virus. A moderate acute alveolitis and bronchiolitis was seen (**g**, orig. mag. 20x; H orig. mag. 40x). Viral antigen was observed in alveolar lining cells (**i** orig. mag. 20x) [37]

Two recent studies have shown that influenza HA proteins make direct contacts with many sub-terminal carbohydrates in sialic acid containing glycans that contribute to their α2–3 and α2–6 binding specificity [40,41]. Moreover, Stevens, et al. used glycan arrays to demonstrate that the same influenza viral HA proteins show differential binding to a variety of branched and unbranched oligosaccharides with the identical terminal disaccharide, whether α2–6 or α2–3 [18]. Thus, the model that influenza virus binding is determined simply by specificity for terminal disaccharides of sialic acid-terminating complex glycans is an over-simplification.

These studies demonstrated that α2–6 binding by 1918 HA does contribute to virulence, but raise new questions about the nature of non-RBD defined virulence of the 1918 HA protein. HA proteins from highly pathogenic avian influenza H5 and H7 subtype viruses, including recent Eurasian H5N1 lineages, possess polybasic amino-acid insertional mutations near the HA_1/HA_2 cleavage site that have been shown to be an important virulence factor. This polybasic region broadens protease specificity and leads to enhanced HA processing and expanded cellular tropism [42]. The 1918 HA protein does not possess such a cleavage site mutation and does not lead to replication outside the respiratory tree in mouse models; while it has been reported to have trypsin-independent growth properties in cell culture [16]. Analysis of viral antigen-distribution in lungs of infected mice showed that 1918 HA-expressing viruses were dominantly found in both bronchiolar and alveolar epithelial cells with a distribution that appeared to be independent of sialic-acid binding specificity. However, these studies could not rule out subtle differences in sialic acid-mediated cellular tropism, including differences in infection of type I and II alveolar epithelial cells or macrophages. Studies are underway to address the question of which bronchiolar and alveolar epithelial cells are infected by different 1918 HA-expressing viruses. A possible mechanism of the virulence of the 1918 HA could be tropism for immune cells, such as alveolar macrophages and dendritic cells which might lead to inhibition of protective immune responses. Recent work showed that the 1918 PB1-F2 protein sensitized immune cells to pro-apoptotic stimuli [43]. Thus, if the 1918 HA protein increases infection of alveolar macrophages, effects of the 1918 PB1-F2 protein could lead to suppression of early protective-immune responses that may help explain the virulence of the fully reconstructed 1918 influenza virus in mice and macaques [16,28,44].

The results from these experiments demonstrate that while sialic acid binding-specificity of the 1918 HA protein plays an important role in pathogenesis in mice, additional virulence determinants must likely reside outside of the RBD [37]. Given the differences in reported primary isolate 1918 RBD HA sequences, it will be of interest to examine viral antigen distribution in the autopsy tissue sections from human 1918 influenza cases with known differences in HA RBD amino acid sequences to determine if the pattern of infection in human alveolar lining cells is affected by changes in HA RBD sequence and sialic acid binding-specificity. Future studies will be required to identify these virulence determinants and to understand their potential roles in affecting host range, cellular tropism, replication, transmission and pathogenesis.

36.4 Current Concerns and Future Work

Even with modern advances in vaccines and antiviral therapies, influenza A viruses remain significant pathogens for people and agriculturally significant animals. The evidence that >95% of deaths during the 1918 influenza pandemic resulted from secondary bacterial pneumonias and that primary infection with the 1918 virus results in significant activation of inflammatory responses underscore the relationship between viral and bacterial respiratory pathogens. *Staphylococcus aureus* and methilcillin-resistant *Staphylococcus aureus* (MRSA) are major causes of serious bacterial infections in the United States. Of significant concern is the emergence of community-associated methicilin-resistant *Staphylococcus aureus* (CA-MRSA) that is now epidemic in the United States. CA-MRSA causes infections in healthy individuals and has the ability to cause severe invasive disease, including fatal pneumonia that often is preceded or concurrent with influenza virus infection. Thus the emergence and spread of drug-resistant bacteria increases the need for studies of the synergistic relationship between influenza viral and bacterial infections and for pandemic preparedness. Unfortunately, much remains to be learned about the molecular basis for how influenza A virus-infection facilitates bacterial infections in natural and experimental models [25]. Identifying the molecular determinants of virulence encoded by pandemic influenza viruses and how both pandemic and seasonal influenza viruses increase susceptibility to severe secondary bacterial pneumonia is an important goal for our laboratory. Such studies will be of great importance for responding to future influenza pandemics that have been predicated to cause as many as a million deaths in the United States alone [45].

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Part XI
Clinical Medicine: Autoimmune Diseases

Chapter 37
Mind Your Xs and Ys: Genetics of the Autoimmune Disease Systemic Lupus Erythematosus

Steve P. Crampton and Silvia Bolland

37.1 Introduction

Systemic autoimmune diseases are disorders in which a variety of genetic and environmental factors interact to result in a loss of tolerance to self antigens and emergence of pathogenic autoantibodies. Multiple genes contribute to the development of these diseases, modulated by hormonal, environmental and infectious factors. Determining these interactions and their consequences is a fundamental step in elucidating the mechanisms that give rise to disease and will provide insights into methods for effective treatment. Systemic lupus erythematosus (SLE) is one of the most prevalent autoimmune diseases, affecting two million people in the USA. This disease has a 9:1female-to-male bias; symptoms usually arise during women's child-bearing years [1,2]. Lupus is most common in minority populations, such as African Americans, Latinos and Native Americans. Lupus disease is associated with a wide range of symptoms. In its mildest form, this disease causes general fatigue, fevers and skin rashes. In its most severe form, lupus causes a lethal inflammatory disease that leads to kidney, heart and lung failure. A hallmark of this disease is the presence of anti-nuclear antibodies (ANA) in the serum, which is an indication of B-cell reactivity against common self antigens. Our laboratory is conducting research that aims to understand genetic and environmental causes of lupus disease. We are investigating animal models of lupus disease in order to identify novel susceptibility factors and to discover mechanisms underlying the onset of systemic autoimmune disease.

37.2 The FcγRIIB-Deficient Mouse as an Animal Model for Lupus Disease

FcγRIIB is an immune receptor expressed on B cells and myeloid cells, which transmits a negative signal when ligated to the Fc region of IgG-containing immune complexes [3]. Mice deficient in this molecule develop spontaneous anti-nuclear antibodies and fatal glomerulonephritis, similar to human lupus [4,5]. Thus, *FcγRIIB* is a potent lupus-susceptibility gene capable of interacting with a variety of other loci to modify both the induction and progression of autoimmune disease [6]. Additionally, FcγRIIB upregulation has been found to be impaired on memory B cells from lupus patients compared to normal controls, showing unequivocally that this gene is an important regulator of autoimmunity in both murine and human lupus [7].

The identification of genetic modifiers of autoimmune disease in murine models of systemic lupus is a powerful method for discovering key pathways and molecular mechanisms that lead to increased disease susceptibility. In the characterization of the autoimmune-prone FcγRIIB-deficient mice, we have observed that their disease can be aggravated or attenuated by crossing to certain genetic modifier strains. By comparing these new models of disease, we can then systematically dissect the requirements for the autoimmune pathology.

37.3 Identification of a BALB/c-Derived Lupus Suppressor Locus

Homozygous deficiency of *FcγRIIB* on the B6 background results in anti-nuclear antibody production and eventually fatal glomerulonephritis [4]. Mice containing the same deletion on the BALB/c background are completely protected from lupus, indicating the existence of BALB/c-specific suppressor loci which restrict the development of autoimmunity [4]. To study the impact of the B6/BALB background modifiers, we performed an F2 linkage analysis from a cross between B6.*FcγRIIB*$^{-/-}$ and BALB.*FcγRIIB*$^{-/-}$ mice. These studies indicated that chromosomes 12 and 17 were likely to contain regions with positive linkage for ANA and glomerulonephritis [5]. The strongest effect came from a proximal region on chromosome 17 near the MHC complex. MHC genes have already been described as important genetic factors for autoimmunity, both in mice and humans, so we chose to concentrate our attention to the locus on chromosome 12, which we have named *sbb2*. This interval does not contain obvious candidate genes,

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thus increasing the likelihood that fine mapping of this locus will lead to the identification of novel genes that modify susceptibility of autoimmune disease.

In order to identify more narrowly the BALB/c-specific region on chromosome 12 that confers protection against lupus, we generated a congenic strain in which the BALB/c resistant allele on chromosome 12 was transferred to the B6.*FcγRIIB*$^{-/-}$ lupus-prone strain [8]. Thus, the centromeric portion of the BALB/c chromosome 12 was transferred to the B6.*FcγRIIB*$^{-/-}$ background, using a marker-assisted selection protocol. After seven generations of backcrosses, it was determined that over 99% of the genome in these mice was of B6 origin. From the analysis of six different congenic lines, the genomic region between 15.3 Mb and 25.6 Mb on chromosome 12 was defined as the *sbb2* locus, the BALB/c allele being *sbb2*^{a} and the B6 allele being *sbb2*^{b}.

Our analysis of the inheritance of the s*bb2* locus among F2 progeny suggested that it contributes to susceptibility in an allele-dose manner, intermediate for heterozygotes and complete for homozygotes [5]. Exactly as predicted, the presence of *sbb2*^{a}, even as heterozygote BALB/B6, rendered B6.*FcγRIIB*$^{-/-}$ mice resistant to lupus disease: none of the 12 B6.*FcγR2IIB*$^{-/-}$*sbb2*$^{a/a}$ mice tested developed ANA or glomerulonephritis, compared with 47 out of 55 [85%) for the B6.*FcγRIIB*$^{-/-}$*sbb2*$^{b/b}$ mice [8]. The primary effect of the *sbb2*^{a}-mediated lupus suppression seems to be to counterbalance the increase in the number of IgG-producing cells due to FcγRIIB deletion.

Our initial results using mixed bone-marrow reconstitutions excluded B cells and myeloid cells as candidates for the *sbb2*^{a}-mediated suppression [8]. T-cells seemed to be a good candidate because the most noticeable difference between *sbb2*^{a} and *sbb2*^{b} strains is the disparity in the number of effector/memory T cells. Because this phenotype is apparent in mice before the inflammatory pathology is evident, most likely it is a primary result from the different *sbb2* alleles. A reduction in the number of activated T cells could have an impact in the serum IgG levels, either by limiting the formation of germinal centers, by changing the cytokine environment, or by reducing the amount of tissue destruction and concomitant exposure to new antigens.

37.4 The Y Chromosome-Linked Autoimmune Accelerator Locus Potentiates Autoimmunity in FcγRIIB-Deficient Mice

The Y chromosome-linked autoimmune accelerator (*Yaa*) was first identified as an enhancer of autoimmunity in males of the BXSB strain [13]. Beside the hybrid BXSB strain, the *Yaa* Y chromosome exacerbates disease in a number of lupus-prone strains, such as NZW/B6 F1, B6.*Sle1* and B6.*CD22*$^{-/-}$, but it does not induce autoimmune disease in wild-type B6 mice [14]. The identity of the *Yaa* gene product and the mechanism by which *Yaa* modifies autoantibody specificity was unknown until recently. Several pieces of evidence suggested that the *Yaa* defect might be involved in the excessive activation of B cells and myeloid cells: *Yaa* B cells showed increased spontaneous IgM secretion and lacked the marginal-zone population [15]. Monocytosis and CD11c cell expansion were also detected in the *Yaa* mice from an early age [16].

We have analyzed the effect of the *Yaa* genetic modifier in the context of the lupus-prone, FcγRIIB-deficient mice. Addition of the *Yaa* modifier to the FcγRIIB-deficient model not only aggravates the kidney pathology and reduces the animal's lifespan, but it also leads to a switch of specificity from anti-chromatin to anti-nucleolar autoantibodies [5] (Fig. 37.1). The specificity of autoantibodies against nuclear antigens is quite often used to diagnose and prognose systemic autoimmune diseases. Additionally, ANA specificities seem to be an important factor determining success in B-cell depletion therapies [17]. Presumably, these different antibody specificities reflect a discrete breach in immune regulation, which we aimed to uncover with the identification of the *Yaa* gene product and the elucidation of the

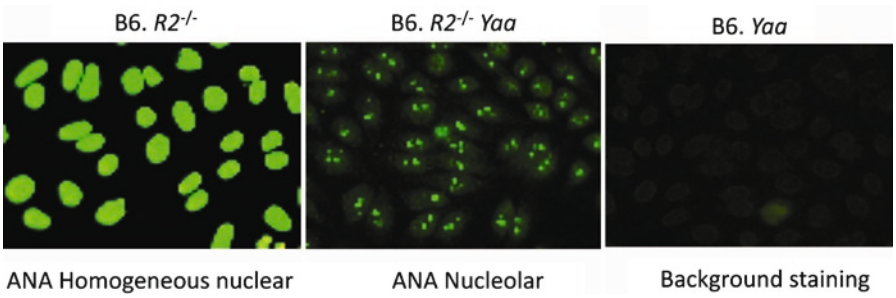

Fig. 37.1 The presence of the *Yaa* genetic modifier changes autoantibody specificity from anti-nuclear to anti-nucleolar. Representative images displaying the staining pattern of serum autoantibodies to a Hep-2 human cell line. Serum was obtained from B6.*FcγRIIB*$^{-/-}$(R2$^{-/-}$), B6.*FcgRIIB*$^{-/-}$*Yaa*, or B6.*Yaa.* From left to right: homogenous nuclear staining indicative of anti-histone or anti-DNA serum autoantibodies, nucleolar staining representing anti-RNA or anti-RNA-binding protein autoantibodies and non-specific background staining

mechanism by which *Yaa* modifies autoantibody specificity towards nucleolar antigens.

In mixed bone-marrow reconstitution experiments, *Yaa FcγRIIB*$^{-/-}$ B cells produced anti-nucleolar antibodies while non-*Yaa FcγRIIB*$^{-/-}$ B cells yielded a homogeneous nuclear pattern. This experiment established that, in the context of a general loss of tolerance promoted by *FcγRIIB*-deficiency, *Yaa* B cells produce antibodies with nucleolar specificity in a cell-autonomous manner [18].

Considering the overall hyperresponsive phenotype in *Yaa*, we reasoned that the marginal-zone defect in B6.*Yaa* mice might be rescued by a mutation that reduces B-cell responsiveness, such as a *Btk* deficiency. If so, this would suggest that the *Yaa* effect would be directly related to a B-cell signaling phenotype. To test this hypothesis, we generated B6.*Btk*$^{-/Yaa}$ mice and control B6.*Btk*$^{+/Yaa}$ littermates. We observed that the absence of Btk both corrected the marginal zone defect in B6.*Yaa* mice and rescued the autoimmune phenotype in the *FcγRIIB*$^{-/-}$*Yaa* mice, confirming that the Btk signaling pathway is essential for the loss of tolerance to nucleolar antigens in *FcγRIIB*$^{-/-}$*Yaa* mice [18].

37.5 Yaa-Mediated Autoimmune Acceleration in FcγRIIB$^{-/-}$ is Due to a *TLR7* Gene Duplication

Using microarray analysis on follicular B-cell RNA, we compared the gene expression profile in B6 versus B6.*Yaa*. Four of the genes that showed a two-fold increase of expression in *Yaa* B cells (*Msl31*, *Tlr7*, *Tmsb4x* and *Rab9*) were located on the X chromosome and their sequences were consecutive in the genome. This result suggested to us that the four genes contained in this genomic region of chromosome X were duplicated in the *Yaa* genome, and predicted that the DNA duplication would be located on the Y chromosome. This was confirmed by fluorescence *in situ* hybridization, using BAC probes that expanded a 4.5 Mb region at the distal end of the X chromosome. Overall, this analysis uncovered a translocation of a 4 Mb region of the X chromosome onto the Y-chromosome in *Yaa* males that results in the duplication of at least 13 known and four unknown genes [18].

Among the genes duplicated to the Y-chromosome of *Yaa* mice, Toll-like receptor 7 (*TLR7*) was the most interesting to us because it shared several characteristics predicted for the *Yaa* locus: it is expressed in mouse dendritic cells and B cells, it binds to Btk, and its ligand is ssRNA, a likely component of antigenic particles of nucleolar origin [19–21]. We confirmed that the genomic duplication in *Yaa* males resulted in increased activation of splenocytes by the TLR7 agonist imiquimod [18]. Just as TLR9 has been suggested to promote B-cell activation by DNA-containing self-antigens [22], our data indicated that TLR7 might also contribute to autoimmunity by inducing

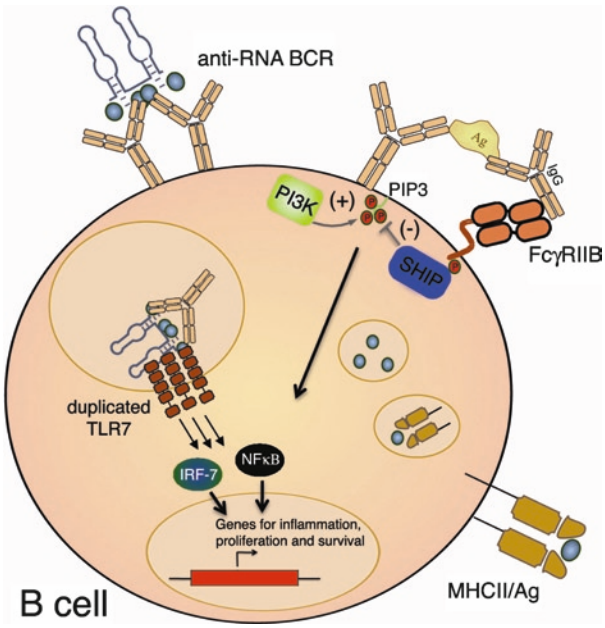

Fig. 37.2 Multiple contributions to systemic autoimmunity. FcγRIIB inhibits PI3K signals downstream of B cell receptor signaling by recruiting SHIP to the plasma membrane. In the absence of FcγRIIB, and the presence of duplicated TLR7, as seen in the *Yaa* mouse, anti-self-specific B cells are allowed to persist unchecked. Increased *TLR7* gene dosage in B cells causes the specificity of autoantibodies to switch from anti-DNA to anti-RNA, presumably by selecting for RNA-containing antigens, which will bind to and activate TLR7

the activation and selection of B cells with anti-RNA B-cell receptor specificity (Fig. 37.2). Indeed, *in vitro* B-cell activation by RNA-associated autoantigens has previously been demonstrated [23].

To determine if *TLR7* duplication was necessary for the *Yaa* phenotype, we bred *Yaa* mice to *TLR7*-ko mice, in order to eliminate the copy of this gene from the X chromosome of *Yaa* males. *FcγRIIB*$^{-/-}$*Tlr7*$^{-/Yaa}$ male mice had an increased survival rate with decreased kidney pathology compared to *FcγRIIB*$^{-/-}$*Tlr7*$^{+/Yaa}$ mice [24]. Additionally, serum autoantibodies did not switch from anti-nuclear to anti-nucleolar specificity. These data unmistakably show that an increase in *TLR7* gene dosage can potentiate systemic autoimmunity, as the phenotype of the *Yaa* mouse was completely restored through lowering *TLR7* gene dosage. *TLR7* has also been shown to be an important contributor to the *Yaa* phenotype in the B6.*sle1* mouse model of lupus [25].

37.6 Transgenic Expression of TLR7 Drives Systemic Autoimmunity

Although we determined that duplication of *TLR7* onto the Y-chromosome was the sole requirement for the accelerated autoimmune phenotype in *FcγRIIB*$^{-/-}$*Yaa* mice, we felt it important to ask whether increased *Tlr7* gene dosage was

sufficient to drive autoimmunity in the absence of the ~16 other genes duplicated in *Yaa*. In order to answer this question, we used a BAC transgenic approach [24]. The transgenic construct contained approximately 40kb, both upstream and downstream of *TLR7*, as it was designed to maintain tissue-specific expression comparable to that of the endogenous gene. Expression at the mRNA level ranged between four- and 16-fold higher than the wild-type level, as judged by quantitative PCR on follicular B cell cDNA. Increased expression of *TLR7* was also detected in purified dendritic cell mRNA, while CD4 T-cells did not show similar patterns of overexpression. Transgenic overexpression of *TLR7* resulted in the development of acute systemic autoimmune disease characterized by splenomegaly; anti-RNA autoantibody production; glomerulonephritis; dendritic cell expansion; anemia, and a pro-inflammatory interferon signature in the dendritic cells [24]. While moderately increased *TLR7* gene dosage recapitulates the Yaa phenotype and promotes activated lymphocytes, a higher level of *TLR7* expression produces a massive expansion of inflammatory dendritic cells and anemia. Thus, while TLR7 has been described as a modifier of systemic autoimmunity, we demonstrated that dysregulation of TLR7 on its own can break tolerance.

37.7 Possible Role for Autophagy in the Generation of Autoimmunity

Many questions remain about how BCR and TLR signals contribute to autoimmune phenotypes. It is well established that co-engagement of the BCR and TLR9 (using hypomethylated CpG DNA) promotes effective activation of autoreactive B cells with anti-DNA specificity [22,26]. A. Chaturvedi from the Susan Pierce Group established that internalized BCR-CpG-complexed antigen traffics to autophagosome-like structures, where it recruits TLR9 from endocytic vesicles for synergistic activation of MAP kinases [27]. In collaboration with the above-mentioned investigators, also at NIAID, we are trying to determine whether similar mechanisms exist for TLR7 and ssRNA-containing immune complexes. Indeed, it has been demonstrated that, similar to TLR9, co-engagement of the BCR and TLR7 leads to enhanced activation of autoreactive B cells [28]. Interestingly, this effect was further enhanced by administration of IFN-α, a type I interferon. Plasmacytoid Dendritic cells (pDCs) are a major source of type I interferon after viral infection and also express high levels of both TLR7 and TLR9 [29,30]. DNA-containing immune complexes have been shown to activate pDCs to secrete type I interferons through the synergistic activation of the Fc receptor FcγRIIA and TLR9 [31]. It is interesting to speculate that inappropriate activation of pDCs through immune-complexed DNA or immune-complexed RNA may lead to the IFN-α-dependent survival and Ig production of autoreactive B cells. Similar autophagosome-mediated events may exist in pDCs, as in B cells, which bring together immune-complexed DNA or immune-complexed RNA with TLRs in the endosome and ultimately amplify autoimmunity [27]. Indeed, autophagy (the process of autophagosome-formation) is required for pDC-mediated IFN-α production in response to an infection of a virus containing ssRNA [32].

37.8 SHIP Acts Downstream of FcγRIIB to Inhibit Immune Responses

Signaling events upon FcγRIIB engagement include the recruitment of the inositol phosphatase SHIP [33]. SHIP is a tyrosine phosphoprotein that breaks down PI[3,4,5]P_3 (PIP3), and regulates many PI3K-induced events, including proliferation; differentiation; apoptosis, and cell adhesion [34]. By removing PIP3 from the membrane, SHIP prevents membrane localization of several PH-domain containing factors, such as Btk and PLCγ. The net effect is to block calcium influx, which prevents sustained cellular activation [33,35,36]. SHIP is also a modulator of both growth factor and cytokine signaling [34,37]. Germline deletion of SHIP results in a lethal myeloproliferative syndrome characterized by splenomegaly, extramedullary hematopoiesis and massive myeloid-cell accumulation in the lungs [38]. The increased myeloid-cell proliferation in the SHIP-deficient mice is associated with both increased PIP3 production and the activation of the kinase Akt/PKB [39,40]. These SHIP-deficient myeloid cells are also less sensitive to apoptosis. In the lymphoid cell compartment, SHIP-deficient B-cell numbers are decreased and T cells express higher levels of activation markers, as well as increased regulatory function. Due to the pleiotropic phenotype generated from germline deletion of SHIP, the direct effect on each hematopoietic lineage is unclear. To delineate the *in vivo* function of SHIP in specific cell types, while avoiding the multi-cellular effect in germline-deleted SHIP mice, we have characterized tissue-specific SHIP deficiencies in the mouse.

37.9 Conditional Deletion of SHIP in Specific Immune Populations Reveals Its Role in Regulating Cytokine Responses

We generated mice with T-cell-specific deletion of SHIP by crossing SHIP-floxed mice with CD4-Cre mice. In the absence of SHIP in T cells, we found no differences in thymic selection or in the activation state or numbers of regulatory T cells in the periphery [41]. In contrast, SHIP-deficient T cells

did not skew efficiently to a Th2 phenotype *in vitro*. Mice with T-cell-specific deletion of SHIP showed poor antibody responses upon Alum/NP-CGG immunization and diminished Th2 cytokine production when challenged with eggs from the parasite *Schistosoma mansoni*. The failure to skew towards Th2 may be the consequence of increased basal levels of the Th1-associated transcription factor T-bet, resulting from enhanced sensitivity to cytokine-mediated T-bet induction. SHIP-deficient $CD8^+$ T-cells showed enhanced cytotoxic responses, consistent with elevated T-bet levels in these cells. Overall, the data indicate that in T cells, SHIP negatively regulates cytokine-mediated activation in a way that allows effective Th2 responses and limits T-cell cytotoxicity.

As mentioned earlier, in SHIP germline-deficient mice, there is an expansion of cells in the myeloid compartment [38]. In order to understand SHIP's direct role in myeloid cells and on the whole phenotype, we next generated mice deficient for SHIP in the macrophage/granulocyte lineage by crossing SHIP-floxed mice with LysM-Cre mice. In contrast to the unaltered T-cell development observed in mice with T-cell-specific deletion of SHIP, we detect an increased number of activated and regulatory T cells in mice with macrophage-specific deletion of SHIP (T. Tarasenko, manuscript in preparation). Consistent with an elevated inflammatory environment, $CD4^+$ T-cells primed in the presence of macrophage-specific SHIP-deleted splenocytes produced higher levels of IL17 message. We then tested these mice for sensitivity to the multiple sclerosis mouse model: experimental autoimmune encephalomyelitis (EAE), which is induced by the MOG peptide and has been reported to be dependent on IL17 and T cells [42,43]. We found that mice with macrophage-specific deletion of SHIP were more sensitive to EAE induction and developed a larger number of MOG-specific IL17-producing T cells, in addition to an expanded T-regulatory cell population. These results suggest that alterations in the macrophage-activation state resulting from SHIP deletion can lead to enhanced T-cell activation, IL17 production, elevated numbers of regulatory T cells and an increased sensitivity to an experimental autoimmune disease.

37.10 Systemic Autoimmunity Is a Multifaceted Autoimmune Disease With Both Genetic and Environmental Contributions

It is well appreciated that multiple genetic and environmental factors contribute to the onset, as well as the pathogenesis of lupus [1,2,44–46]. We have used the $Fc\gamma RIIB^{-/-}$ mouse model of lupus to investigate other loci that modify, exacerbate or protect against disease [4,5,8,18]. Molecular mimicry, and/or uncontrolled inflammation due to viral infection may underlie some of the causes or contribute to the pathogenesis of lupus. We are investigating the role of viral infection in our lupus-prone $Fc\gamma RIIB^{-/-}$ mice. We are also extending our observations on the role of increased dosage of *TLR7* in exacerbating lupus to other innate immune-receptors important for inflammation. Ultimately, an understanding of the complex genetic makeup of a lupus-susceptible individual, in addition to the role of environmental triggers, will be important to treat the disease effectively.

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Chapter 38
A Bench-to-Bedside Trail of Research Leading to the Understanding and Treatment of Ulcerative Colitis

Warren Strober and Ivan Fuss

38.1 Introduction: The Nature of Inflammatory Bowel Diseases and Their Study by the Mucosal Immunity Section of NIAID

The inflammatory bowel diseases (IBDs), Crohn's disease and ulcerative colitis, are fairly common chronic inflammatory diseases of the gastrointestinal tract that are generally thought to be due to abnormal immune responses to specific antigens and innate ligands in the normal bacterial flora of the gastrointestinal tract. Inasmuch as such antigens have access to the internal milieu, they are immunologically equivalent to self antigens. On this basis the IBDs are properly considered autoimmune diseases. Research conducted over the last two decades in the Mucosal Immunity Section (MIS) of the Laboratory of Host Defenses of the NIAID has played a leading role in the unraveling of the causes of these diseases, as well as in the development of agents that can conceivably bring these diseases under control [1]. Such research has centered on the development and study of experimental models of mucosal inflammation, as well as the underlying and basic immunologic processes that underlie such inflammation [2]. In addition, they have involved studies of patients with IBD, including studies that probe steady-state immunologic responses in patients with ongoing inflammation, as well as studies that gauge patient responses to various forms of therapy. In the following review of the work of the Mucosal Immunity section we will begin with a short discussion of our work addressing the basic immunology and treatment of Crohn's disease. We will then move to our main focus, our ground-breaking and unique studies of ulcerative colitis.

38.2 Th1/Th17 Inflammatory Bowel Disease and Crohn's Disease

Crohn's disease is a bowel-wall inflammation usually centered in the terminal part of the small bowel and adjacent ascending colon; however, it can involve virtually any part of the alimentary canal, in the form of isolated lesions randomly affecting some areas of bowel and not others. Histologically, Crohn's disease is characterized by a dense transmural infiltration of inflammatory cells that frequently contains granulomas. As a result, the lesion leads to bowel obstruction and the sequelae of obstruction: fistula and abscess formation. Mucosal ulceration can also occur but, in contrast to ulcerative colitis, it is a secondary event [3].

MIS scientists have made many important contributions to the study of Crohn's disease. Among these was the demonstration that this condition conforms to the Th1/Th2 paradigm, in that it is mediated in part by a Th1 T-cell response driven by IL-12p70 [4,5]. In addition, using a murine model of the disease developed in the Section, trinitrobenzene sulphonic acid (TNBS)-induced colitis (TNBS-colitis), we were the first to show that a Crohn's-like inflammation would respond to treatment with anti-IL-12p40, an antibody that simultaneously targets a molecular component common to both IL-12 and IL-23; thus, therapy based on anti-IL12p40 addresses the "master" cytokine inducers of both the Th1 and Th17 responses [6]. MIS scientists went on to conduct studies of the use of anti-IL-12p40 in patients with Crohn's disease, which showed that this antibody was a highly potent therapeutic agent [7]. Currently, further clinical evaluations of this antibody in the treatment of Crohn's disease are being tested by two pharmaceutical companies.

38.3 Genetic Factors and CARD15 Polymorphisms

A notable feature of both Crohn's disease and ulcerative colitis is that they are, at least in part, genetically determined diseases and as such are associated with genetic polymorphisms involving a panoply of genes that are likely to play a role in disease susceptibility [8]. MIS scientists have taken a leading position in determining how these polymorphisms lead to gut inflammation. In particular, they have conducted extensive studies relating to the function of *CARD15*, a gene on chromosome 14 that exhibits homozygous (or mixed

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heterozygous) polymorphisms in some 15% of Caucasian Crohn's disease patients [9–12].

CARD15 encodes NOD2 (**n**ucleotide-binding **o**ligomerization **d**omain **2**), an intra-cellular sensor of a component of the bacterial-cell wall peptidoglycan, muramyl dipeptide (MDP). It is expressed in antigen-presenting cells such as macrophages and dendritic cells, as well as in defensin-secreting epithelial cells (Paneth cells) located at the base of intestinal crypts of the terminal small intestine. Early studies of NOD2 showed that upon activation by MDP NOD2 causes the activation of NF-κB and thus the production of large numbers of pro-inflammatory cytokines that are produced by NF-κB target genes [13]. Paradoxically, however, the polymorphisms associated with Crohn's disease led to a reduced capacity to activate NF-κB and a reduced inflammatory response [14]; thus, ability of these polymorphisms to cause increased susceptibility to an inflammatory disease was difficult to understand.

Research conducted in the Mucosal Immunity Section solved this enigma. In initial *in vitro* studies of cells from NOD2-deficient mice, we showed that NOD2 deficiency led to increased responses to the TLR2 ligand, peptidoglycan (PGN), the molecule that gives rise to MDP and, in addition, that normal cells exhibited suppression of responses to PGN upon exposure to MDP [9]. These findings thus suggested that NOD2 had a negative regulatory effect. We then confirmed this conclusion in studies of mice over-expressing NOD2 due to the presence of a NOD2 transgene or, in mice administered MDP, both instances where NOD2 signaling is increased [11,12]. We showed that under these conditions TLR responses were decreased and, in fact, experimental colitis induced by TNBS or dextran sulfate was greatly diminished. Based on this information, we conducted further studies that showed that the TLR responses were decreased because NOD2 signaling leads to the generation of an inhibitory molecule IRF-4 (interferon regulatory factor 4) which inhibits NF-κB activation by binding to and inhibiting the function of a down-stream product of NOD2 signaling, RICK (a receptor-interacting serine/threonine kinase) [12]. Finally, we related these findings to the *CARD15* polymorphism found in Crohn's disease by showing that while MDP treatment of NOD2-deficient mice repleted with a plasmid expressing normal NOD2 inhibit experimental colitis, MDP treatment of NOD2-deficient mice repleted with a plasmid expressing NOD2 with a polymorphism fail to inhibit experimental colitis [12]. In other words, NOD2 signaling downregulates TLR responses whereas NOD2 expressing a Crohn's disease polymorphisms does not.

The significance of these findings is twofold. First they provide at least one molecular mechanism underlying the ability of commensal organisms in the gastrointestinal tract (which contain peptidoglycan in their cell walls and which gives rise to MDP) to cause a form of IBD (Crohn's disease). In the absence of normal NOD2, exposure to TLRs, expressed by commensal organisms, induces excessive immune responses. Second, they suggest a novel form of treatment for Crohn's disease: the administration of an MDP-like compound that activates NOD2 and induces substances that inhibit excessive TLR responses.

38.4 Ulcerative Colitis: A Trail of Research

Ulcerative colitis (UC), the second major type of inflammatory bowel disease, has also been a major focus of the Mucosal Immunity Section investigation. This form of IBD differs from Crohn's disease in both its clinical manifestations and its underlying immunopathogenesis. Thus, unlike Crohn's disease, it is an inflammation limited to the large bowel (colon) and in this organ it is manifests as a relatively superficial inflammation, whose main pathologic effect is visited on the colonic epithelium. Thus, its main features consist of neutrophil-rich accumulations at the base of the intestinal crypts (crypt abscesses), ultimately leading to crypt distortion, ulcerations of the epithelium, and gastrointestinal bleeding [3]. Also, unlike Crohn's disease, ulcerative colitis is not associated with granuloma formation or transmural inflammation and thus does not result in obstruction; however, over time the colon wall becomes fibrotic and stiff, exhibits poor peristaltic activity and loses absorptive and reservoir function.

Although ulcerative colitis is also marked by lymphocyte/macrophage infiltration, its immunopathogenesis has been more difficult to categorize than that in Crohn's disease. This arises from the fact that that the infiltrating cells do not fall into the usual category of either a Th1/Th17 T-cell–mediated disease (dominated by IFN-γ and IL-17 secretion) or into a Th2 T-cell–mediated disease (dominated by IL-4 secretion); on the contrary, IL-4 secretion is generally decreased [4]. Moreover, murine models of colitis in which the lymphocyte infiltrate was in fact a Th2 T-cell accumulation and was thus composed of cells that did produce IL-4, displayed a histopathologic picture that did not resemble that in ulcerative colitis [2]. Only one observation favored the idea that UC might be a kind of Th2 T-cell disease: in early studies of cytokine secretion in UC it was shown that cells extracted from UC specimens and stimulated *in vitro* produced increased amounts of IL-5 [4]. Despite this "clue," for many years the immunologic factors underlying ulcerative colitis remained poorly understood.

38.5 Oxazolone-Colitis

A breakthrough in the understanding of ulcerative colitis came with the development of a murine model of colitis known as oxazolone-induced colitis [oxazolone-colitis) [15].

The discovery of this model arose from previous studies of TNBS-colitis, in which it had been shown by members of the Mucosal Immunity Section that feeding of mice TNP-substituted protein prevented colitis development by inducing mucosal production of cells producing TGF-β. Thus, these studies of TNBS-colitis established that a Crohn's inflammation could be prevented by induction of mucosal unresponsiveness (tolerance), since, in all likelihood, the regulatory cells mediating the unresponsive were likely to be cells with anti-self specificity. The motivation to find another contactant-induced colitis was driven by the question of whether feeding of one contactant could prevent colitis due to another contactant, i.e., whether the regulatory cells induced by feeding would ameliorate colitis driven by a non-cross-reactive antigen. To our surprise, however, the colitis induced by oxazolone was far different from the one induced by TNBS. Oxazolone induced an intense, short-lived inflammation characterized by superficial, edematous inflammation of the gut wall marked by the presence of ulcerations and a neutrophil infiltration. Moreover, the inflammation was limited to the distal half of the colon. This picture exhibited far more similarity to ulcerative colitis than to Crohn's disease and, as we shall see, resembled UC in other ways as well.

Analysis of the cytokine profile of oxazolone-colitis likewise revealed that it was distinct from TNBS-colitis. The lamina propria of oxazolone-colitis was populated by T cells that, upon *ex vivo* stimulation with T-cell stimuli, produced Th2 cytokines, IL-4 and IL-5, compared to similar cells from TNBS-colitis tissues which produced mainly interferon-γ (IFN-γ) (and to a lesser extent, IL-17) [15]. In addition, as shown in further studies, this Th2 cytokine response was responsible for the inflammation, since administration of anti-IL-4 completely prevented the disease, whereas as administration of anti-IL-12p70 exacerbated the disease. Interestingly, LP cells from oxazolone-colitis lesions also produced copious amounts of TGF-β and administration of anti-TGF-β led to expansion of the disease to the entire colon. This correlated with the fact that cells extracted from the distal half of the colon in oxazolone colitis produced a greater amount of TGF-β than cells extracted from the proximal half [15]. Whether this accounts for the fact that UC likewise has a predilection for the distal bowel because of TGF-β production gradient remains to be seen.

This initial study of oxazolone colitis was a promising model of human ulcerative colitis, but was inadequate because it was so short-lived. In addition, it differed from the human disease by the fact that it appeared to be caused by IL-4, a cytokine not upregulated in ulcerative colitis. However, in a second study of this model, this problem was overcome by a skin-pre-sensitization step which led to a considerably longer disease course: whereas in the initial model the inflammation subsided after three to four days, in the model in which the mouse was pre-sensitized the inflammation persisted for seven-12 days [16]. In this more prolonged model it was found that the initial IL-4 response of the lamina propria T cells subsided after three to four days and was replaced by a more persistent IL-13 response. Moreover, the latter response became the driving force of the inflammation, since administration of IL-13R2α-Fc, an agent that blocks IL-13 with its receptors or, indeed, anti-IL-13, led to complete amelioration of the inflammation. These new data showing that oxazolone-colitis did not rely on IL-4 established this model as a *bone fide* model of ulcerative colitis.

The fact that IL-13 was produced by lamina propria cells stimulated by anti-CD3 strongly suggested that the origin of the IL-13 was some type of T cell. We, therefore, undertook various studies to identify which type of T cell was in fact involved. In one such study we found that the cell could be removed from extracted whole-cell populations by passage over an column containing bound Ig, suggesting that the cell bore an avid Fcγ receptor (CD16 or CD32) [16]. On this basis we performed several studies to determine if the cell involved was one that usually displays such a receptor such as an NKT cell (natural killer T-cell). To investigate this possibility, we treated mice with an anti-NK1.1-depleting antibody and found that, indeed, by such treatment we could prevent the development of oxazolone-colitis. In further studies along these lines we determined if oxazolone-colitis could be established in mice that lacked expression of either CD1 or a component of CD1, β2m, recognizing that CD1 is the MHC molecule that is required for activation of NKT cells (see below) [16]. The result was that oxazolone-colitis could not be induced in either CD1- or β2m-deficient mice. Finally, in the knowledge that most NKT cells bear "invariant" T-cell receptors that utilize a T-cell receptor containing the Jα281 J chain segment, we attempted to establish oxazolone-colitis in mice in which Jα281 had been deleted and found that deletion of this usual component of NKT-cell activity led to major attenuation of oxazolone-colitis [16]. Thus, on several grounds, the cell responsible for the development of oxazolone-colitis was in fact an NKT cell.

Further studies of oxazolone-colitis centered on the NKT cell and helped establish how this cell may be causing the colitis. First, we found that oxazolone-colitis was accompanied by a major expansion of the NKT cells; particularly in the lamina propria, where about a 10-fold increase in NKT cells was seen. Second, we found that stimulation of lamina propria cells with α-galactosyl ceramide (αGalCer), a glycolipid antigen that had been shown previously to stimulate NKT cells but has little capacity to stimulate conventional T cells or NK cells, led to substantial production of IL-13 (as well as a lesser amount of IL-4) [16]. In these studies, the antigen-presenting cell employed was a fibroblast [an L cell) transfected with a CD1 construct, thus fulfilling the condition that NKT cells require stimulation by antigen presented by

CD1. Thus, these studies provided additional evidence that NKT cells were the source of the IL-13 so critical to the development of oxazolone-colitis.

38.6 NKT Cells

The above studies of oxazolone colitis led to the obvious questions of whether this model was representative of human ulcerative colitis and whether the latter disease was also caused by NKT cells producing IL-13. Before we address these questions, however, it will be useful to review certain salient features of NKT cells, which might explain their relation to oxazolone-colitis and their possible connection to ulcerative colitis revealed in the discussion below.

As its name implies, an NKT cell is a "hybrid" cell that has properties of both conventional T cells and NK cells [17]. It is like a T cell in that it bears a functional T-cell receptor (unlike NK cells) and it is like an NK cell in that it bears surface antigens that are specific for NK cells. This definition implies that NKT cells can be activated/regulated by both specific antigens and by cells bearing ligands for NK surface molecules; in addition, this definition indicates that NKT cells straddle the adaptive and innate arms of the immune system.

A unique and defining feature of NKT cells, and one relating to its activation by antigens, is that these cells recognize antigens presented by an atypical MHC molecule (CD1 in mice and CD1d in humans) that present glycolipid antigens to the NKT cell T-cell receptor (TCR) [18,19]. This highly restricted set of antigens is derived from both environmental microorganisms and host cells—the latter accounting for the fact that presenting cells bearing CD1d can activate NKT cells in the absence of exogenous antigen, presumably because the CD1 may contain self antigens in its antigen-presenting groove [19,20]. Another unique feature of NKT cells is that they usually utilize a distinctive (invariant) T-cell receptor that contains a specific α chain component (Vα14-Jα281 in mice: Vα24-Jα18 in humans) linked to a restricted set of Vβ chains. However, not all NKT cells conform to this rule. A minor subset has either atypical invariant T-cell receptors or non-invariant T-cell receptors that can conceivably allow it to respond to a various types of glycolipid antigens, as long as the latter are still presented in association with CD1 [19,20]. NKT cells are heterogeneous with regard to expression of CD4 and CD8 and may display either neither or both. In addition, while most NKT cells produce mainly Th1 cytokines, some produce Th2 cytokines and their polarization in this regard appears to depend on local conditions [20]. Finally, NKT cells share with NK cells an ability to act as cytotoxic cells and it is this quality that accounts for their anti-tumor function or effector activity in certain autoimmune states.

The fact that oxazolone-colitis is associated with the expansion of NKT cells rather than another T-cell subset suggests that oxazolone provokes a unique response that is quite different from that induced by TNBS. One possibility is that oxazolone induces epithelial cells to produce certain inductive cytokines that have been shown to result in NKT cell development. Recent studies of airway hypersensitivity in which NKT cells have been shown to play an etiologic role suggest that epithelial cell production of IL-25 induces NKT cells with IL-25 receptors [21]. Thus, the association of NKT cells with oxazolone colitis results from the juxtaposition of the inflammation to the epithelial cell layer.

38.7 NKT Cells and IL-13 in Ulcerative Colitis

With this knowledge of NKT cells in hand we are now in a position to describe our studies of ulcerative colitis that, as alluded to above, were motivated by the findings in oxazolone-colitis. In initial studies we first ascertained the ability of cells extracted from patients to support the production of various cytokines. As expected from previous studies, lamina propria (LP) cells from UC patients stimulated *in vitro* with T-cell stimulants produced increased amounts of IL-5 but not IL-4 and IFN-γ [22]. In addition, however, they also produced increased amounts of IL-13 and thus satisfied an initial similarity to oxazolone-colitis. Of interest, while LP cells from patients with Crohn's disease differed from those with UC in that they produced greatly increased amounts of IFN-γ, these cells also produced increased amounts of IL-13, although not nearly as much as that produced by UC cells; thus IL-13 in this context appears to be increased under Th1/Th17 conditions that prevail in Crohn's disease tissues. Finally, it is important to add that increased IL-13 production was found in cells from inflamed areas of UC tissue but not uninflamed areas, suggesting an etiologic relation between IL-13 and disease.

In related studies we determined the cellular origin of the IL-13. Here we relied on the CD161 (NK1.1) surface marker, recognizing that while this marker was indeed found on NKT cells, it was also found on a subpopulation of activated $CD4^+$ T-cells. Using three-color flow cytometry with an intra-cellular staining procedure, we showed that while, as expected, the number of CD161 T cells was increased in both Crohn's disease and UC, in the former case this increase was associated with cells producing IFN-γ and not IL-13, whereas in the latter case the increase was associated with cells producing IL-13 and not IFN-γ [22]. In addition, depletion of LP cell populations of $CD161^+$ cells prior to cell activation (which downregulates this marker) led to a >85% depletion

of IL-13$^+$ cells. Thus, most of the IL-13$^+$ cells do bear a marker associated with NKT cells.

As noted above, NKT cells usually bear invariant TCRs (T-cell receptors) and respond to a limited number of glycolipid antigens, most notably αGalCer; therefore, it was logical to assume that the putative NKT cells producing IL-13 in UC bear an invariant TCR, as determined by the presence of cells that express cell surface Vβ11, the Vβ chain subtype of the human invariant chain NKT TCR and/or express a TCR that binds a fluorescence-labeled tetramer loaded with αGalCer. However, NKT cells in LP populations from UC patients fulfilled neither of these criteria for the identification of invariant NKT cells. We, therefore, concluded that the NKT cells belonged to the minority of NKT cells that are either invariant NKT cells that belong to an sub-family which binds a glycolipid other than αGalCer or non-invariant NKT cells which bind a large array of antigens (as do conventional T cells [22]. To buttress this possibility, we determined the ability of cells present in the UC LP T-cell population to undergo stimulation by antigen-presenting cells that express high levels of CD1d, assuming that, as mentioned above, NKT cells are defined by such stimulation. Accordingly, we cultured CD161$^+$ T-cells from the lamina propria of UC and CD patients with Epstein-Barr virus-transformed B cells expressing high levels of CD1d and then measured IL-13 production as a read-out of stimulation. As shown previously, this form of NKT-cell stimulation does not require the presence of exogenous antigen because the T cells are simultaneously exposed to substimulatory doses of phorbol ester (PMA). We found that, indeed, LP NKT cells from UC patients, but not CD patients, are stimulated to produce IL-13 (but not IFN-γ) under these conditions [22]. Thus, despite the absence of an NKT cell-associated invariant TCR, the cell producing IL-13 in ulcerative colitis is in fact an NKT cell and one that produces IL-13 upon appropriate stimulation.

In a final series of studies of NKT cells in UC patients, we evaluated the potential of the UC NKT cells to act as cytotoxic cells with respect to epithelial cell target cells bearing CD1d. We found that NKT cells extracted from UC tissues (but not CD tissues) are cytotoxic to HT-29 epithelial cells, particularly when they are cultured with the epithelial cells in the presence of IL-13. In addition, such cytotoxicity is greatly diminished if antibody to CD1d is present, indicating that the NKT cells are indeed recognizing the epithelial cells via CD1d (Fig. 38.1) [22].

38.8 IL-13 and Epithelial Cells

Since, in the above studies IL-13 proved to be so important in the pathogenesis of both experimental and human ulcerative colitis, we next conducted extensive studies of the effect of IL-13 on epithelial cell monolayers [23]. In this case, we again studied HT-29 cells, since these cells also bear receptors for IL-13 and thus can conceivably respond to this cytokine. We found that IL-13 does indeed have a detrimental effect on HT-29 cell monolayers, which is manifest as a rapid and persistent decrease in transepithelial resistance. In contrast, IL-4 did not have a similar effect, despite the fact that IL-4 and IL-13 share a receptor: IL-13Rα1 also expressed by HT-29 cells. This suggests that the detrimental effect of IL-13 is mediated by a non-shared IL-13 receptor: IL-13Rα2. It is also worth mentioning that TNF-α synergized with IL-13 in causing detrimental epithelial effects, thus providing a rationale for the treatment of UC patients with anti-TNF-α.

Investigation of this effect of IL-13 suggested that the decrease in epithelial resistance was due to an increased paracellular permeability. This was supported by the fact that IL-13-treated monolayers exhibited increased tracer fluxes and an increased number of areas exhibiting elevated conductivity. This increase in paracellular permeability was probably due, at least in part, to increased apoptosis and local disruption of epithelial cell integrity around apoptotic cells. Indeed, we observed that IL-13 exposure was associated with a five-fold increase in the number of apoptotic cells and the resistance change was decreased by about 50% with the addition of exogenous caspase inhibitors. A second factor contributing to the increased paracellular permeability was a change in the composition of the tight-junction protein network. Thus, IL-13 treatment was associated with three-fold increase in the expression of claudin-2, a tight-junction protein that increases epithelial layer permeability, but no changes in occludin, claudin-1 or claudin-4. Importantly, these changes in claudin-2 were mirrored by similar changes in the inflamed mucosal tissues of ulcerative colitis patients where the increase in claudin-2 levels was even more pronounced and occludin and claudin-1 levels were increased. A final abnormality associated with IL-13 exposure was that treated cells exhibited decreased restitution of standardized gaps in the monolayer, suggesting that the cell monolayer had a reduced capacity to recover from injury [23].

Taken together, these studies offer ample evidence that IL-13 has a direct effect on epithelial cells that leads to profoundly altered epithelial barrier function and possibly to ulceration (Fig. 38.1).

38.9 A Clinical Study

While the studies described above strongly implicate NKT cells producing IL-13 in the pathogenesis of ulcerative colitis, the ultimate proof of this concept awaits clinical studies in which it is shown that the disease is ameliorated by agents which block NKT cell activity and/or IL-13 production.

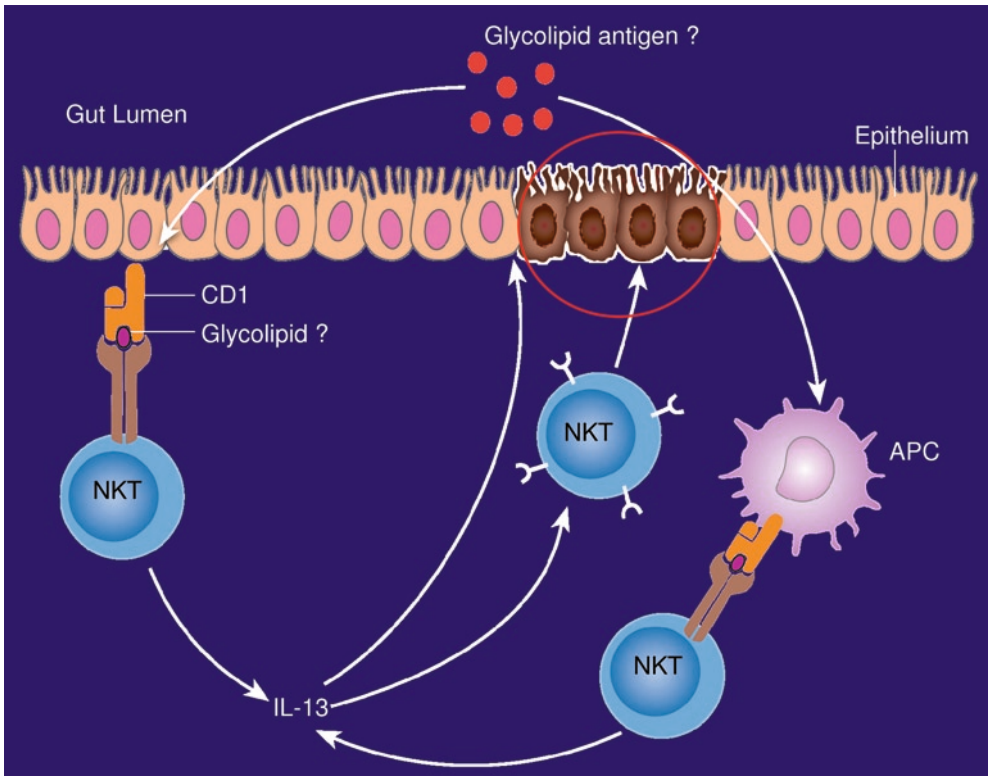

Fig. 38.1 Proposed pathogenesis of human ulcerative colitis. Innate or self -antigen(s) is(are) presented to NK T cells by lamina propria antigen-presenting cells in the context of CD1. The NKT cells activated in this way secrete IL-13, a cytokine with the potential to degrade epithelial barrier function. Alternatively, the NKT themselves are damaging to epithelial cells through their cytotoxic activity, particularly when they are stimulated in the presence of IL-13. The combined effect of IL-13-secreting NKT cells is the loss of epithelial integrity, ulceration and the entry of commensal bacterial, which cause further inflammatory chanes, into the lamina propria

We obtained partial evidence of this type in a study conducted by Dr. Peter Mannon and other members of the Mucosal Immunity Section, in which patients were treated with IFN-β, an agent previously shown to have some efficacy in UC [24,25]. In these studies, we showed that weekly administration of IFN-β for 12 weeks led to a clinical response in about 80% of 16 patients and in these patients, the response persisted for 18 weeks after cessation of treatment. Among these responding patients, 10 had experienced clinical remission, which, in most cases, persisted throughout the observation period of 24 weeks. Finally, four patients with initial remission from disease who eventually had recurrent disease, all experienced a second remission upon retreatment.

These studies established that IFN-β is a possible treatment of UC and, in addition, provided a platform to ask the question of whether or not successful therapy might be accompanied by a normalization of IL-13 secretion. To explore this possibility, the study was accompanied by an extensive evaluation of patient lamina propria mononuclear-cell, cytokine-secretion profiles before and after treatment. We found that, consistent with previous studies mentioned above, all patients exhibited elevated IL-13 secretion. More importantly, however, we found that these increased secretion values fell over the course of treatment in rough proportion to the level of clinical response: those with frank remission manifested a return to normal IL-13 secretion levels, whereas those with only partial responses exhibited reduced IL-13 secretion levels that nevertheless remained above normal secretion levels; finally, those without clinical responses showed no drop in IL-13 secretion. This correlation of disease activity with a decrease in IL-13 secretion provided some validation of the view that elevated IL-13 secretion is indeed a major factor in the immunopathogenesis of UC. It should be noted, however, that it is still possible that the IL-13 decrease noted in this study was a "bystander" effect that accompanies remission but is not responsible for the latter. Thus, definitive proof awaits treatment of patients with agents that specifically target IL-13.

38.10 Summary and the Future

The studies described above by members of the Mucosal Immunity Section of the Laboratory of Host Defenses represent a bench-to-bedside trail of research that not only provides insight into the nature of a major inflammatory disease, ulcerative colitis, they have led to a new treatment option that

holds promise of providing definitive control of this disease. We can now say with considerable confidence that ulcerative colitis is a disease associated with the appearance of lamina propria NKT cells producing IL-13. Furthermore, the prime inflammatory features of the disease, epithelial cell disruption and ulceration, are likely to be the result of these immune elements, in that NKT cells have demonstrable cytotoxic activity for epithelial cells and IL-13 has profound effects on epithelial barrier function, apoptosis and restitution.

These major advances set the set the stage for a new round of research that addresses major questions raised by this new knowledge. First, what are the factors that result in the expansion of NKT cells and, more particularly, NKT cells that produce IL-13? As alluded to above, it seems likely that the juxtaposition of NKT cells and epithelial cells in ulcerative colitis is not fortuitous, but instead, is the result of an etiologic relationship. It is possible, for instance, that epithelial cells in ulcerative colitis are genetically conditioned to produce cytokines that induce IL-13-producing NKT cells under certain circumstances. Evidence for this possibility comes from studies of experimental asthma, in which it has been shown that IL-25 plays a strategic role in such induction [21]. The availability of the oxazolone-colitis model offers an opportunity to examine this possibility. Another key research question is whether or not specific anti-IL-13 therapy offers a new and more powerful way of treating ulcerative colitis. The promise of such treatment is vested in the fact that IL-13 not only has detrimental effects on epithelial cells, but also enhances both cytotoxic activity of NKT cells and the tendency of these cells to produce IL-13. This ability of IL-13 to signal NKT cells and thereby enhance their pathologic potential also requires additional investigation, inasmuch as the receptor IL-13 uses in such signaling may be an additional target of rational treatment.

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Part XII
Clinical Medicine: Vaccines

Chapter 39
Structural Biology and the Design of Effective Vaccines for HIV-1 and Other Viruses

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Structural biology provides a wealth of information about the three-dimensional organization and chemical makeup of proteins. An understanding of atomic-level structure offers enormous potential to design rationally proteins that stimulate specific immune responses. Yet current vaccine development efforts makes little use of structural information. At the Vaccine Research Center, a major goal is to apply structural techniques to vaccine design for challenging pathogens, that include human immunodeficiency virus type 1 (HIV-1) and other enveloped viruses such as influenza, Ebola, and respiratory syncytial viruses. Our three-part strategy involves 1.) the definition of the functional viral spike at the atomic level 2.) achieving a structural understanding of how neutralizing antibodies recognize the spike, and 3.) rational development of proteins that can elicit a specific antibody response. Overall, our strategy aims to incorporate information about viral spike-antibody interactions, to assimilate immunogenic feedback, and to leverage recent advances in immunofocusing and computational biology.

For an elusive pathogen such as HIV-1, whether such a strategy will succeed depends both on the existence of sites of envelope (Env) vulnerability susceptible to neutralizing antibody and on the ability of the human immune system to generate high titers of such antibodies. With HIV-1, the atomic-level definition of one such vulnerable site [1] and the discovery of individuals with broadly neutralizing sera that target this site [2] bodes well for our informatics-based approach. With Ebola and influenza viruses, structures of functional viral spikes have been determined [3, 4], and sites of antibody vulnerability and elicitation of appropriate antibodies are under investigation. With respiratory syncytial virus, structures of closely related spikes have been determined [5], and issues center on the elicitation of antibodies that defuse rather than exacerbate disease.

39.1 Enveloped Viruses and Neutralizing Antibodies

A hallmark of enveloped viruses is the presence of a lipid membrane that surrounds the viral core structural proteins (Fig. 39.1). These membranes are derived from the host cell, and among other purposes, serve to protect the internal components of the virus from immune recognition. While protective, the membrane is also a barrier: to infect a cell, enveloped viruses must breach not only the target host membrane, but also their own membranes. To do so, they utilize energy stored in metastable viral spikes that protrude through the viral membrane [6]. These spikes also target the virus to specific receptors on host cells, prior to the fusion event. Together, these processes facilitate entry of the viral genome to the host cell.

In the case of type 1 fusion machinery (Fig. 39. 1), the viral spike is composed of a trimer of glycoproteins, each with a single transmembrane domain. The glycoproteins are cleaved during the maturation process into exterior N-terminal and transmembrane C-terminal components. The N-terminal component typically interacts with appropriate host receptors. The C-terminal component is involved in membrane fusion, which it accomplishes by inserting a hydrophobic "fusion peptide" into the target cell membrane, and then refolding to bring fusion peptide and transmembrane region/viral membrane into close proximity [7].

Because it protrudes through the protective membrane, the viral spike is a target for neutralizing antibody, which either binds to the spike to prevent cell attachment or binds and prevents conformational changes required for fusion. Vaccine efforts to elicit neutralizing antibodies that target the viral spike are currently underway for a number of

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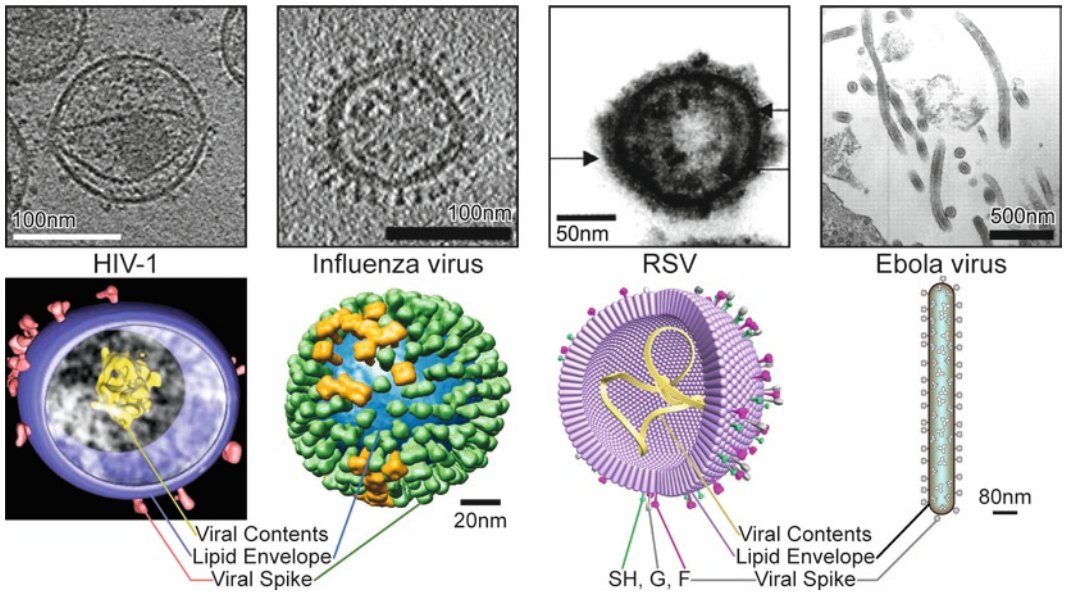

Fig. 39.1 Enveloped viruses with type 1 fusion machinery. Cryo-electron tomograms or electron micrographs (top row) are shown for HIV-1, influenza virus, RSV and Ebola virus. Schematics (bottom row) point out locations of viral spike, lipid envelope, and viral contents. HIV-1 cryo-electron tomogram and rendering reproduced with permission from the work of Sriram Subramaniam and colleagues, NCI/NIH (2008) Molecular architecture of native HIV-1 gp120 trimers. Nature 455:109–113. Influenza cryo-electron tomogram and rendering reproduced with permission from Harris, A., Cardone, G., Winkler, D.C., Heymann, J.B., Brecher, M., White, J.M. and Steven, A.C. (2006) Influenza virus pleiomorphy characterized by cryoelectron tomography. Proc Natl Acad Sci USA 103:19123–19127, copyright (2006) National Academy of Sciences, U.S.A. RSV electron micrograph reproduced with permission from Brown G, Aitken J, Rixon HW, Sugrue RJ. (2002) Caveolin-1 is incorporated into mature respiratory syncytial virus particles during virus assembly on the surface of virus-infected cells. J Gen Virol. 83(Pt 3):611–21, copyright (2002) Society for General Microbiology. Ebola electron micrograph reproduced with permission from Halfmann, P., Kim, J.H., Ebihara, H., Noda, T., Neumann, G., Feldmann, H. and Kawaoka, Y. (2008) Generation of biologically contained Ebola viruses. Proc Natl Acad Sci USA 105:1129–1133, copyright (2008) National Academy of Sciences, U.S.A

human viral pathogens. Here we describe efforts with four enveloped viruses, all of which utilize type 1 fusion entry machines.

39.2 HIV-1

HIV-1 is an enveloped lentivirus. Since its crossover from chimpanzees to humans at the end of the 19th or early part of the 20th centuries [8], the human immunodeficiency virus has killed more than 20 million individuals, and an additional ~30–35 million are currently infected [9].

Significant effort has been expended to develop an effective HIV-1 vaccine. To date, however, no vaccine against HIV-1 has been developed that elicits a fully protective response in humans. In large measure, this lack of progress is related to the elusive properties of the HIV-1 Env, which is highly variable, silenced by carbohydrates, and flexible in conformation [10]. We have therefore sought to understand the structure of the HIV-1 Env in an effort to reveal vulnerabilities on the Env glycoproteins, gp120 and gp41.

Three gp120 envelope glycoproteins associate non-covalently with three gp41 transmembrane molecules to form the functional viral spike (reviewed in [11]). Despite extensive effort, by our group and others, the trimeric functional spike has so far resisted atomic-level structure determination. Recent low resolution cryo-electron microscopy (EM) studies have provided insight into possible gp120-gp41 subunit arrangements within a functional viral spike [12, 13]. Information from cryo-EM studies is being used in our efforts to design candidate Env molecules amenable to high resolution crystallographic analysis. Part of the difficulty in structurally characterizing the functional spike relates to the biology of HIV-1. Both gp120 and gp41 components of the viral spike undergo conformational rearrangements during viral entry (Fig. 39.2). The binding by the gp120 component to the CD4 receptor is believed to induce large structural rearrangements [14, 15], which are required for the formation of the binding site for a requisite coreceptor [16], generally CXCR4 or CCR5 (reviewed in [17, 18]). The gp41 component then utilizes a large conformational change, involving formation of a trimeric "post-fusion" coiled-coil [19-23], to fuse the viral and target cell membranes. Since the "pre-fusion" conformation of the viral spike [24] is believed to be the primary target for elicitation of neutralizing antibody, we have sought to understand its conformation in atomic-level detail.

Our current approaches focus on developing strategies to overcome the inherent flexibility of the envelope spike and to

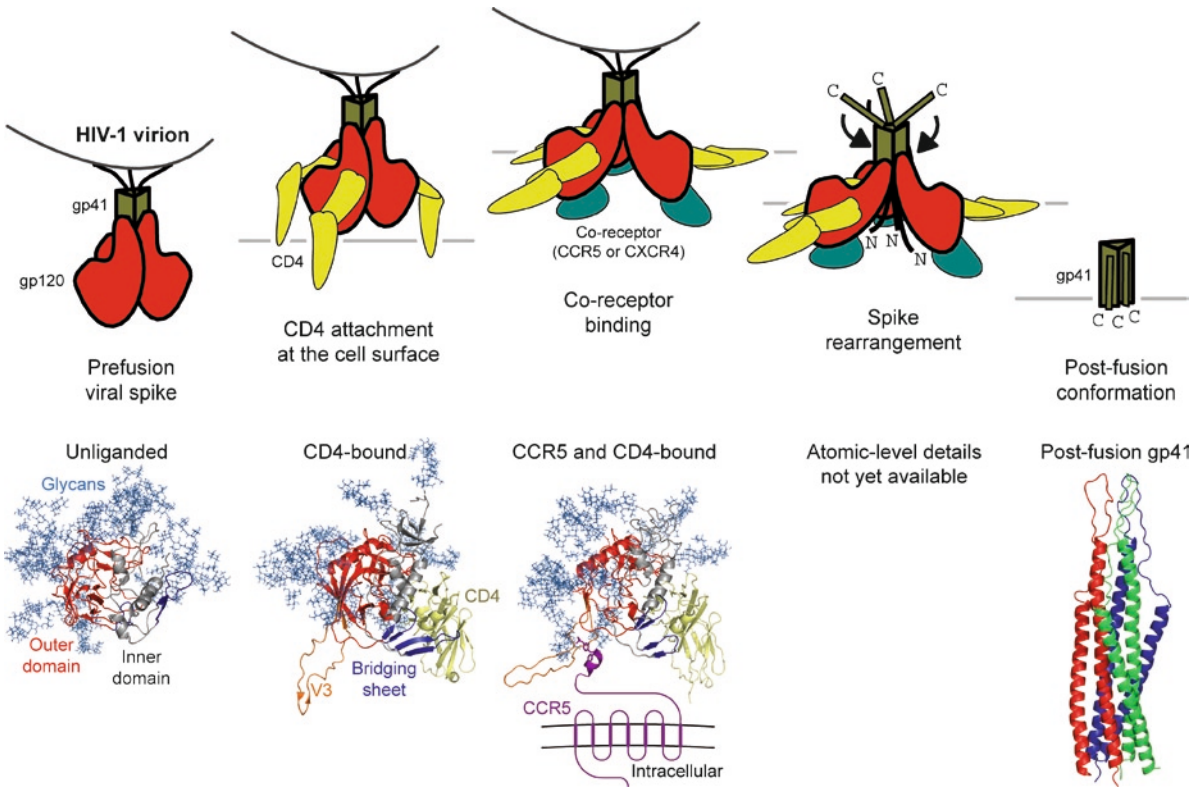

Fig. 39.2 HIV-1 entry pathway and atomic-level structures. (Top row) Schematics of the viral spike (gp120 in red and gp41 in tan) are shown, first in the context of free HIV-1 virion (left-most image), then binding to CD4 (yellow) at the target cell surface. Binding to CD4 induces conformational changes that assemble the binding site for a requisite co-receptor (teal). Binding to co-receptor results in further conformational changes: the fusion peptide at the N-terminus of gp41 is thrown into the target cell membrane; the gp41 C-terminus rearranges to form the final coiled-coil structure with gp41 N and C termini in close proximity (right-most image). (Bottom row) Atomic-level structure of monomeric gp120 and trimeric gp41 are shown in Cα-ribbon representation. Structures are arranged to correspond with conformational states shown in the schematics. Unliganded gp120 coordinates from (15); PDB ID 2bf1. CD4-bound gp120 coordinates from (29); PDB ID 2b4c. CCR5- and CD4-bound gp120 coordinates from (30); PDB IDs 2qad & 2rll. Post-fusion gp41 coordinates from (19); PDB ID 2ezo

isolate sufficient amounts of conformationally stable trimeric proteins for high resolution structural studies. In an effort to obtain well-diffracting crystals of the "pre-fusion" trimeric viral spike, we have utilized heterologous trimerization domains as well as ligands and chemical/mutational modifications to enhance protein stability. Most of the viral spikes that we have constructed have mutations that prevent the proteolytic cleavage of gp41 from gp120, which increases protein stability and ease of production. However, since cleaved viral spikes are antigenically and structurally distinct from uncleaved spikes, and represent the functional form of the molecule, methods for creating cleaved viral spikes are being optimized. gp41 peptides are currently being developed that bind to the N and C termini of gp120 and the co-crystallization of other published peptides is also being pursued [25].

In a further attempt to increase the number of crystallization variants [26], the methods described above are being applied to envelope proteins from the closely related primate lentiviruses, HIV-2 and SIV. HIV-1, HIV-2 and SIV envelope glycoproteins share a high degree of sequence conservation and common structural elements. Like HIV-1, HIV-2 and SIV use CD4 and chemokine co-receptors, thereby suggesting the use of a common mechanism for viral entry. High resolution structure determination of a trimeric HIV-2 or SIV envelope spike could provide a useful prototype for HIV-1 immunogen design.

We and others have obtained structural information on the individual gp120 and gp41 components (Fig. 39.2). For gp41, only post-fusion structures have been determined [19, 21–23]. For gp120, structures of several proposed intermediates in the entry pathway have been determined. These structures include a flexible, unliganded gp120-core for a simian immunodeficiency virus isolate [15], the CD4-bound gp120 core for several different HIV-1 isolates [27–29], and HIV-1 gp120 bound to both CD4 and the N-terminus of CCR5 that was obtained by a combination of X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and molecular docking [30]. These structures reveal the conformational flexibility that gp120 must adopt to facilitate HIV-1 entry.

In addition to the entry mechanism, the structural information reveals additional mechanisms of humoral immune evasion. Mapping antibody epitopes onto atomic-level structures

showed that a large portion of the gp120 surface is not recognized by antibody [10]. This surface corresponds to regions with high concentrations of N-linked carbohydrate (glycan), suggesting that carbohydrate is seen as "self" by the humoral immune system and can form an immunologically "silent" face [10]. In the context of the functional viral spike, this glycan can protect neighboring epitopes through an "evolving glycan shield" [31]. These and other studies, defining for example protective variable loops [32] and conformational masking of antibody epitopes [33], have led to an understanding of the molecular mechanisms that protects HIV-1 from the humoral immune response.

39.3 Structure-Assisted Vaccine Design for HIV-1

Currently, two lines of investigation have shown promise in our effort to harness structural biology for vaccine design. One line involves the delineation of functional constraints to identify potential footholds of conservation and exposure. We have investigated antibodies, called CD4-induced antibodies that bind to the co-receptor-binding site on gp120. These were found capable of neutralizing not only HIV-1, but also the more evolutionarily divergent HIV-2 [34]. They also accumulate to high titers in most HIV-1 infected individuals [34]. Unfortunately, the virus effectively hides the site of co-receptor binding prior to CD4 engagement [34, 35]. After CD4 engagement at the cell surface, the close proximity of the target-cell membrane sterically occludes potential sites of vulnerability on the co-receptor-binding site [36]. These studies demonstrate that functional constraints restrict epitope variation but do not necessarily expose epitopes sensitive to antibody neutralization. We are currently exploring whether the functional constraint of binding to the CD4 receptor, which — unlike the co-receptor-binding site — must be available as an initial site of attachment [1], can provide a site of vulnerability to neutralizing antibodies.

A second line of investigation involves structural characterization of monoclonal antibodies that neutralize diverse isolates of primary HIV-1 and comparative analysis to antibodies that do not neutralize the primary isolates. Only four antibodies that neutralize the primary isolates have thus far been characterized-the monoclonal antibodies 2F5, 2G12, 4E10, and b12 [37–40]. We have determined the structures of 2F5 and b12, each with their HIV-1 envelope epitopes [1, 41] (Fig. 39.3). In the case of the broadly neutralizing 2F5 antibody, the structure of the membrane-proximal external region of gp41, when bound by 2F5, was found to adopt an extended

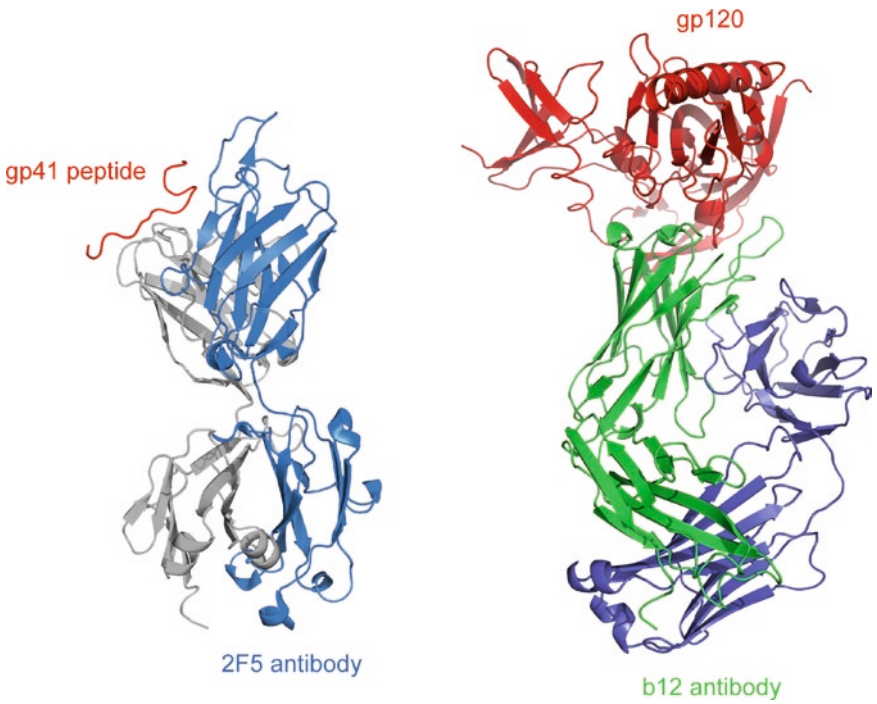

Fig. 39.3 Structure of antibodies 2F5 and b12 in complex with their respective HIV-1 Env epitopes. (Left) 2F5 antibody with gp41 epitope. The antigen-binding portion (Fab) of 2F5 is shown with heavy chain in blue and light chain in gray. A peptide, corresponding to the gp41-linear epitope recognized by 2F5, is shown in red. Coordinates from (41); PDB ID 1tji. (Right) b12 antibody with gp120. The Fab of b12 is shown with heavy chain in green grasping its gp120 epitope, while the light chain in dark blue is positioned over 15 Å away from gp120. Coordinates from (1); PDB ID 2ny7

loop conformation – distinct from unbound structures of this region, which largely adopt alpha-helical conformations [41]. These structural differences prompted the hypothesis that the inability of existing immunogens against this region to elicit 2F5-like antibodies may partly be due to a lack of proper antigenic mimicry of the 2F5-bound form of gp41. In collaboration with Drs. David Baker and Bill Schief at the University of Washington, techniques of computational protein design are being used to develop heterologous epitope scaffolds that possess conformational mimics of the 2F5-bound form of gp41 on their surfaces. These epitope scaffolds not only provide a means for structural mimicry, but also serve to focus the immune response to specific sites within otherwise complex structures.

The broadly neutralizing antibody, IgG1 b12, primarily recognizes the conformationally invariant outer domain of gp120. Focusing on the conserved CD4-binding loop, the b12 epitope overlaps considerably with the CD4 contact surface on gp120 and extends further towards the glycan covered "silent face"; however, b12 has only peripheral interactions with the other conformationally mobile parts of the gp120, such as the residues that form the bridging sheet in the CD4-bound state of gp120. Our research indicated that b12 exploits the conformationally invariant and functionally conserved CD4-contact site to achieve neutralization [1]. With a vulnerable site on HIV-1 gp120 defined, we are pursuing collaborative studies to generate b12-binding immunogens in a number of different protein forms. These alternative forms include trimeric mimics of the viral spike, monomeric variants of gp120, domain versions of gp120 that retain b12 binding, and epitope-scaffold mimics believed to be unencumbered by most Env-based mechanisms of humoral evasion. Tests of these various formats in small animals should reveal their potential to elicit antibodies similar to the template broadly neutralizing ones.

In addition to analysis of the b12 antibody, it may be useful to also analyze antibodies that target the CD4-binding site (CD4BS), but do not effectively neutralize HIV-1. Such antibodies comprise a high percentage of the elicited antibody response in HIV-1 infected individuals. We have succeeded in determining the structure of F105 in complex with gp120 (Lei Chen and Young Do Kwon, personal communication). F105 is a prototypical CD4BS antibody and has been tested in Phase I clinical trials [42]: it shows broad recognition of monomeric gp120, competes with CD4 for binding, and neutralizes laboratory-adapted – but not primary – isolates of HIV-1. Analysis of the epitopes on gp120 for F105, b12 and CD4 shows they all bind to a very closely related site on gp120. Despite their similarity, one large difference we find is that the region that makes up the bridging sheet in the CD4-bound conformation of gp120 opens up in the F105-gp120 structure. Strands β20/β21 open to uncover a conserved hydrophobic surface, which serves as a focus of F105 binding. This suggests that antibodies like F105 need to access the hydrophobic region under the bridging sheet. To define F105 as a prototype for non-neutralizing CD4BS antibodies, we tethered the β20/β21 strands to the inner domain with a disulfide and observed inhibition of binding for most CD4BS antibodies, though not b12 or CD4. Thus, this result highlights the importance of occluding the hydrophobic region under the bridging sheet in vaccine immunogens designed to elicit b12-like antibodies, and also provides another example for how atomic-level structural information can be used in the development of a successful HIV vaccine.

In addition to our efforts to understand how HIV-1 can effectively be neutralized by CD4BS antibodies, we are also studying small molecules and CD4 mimetic miniproteins that inhibit the binding between gp120 and CD4. Small molecules such as BMS806 potently inhibit HIV at nanomolar concentations [43]. Others such as NBD-556 and related compounds do so at micromolar concentrations [44]. Definition of the atomic-level mechanism by which these small molecules inhibit HIV may provide insight into how antibodies might similarly neutralize.

Collaborative efforts with Dr. Joseph Sodroski at Harvard Medical School to define the mechanism of BMS806 neutralization are ongoing. Mutations in gp120 in both inner domain and V3 region affect BMS806, a confusing result as these regions are over 50 Å distal from each other. Efforts to crystallize BMS806 are also underway, but have not yet met with success.

With NBD-556 and related compounds, modeling and exploration of structure-activity relationships suggest an interaction with a deep gp120 pocket (the Phe-43 pocket) [45], and efforts to define the interaction crystallographically are on-going. Currently the results suggest that the narrow entrance to the pocket prevents natural amino acids from fully accessing this site of vulnerability.

In terms of the CD4 mimetic miniproteins [46, 47], we have solved the crystal structures of gp120 with CD4M33/CD4M47, synthetic miniproteins into which the gp120 binding surfaces of the CD4 receptor were transplanted, to understand how precisely these miniproteins mimic CD4 for binding to gp120 [48, 49]. Biphenyl moiety of CD4M33 reaches into the deep Phe43 cavity in a way that no side chains of natural amino acids can emulate [49]. Furthermore these structures show that small molecules and miniproteins bind exactly the same gp120 conformation as CD4 binds and suggest that these molecules are useful not only for therapeutic purpose but also for exposing potential vulnerable sites on gp120 for neutralizing antibodies.

Other methods of focusing the antibody response to a precise site required for broad neutralization are being developed. These include antigenic resurfacing, conformational

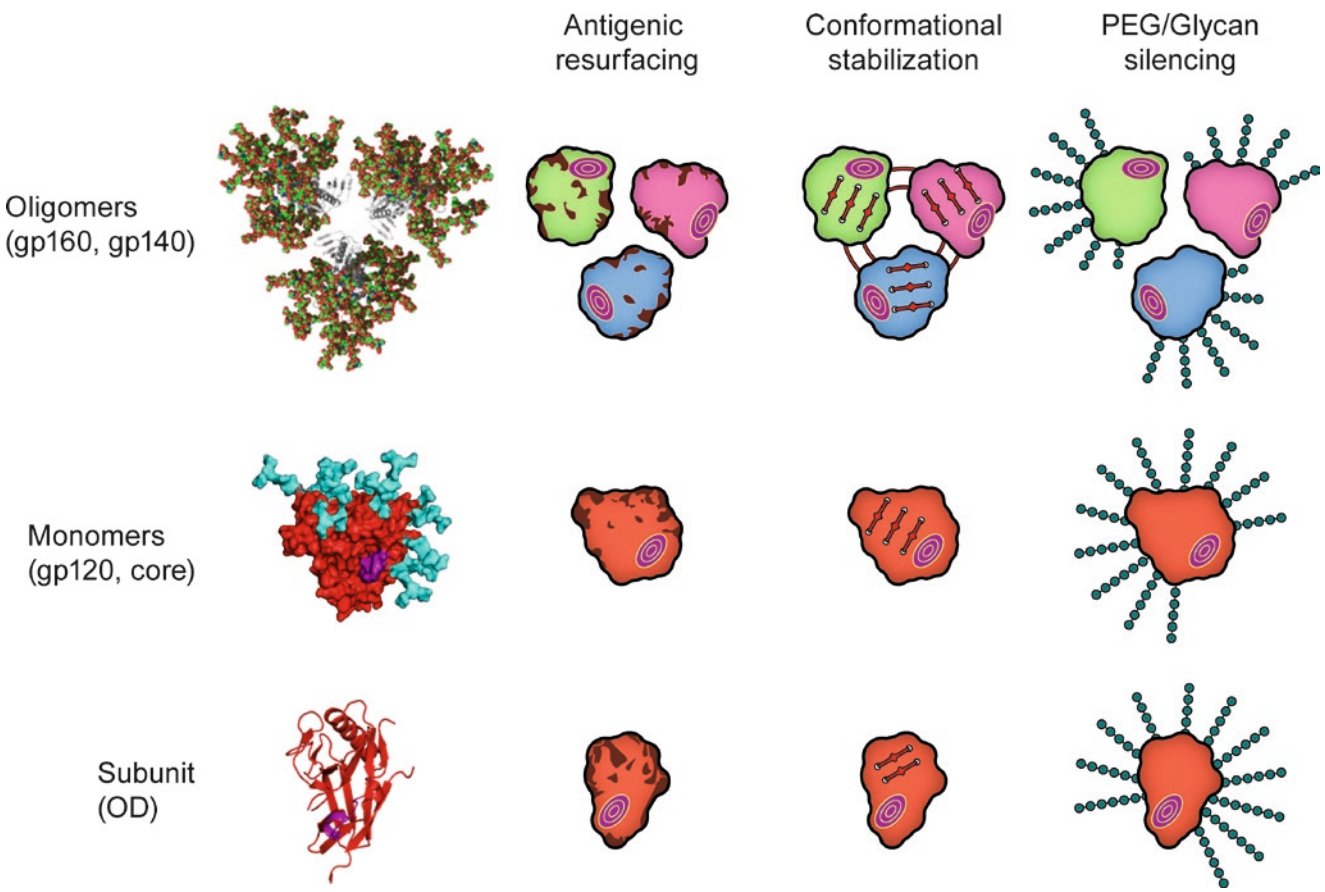

Fig. 39.4 Methods of immunofocusing to enhance the elicitation of b12-like antibodies. Three immunogenic formats of the gp120 Env are shown: as oligomers, corresponding to the trimeric state in the viral spike; as monomers, corresponding to the shed state of gp120; and as a subunit domain. Each of these formats can be modified by antigenic resurfacing, conformational stabilization and/or PEG/glycan silencing, to focus the immune response onto the b12-identified site of vulnerability

stabilization, and PEG/glycan silencing (Fig. 39.4). These methods are in theory independent from each other and could be used combinatorially.

Antigenic resurfacing involves the alteration of all surfaces other than the desired site of recognition. For example, to design antigenically resurfaced immunogens for eliciting b12-like antibodies, the defined b12 epitope was kept in its b12-bound conformation while regions otherwise not covered by glycans were subjected to modification using evolutionary similar sites from SIV or HIV-2. These variants were generated by computational design, expressed and tested for desired antigenic properties, such as binding to b12 and lack of binding to non-non-neutralizing antibodies. To further promote recognition of this specific region, two immunogens antigenically resurfaced with different amino acid modifications can be used in sequential prime boost immunization combinations.

Conformational stabilization involves the introduction of select mutations that reduce the flexibility of gp120 by locking it into a specific conformation [1]. Conformational stabilization can also increase physical stability, a useful property in making proteins sufficiently stable to maintain antigenic fidelity in the presence of adjuvants and immunization conditions.

Immunosilencing, meanwhile, seeks to reduce the immunogenicity of regions outside of the target epitope, through the addition of moieties such as N-linked glycan and or polyethylene glycol (PEG), which inherently have low immunogenicity. N-linked glycan, for example, when added to a particular region of a protein appears to dramatically reduce the immunogenicity of that region [50, 51]. As the sequon controlling N-linked glycosylation (Asn-X-Ser/Thr) can be introduced into an immunogen in a structure-specific manner, glycan-silencing allows for targeted immunosilencing (Fig. 39.5a). A related strategy utilizes PEG-chemical modification to generate protein-PEG adducts [52–54]. Because PEG molecules can be specifically coupled to lysine residues, and lysines can be introduced in a structurally specific manner, such PEG-Lys immunosilencing can also be carried out in a structure-specific manner (Fig. 39.5b).

The toolkit of structure-based design is still evolving. Nonetheless, initial attempts in small animals are beginning

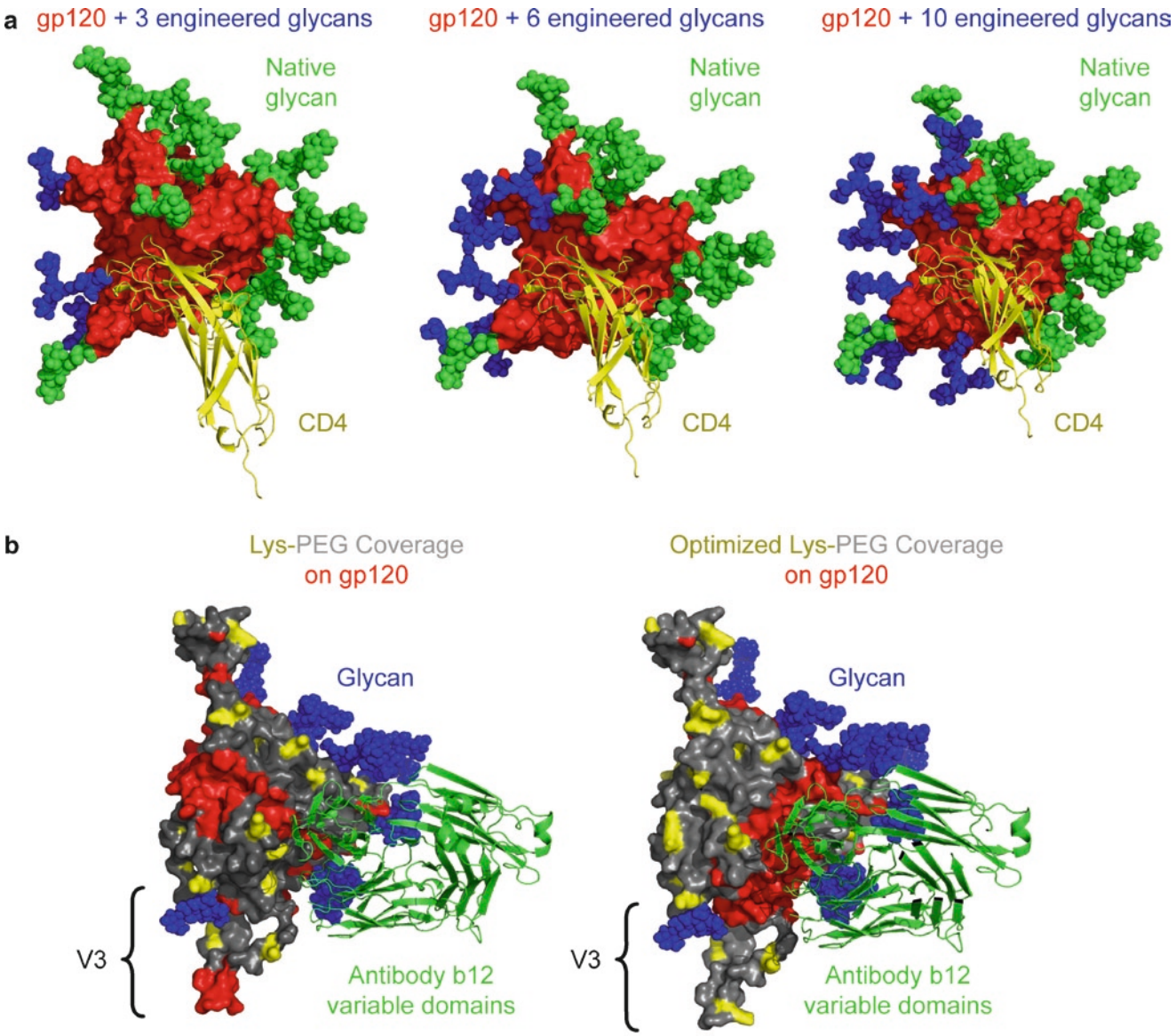

Fig. 39.5 PEG and Glycan silencing of HIV-1 gp120. (**a**) Glycan silencing. The surface of HIV-1 HXBc2 core gp120 is shown in red bound to 2-domain CD4 (yellow; Cα-ribbon representation). Coordinates from (28); PDB ID 1gc1. N-linked glycans are shown in all atom representation at three different densities. Endogenous glycans are colored green and engineered glycans in blue. Experiments with Dr. Richard Wyatt's group at the Vaccine Research Center are currently being carried out to assess the effect of these glycan modifications on expression, antigenicity and immunogenicity. (**b**) PEG silencing. The variable portion of the b12 antibody is shown as a green Cα-ribbon. N-linked glycan are colored blue and the surface of gp120 is colored red, expect for regions corresponding to Lys residues (colored yellow) or within 10 Å of a lysine (colored gray). The HXBc2 core with V3 is shown in the left image, and the Lys-optimized core is shown in the right image. As can be seen, structure-based optimization allows the b12 binding surface to be free of Lys, and to improve coverage of the rest of the gp120 surface (for example, of the V3 loop). Experiments with Dr. Quentin Sattentau's group at Oxford University are currently being carried out to assess the effect of such targeted PEGylation

to yield results. For example, studies done in collaboration with Dr. Richard Wyatt's group at the Vaccine Research Center have demonstrated the ability of conformational stabilization to focus immune response [55]. When gp120 monomers were stabilized in the CD4-bound conformation with multiple disulfides and used for immunization in rabbits, they elicited a stronger response of neutralizing CD4i antibodies than the native monomer. These studies demonstrate that structure-directed conformational stabilization can alter the quality and magnitude of the humoral immune response to a desired epitope. Though the CD4i antibodies elicited in this study are not potent against primary isolates, this proof of concept study bolsters our confidence that structure guided immunogen modification which leads to exposure and stabilization of vulnerable sites on the HIV envelope, elicits high titers of antibodies against a specific epitope.

The precise design of a gp120 monomer in the receptor-stabilized conformation was possible because of the availability of a crystal structure of gp120 bound to CD4 and the CD4i antibody-17b. We expect atomic-level structures of the trimeric spike to enable similar precise design of immunogens with optimal exposure of stabilized vulnerable epitopes of the HIV envelope.

39.4 Methods for Determining Atomic-Level Structure

Critical to the utilization of structural information in HIV-1 vaccine design is the capacity to generate the requisite atomic-level information of the relevant molecules: gp120- and gp41-derived glycoproteins, HIV-1-reactive antibodies, and various immunogens. Two structural techniques –X-ray crystallography and NMR spectroscopy – are capable of generating such information. In our work to date, we have utilized X-ray crystallography almost exclusively, although collaborative efforts to obtain solution NMR structures have progressed [30, 56].

Elucidation of the trimeric HIV-1 Env spike structure is an important, though difficult, step towards rational design of an HIV-1 immunogen. Production of adequate amounts of highly purified protein is a necessary step towards obtaining crystals suitable for atomic-level analysis. We have assessed a number of expression systems for expressing HIV-1 Env glycoproteins suitable for structural analysis (Table 39.1), and currently employ a mammalian cell-based transient transfection system for protein production (Fig. 39.6). However, high-resolution structural analysis has been hindered by difficulties in producing adequate amounts of stable HIV-1 recombinant envelope trimers. Interestingly, poor viral spike expression has also been observed for other members of the class I family of viral fusion proteins, including HIV-2/SIV, coronaviruses, and retroviruses. To address this problem, we have used glycosidase processing inhibitors, multiple transfection reagents and several target cell types to optimize trimer expression, glycan processing, and cost efficiency (Fig. 39.6). At the moment, we are testing the effects of different signal sequences and trimerization domains on viral spike expression.

Even with the current state of the art technology for data collection, only crystals with an ordered internal structure can be used for generating atomic level information. Obtaining suitable crystals for the highly glycosylated, conformationally flexible gp120, is in the best cases demanding and in the worst cases not possible. We have pioneered a number of cutting-edge crystallization strategies to increase our chances of success (Fig. 39.7). These include variational protein crystallization [26], in which a number of closely related proteins are assessed for crystallization. Thus, for example, to obtain a complex of a CD4-induced antibody with gp120, 5 CD4-induced antibodies were combined with 3 different Clade B gp120s to make 15 different complexes. Each of the 15 complexes was then assessed for crystallization, enhancing the overall probability of obtaining crystals suitable for structural analysis [29]. We have also utilized efficient high-throughput methods for screening crystallization conditions, employing factorization [57], sparse matrices [58, 59], and precipitant-focused methods of iterative optimization; and we have also embraced the latest automated structural genomic advances [60] such as crystallization robots [61, 62] and synchrotron-based means of data collection [63, 64].

As protein flexibility may be a critical factor in elicitation, we have used hydrogen-deuterium exchange techniques [65] to quantify the flexibility of gp120 [66]. Information regarding comformational dynamics of a protein in solution can be probed using heteronuclear NMR spin relaxation spectroscopy [67–72]. These experiments require either uniformally or selectively labeled ^{15}N, ^{13}C, and/or ^{2}H proteins, and we are currently optimizing protein expression in eukaryotic systems to obtain sufficient quantities of isotopically enriched HIV-1 envelope glycoprotein domains.

39.5 Ebola Virus

Because HIV-1 has numerous means of immune evasion, a vaccine will likely have to overcome extraordinary hurdles to be effective. To test the feasibility of clearing these hurdles, we have been attempting to apply structural biology to other viruses.

Table 39.1 Production of HIV-1 HXBc2 gp120 core

Expression System	Time required to produce secreted protein starting from DNA construct	Typical yield of core gp120 after affinity purification
Drosophila – S2 cells	*50–70 days**	*5–10 mg/l*
Adenovirus	*40–50 days**	*40–50 mg/l*
Transient transfection – HEK293 Freestyle cells	*3–6 days*	*10–20 mg/l*

*Includes time required to select a stable line and/or virus.

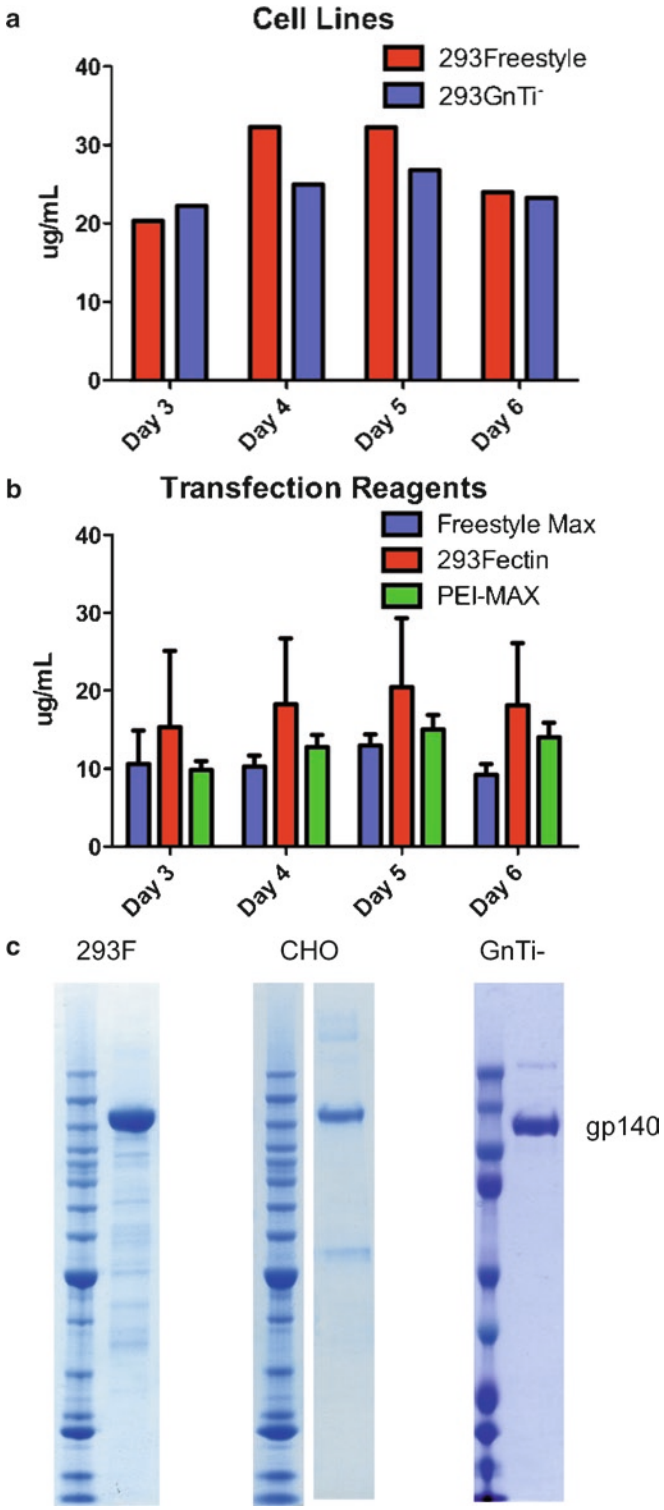

Fig. 39.6 Effects of cell line and transfection reagents on HIV-1 gp140 production and purity. **(a)** Cell line. Transient transfections with 293Freestyle cells (Invitrogen) and 293Fectin (Invitrogen) had ~10% higher CAAN-gp140 yield compared to the 293GnTi-cells (a glycosylation-deficient suspension 293 cell line; HG Khorana) (87). **(b)** Transfection reagent. Transient transfections with 293GnTi-cells and 293Fectin had ~20% higher CAAN-gp140 yield as compared to transfections with Freestyle MAX (Invitrogen) and PEI-MAX (Polysciences) in the same cells. From these results, we conclude that 293GnTi-cells may be the most cost-effective option since glycosidase inhibitors are unnecessary and there is not a significant drop in yield. **(c)** Effect on the purity. A construct encoding a clade A gp140 (fibritin trimerization domain appended C-terminal to the gp41 ectodomain) was tested in transient transfections on 293Freestyle cells (293F), Chinese Hamster Ovary cells (CHO) and 293GnTi$^-$ cells (GnTi-). Transient transfections with 293GnTi-cells resulted in higher purity of the desired protein after a Ni^{2+}-NTA column as compared to the 293Freestyle and CHO (Invitrogen) cells

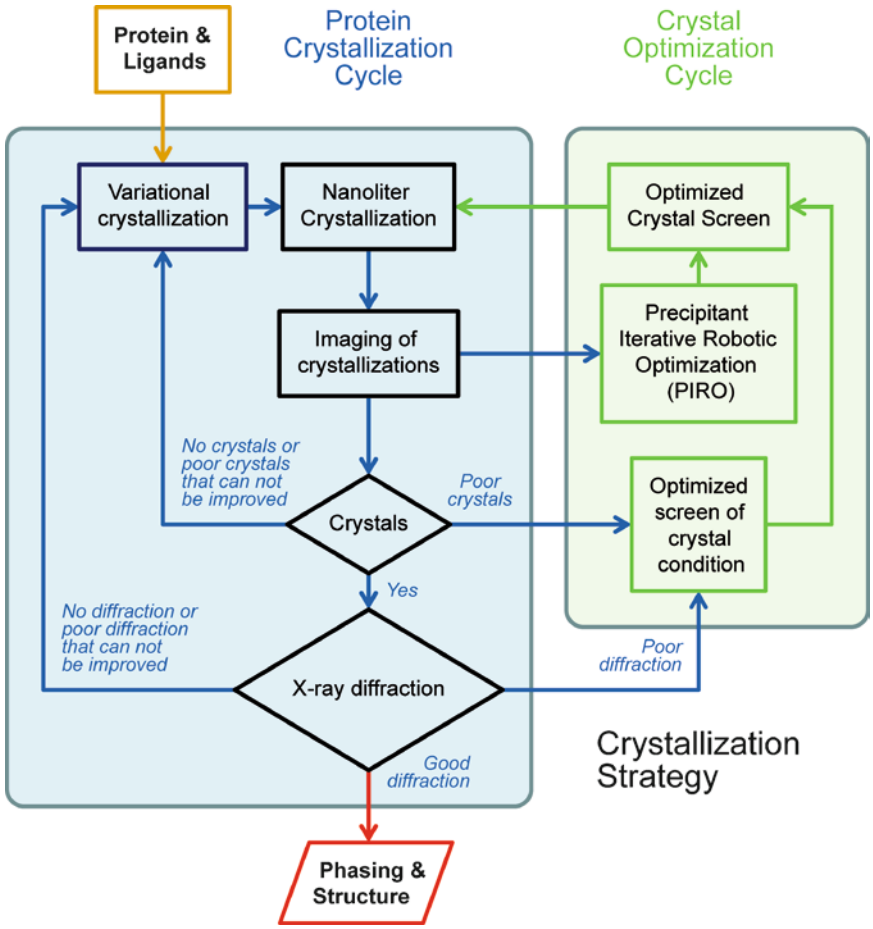

Fig. 39.7 Crystallization strategy. In the Protein Crystallization Cycle, the protein (P1) is complexed with ligands (L1). Variational crystallization, P1 is combined with different ligands (L1, L2, L3…) and/or different variations of the protein (P11, P12, P13…) are combined with ligands. Nanoliter crystallizations with droplets of total volume 200 nanoliters are set up with robots. For each complex, almost 600 different conditions are screened at the start. Data is imaged by robotic imaging systems and scored by the user. Suitable crystals are checked for x-ray diffraction. Good diffraction data is solved for the crystal structure of the protein or protein complex. The Crystal Optimization Cycle is used for optimizing the initial crystallization screening conditions, and for optimizing conditions which result in poor crystals. Imaging data is scored manually, which is used to generate a new crystallization matrix, referred to as an optimized screen. This is based on the optimizing of only the precipitant within each condition of the screen

Ebola virus is an enveloped, negative-strand RNA virus, belonging to the *Filoviridae* family. Ebola virus infection causes severe haemorrhagic fever, and depending on the strain, results in up to 90% mortality. A single envelope glycoprotein on the virion surface is responsible for attachment and entry into permissive cells, presumably via receptor-mediated endocytosis. Following endocytosis, the glycoprotein is cleaved by cathepsin B and/or cathepsin L in the acidic endosome environment and potentially triggers membrane fusion, which subsequently allows for the entry of the Ebola nucleocapsid into the cell cytoplasm [73).

The Ebola glycoprotein is generated as a precursor GP0 protein, which is cleaved at a furin-like site to yield GP1 and GP2. These proteins are linked by a disulphide bond, and a trimer complex of this heterodimer forms the viral spike. Recently, the crystal structure of prefusion Ebola virus strain Zaire was determined in complex with the human neutralizing antibody KZ52 [3]. The structure revealed that GP1 possesses an open chalice-like shape, while GP2 forms a belt around the base to create intimate GP1-GP2 and GP2-GP2 contacts (Fig. 39.8a). While a protective vaccine against Ebola will likely require the elicitation of an appropriate cellular immune response, neutralizing antibody responses against the Ebola viral spike may also play a significant protective role.

The recent structure determination of the Ebola Zaire glycoprotein provides a blueprint to design immunogens that are targeted to biologically relevant regions on the structure (Fig. 39.8c). One of the methods that can be employed to immunofocus the response is silencing regions that are not biologically relevant or known to elicit an unfavorable immune response. For example, the human neutralizing antibody, KZ52, binds to GP1:GP2 residues in the base region

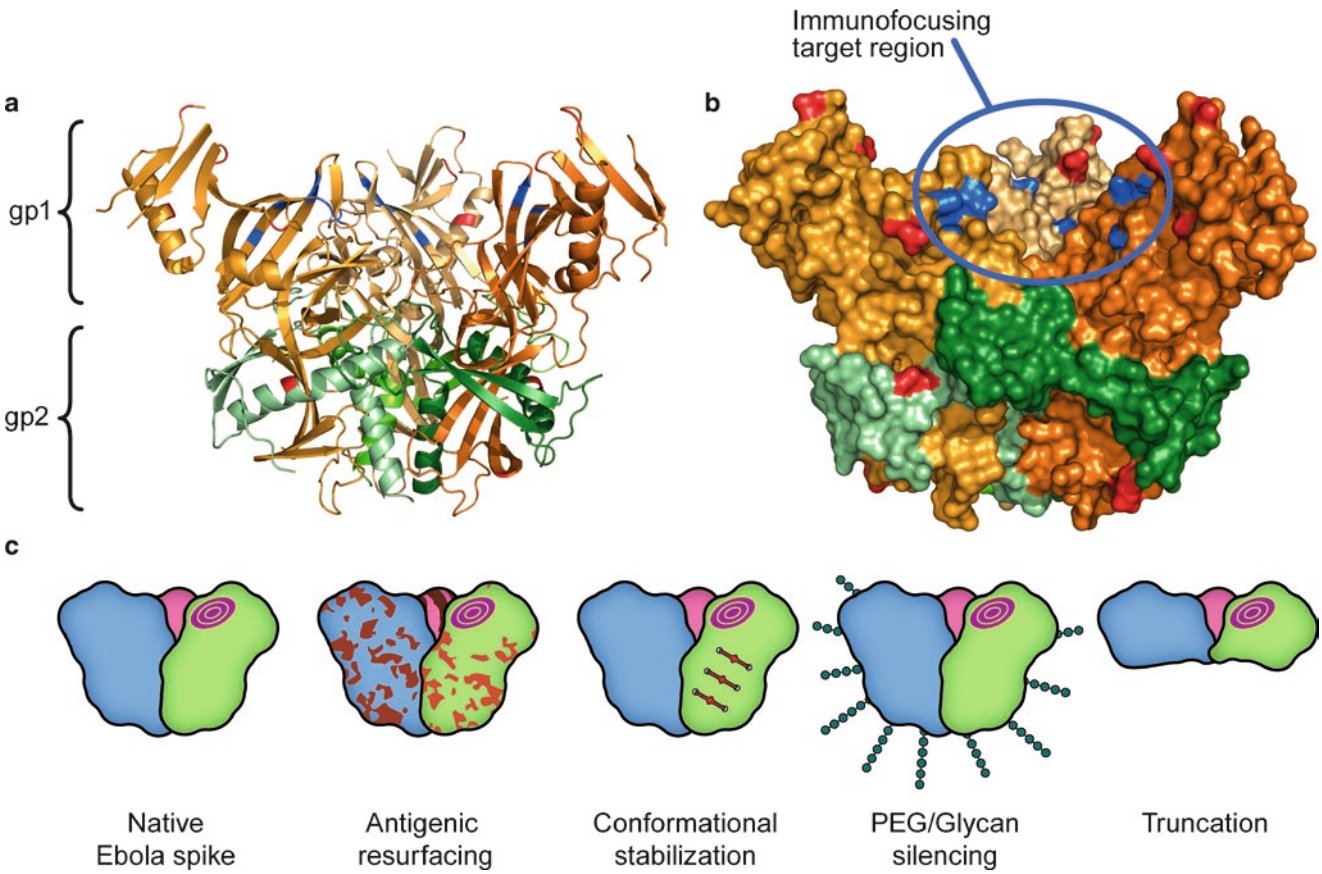

Fig. 39.8 Ebola viral spike: trimeric ectodomain structure and immunofocusing methods. **(a)** Trimeric structure. The crystal structure (3) of the ectodomain of the Ebola Zaire prefusion viral spike is depicted in Cα-backbone representation. It adopts an open chalice-like shape of GP1 (each monomer is a different shade of orange), held together by a belt of GP2 (monomers shown in various shades of green). The receptor-binding domain is localized between residues 54 and 201 of GP1 (88), of which 6 residues of known critical importance for virus entry have been mapped onto the structure (shown in blue). These residues are located in the head region, which itself is surrounded by a glycan cap containing several N-linked glycan sites (shown in red). The mucin-like domain of GP1, which was not included in the protein crystallized, would be modeled to surround the glycan cap and further extensively glycosylate the protein with both N- and O-linked sugars. Coordinates from (3); PDB ID 3csy. **(b)** Target surface. The surface of the Ebola viral spike ectodomain is shown from the same coloring and orientation as in (a), with the putative receptor-binding region highlighted. **(c)** Immunofocusing strategies. The target region for immunofocusing methods is designated by a "bull's eye" in the left-most image, and schematics for four immunofocusing strategies are depicted

of the trimer [3]. We can effectively focus the immunogen to elicit antibodies to the conserved trimer core through the addition of glycans to specific resides to which KZ52 binds. Another approach is to remove the highly glycosylated mucin-like region, which may play a role in providing the ultimate virus immune evasion strategy. Removal of this region also focuses the immune response to the exposed highly conserved receptor binding core. Our current immunogen approach, to target the highly conserved receptor-binding domain, may also result in generating protection against many, if not all, Ebola virus strains. Knowledge of the crystal structure of the prefusion Ebola glycoprotein also allows us to create recombinant proteins that mimic the processed glycoprotein that may represent a conserved but otherwise inaccessible form of the protein that may be sensitive to neutralization.

39.6 Influenza Virus

Influenza virus results in 3–5 million cases of severe illness per year causing up to 500,000 deaths worldwide (WHO EB111/10) with the most severe cases occurring in young children and the elderly. In addition to humans, influenza also infects numerous species of mammals and birds, although wild waterfowl are thought to be the primary reservoir [74]. Influenza is a spherically-shaped single-strand negative sense RNA virus belonging to the *Orthomyxoviridae* family. The outer viral surface comprises three membrane-anchored proteins: hemagglutinin (HA), neuraminidase (NA) and M2. HA is the most abundant and immunogenic of the three. To date, all neutralizing monoclonal antibodies to influenza target HA; no neutralizing antibodies against NA or M2 have been reported.

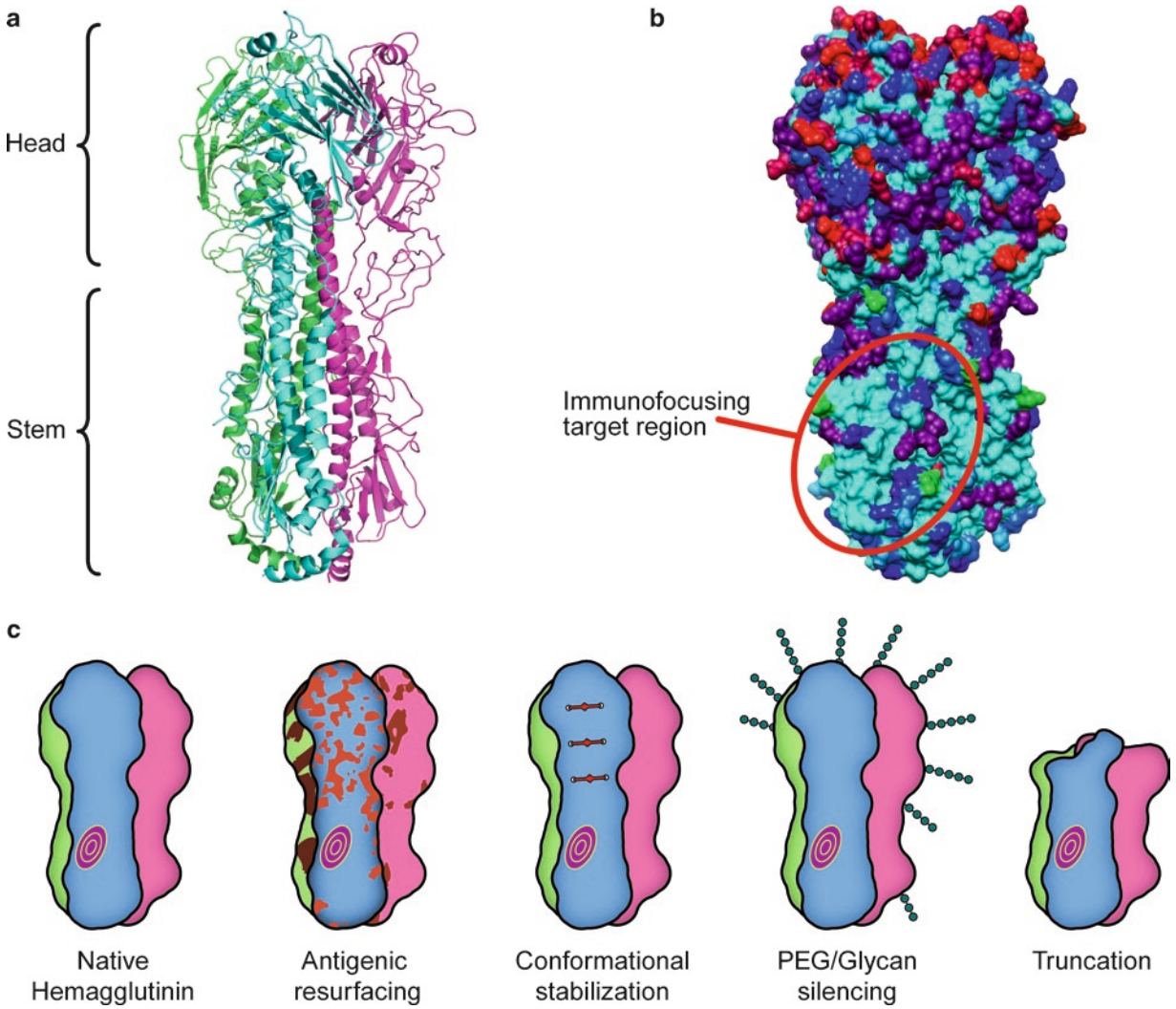

Fig. 39.9 Influenza hemagglutinin: trimeric ectodomain structure and immunofocusing methods. **(a)** Ribbon diagram of an H5N1 hemagglutinin trimer. The HA1-HA2 subunits from each hemagglutinin protomer are colored blue, green and magenta respectively. Coordinates from (75); PDB ID 2fk0. **(b)** Sequence variation between H1, H2 and H5 hemagglutinins mapped onto the molecular surface. Cyan depicts completely conserved and red highly variable amino acids. Color gradations between cyan and red represent increasing degrees of amino acid variability. Green represents Asn residues of conserved N-linked glycosylation sequons. A suggested region for immunofocusing efforts is highlighted with a red circle. **(c)** Immunofocusing strategies. The target region for immunofocusing methods is designated by a "bull's eye" in the left-most image, and schematics for four immunofocusing strategies are depicted

Crystal structures of H1, H3, H5, H7, H9 and B HAs have each revealed a 13.5 nm cylinder-shaped homotrimeric glycoprotein consisting of a mostly β-sheet globular head sitting atop a predominantly helical stem region (Fig. 39.9a) [4, 75, 76]. HA is a class I fusion protein that is cleaved during expression to generate HA1 and an HA2 with an N-terminal fusion peptide. The head region of HA recognizes terminal sialic acid (i.e. the receptor) on the surface of the host cell to facilitate viral internalization. Upon acidification below pH 6.0 in the endosome, HA undergoes a dramatic conformational change that promotes insertion of the fusion peptide into the host membrane and brings the viral and host transmembrane regions of HA into close enough proximity to induce membrane fusion.

Influenza comprises three main viruses: A, B and C, although only A and B cause disease in humans. Type-B has no subtypes. However, influenza A is highly variable and currently consists of 16 known immunological subtypes of HA and 9 subtypes of NA combined in a multitude of reassorted strains. To date, most human infections have resulted from H1N1, H1N2, H2N2, and H3N2 strains, although more recently 390 cases of highly pathogenic H5N1 infections and a handful of H7 and H9 infections of humans have been reported [77]. In total, over 6,000 different strains of influenza

are now known to infect humans [78]. Influenza is highly immunogenic; vaccines created from inactivated or live-attenuated virus are generally effective without the need for a booster shot or the use of adjuvants. Unfortunately, selective pressure from the immune system of hosts combined with an error prone viral RNA polymerase promotes rapid mutation of the influenza viral surface proteins (referred to as antigenic drift) as well as genetic reassortments. As a result, new trivalent vaccines need to be generated each year using the strains of H1N1, H3N2 and B-type influenza that are predicted to be in circulation six months later during the upcoming flu season [79]. Not only does this require a significant outpouring of time and resources every year, but the predictions regarding upcoming influenza strains are not always on target. Furthermore, a large unpredicted human outbreak from a pathogenic avian subtype such as H5N1 would not be covered by current vaccines.

We are therefore focusing our efforts to design a vaccine that can direct the immune response, particularly antibodies, against more conserved regions of the influenza virus that are not likely to mutate quickly. In 1993, Okuno and colleagues reported the generation of a broadly neutralizing murine antibody that could neutralize H1, H2, H5 and some H6 HAs [80]. More recently Kashyap and colleagues reported on broadly neutralizing human monoclonal antibodies that neutralize divergent H1 and H5 strains [81]. These reports suggest that conserved regions of HA can elicit broadly neutralizing antibodies. When sequence conservation is mapped onto the three-dimensional structure of HA, it is evident that the stem region consisting mostly of HA2 is much more conserved than the HA1-head region (Fig. 9b). Since most neutralizing HA epitopes are located on the highly variable head region, we are presently exploring methods to immunofocus antibodies to the conserved HA-stem. The methods we are employing include PEG/glycan silencing, antigenic resurfacing and protein truncation of immunodominant regions as well as removal of native glycosylation and conformational stabilization (Fig. 9c). Our ultimate goal is to design an influenza immunogen that will elicit antibodies that broadly neutralize not only against multiple strains but even against multiple subtypes of influenza over time spans of many years.

39.7 Respiratory Syncytial Virus

Human respiratory syncytial virus (RSV) is a highly contagious pathogen, infecting almost the entire US population by the second year of life [82]. It is the leading cause of bronchiolitis and pneumonia in children less than 12 months old, and it is responsible for ~100,000 hospitalizations each year in the U.S. for children in this age group [82]. RSV can lead to severe disease and death for premature infants and young children with congenital heart disease or compromised immune systems. The elderly are also susceptible to RSV infection, which results in more than 9,000 deaths per year in the U.S. [83].

Currently, there is no approved vaccine for RSV. A formalin-inactivated virus preparation was found to elicit an immune response that enhanced, rather than reduced, disease symptoms [84]. Infections in the highest risk groups can be prevented with monthly injections of neutralizing monoclonal antibodies during the RSV season, but this is an expensive treatment that is not readily available to all people in the world. As RSV shares some similarities with HIV-1 at the molecular level, it is an appealing target for structure-based vaccine design.

The F protein of RSV is related to the gp120/gp41 viral spike protein of HIV-1, and mediates fusion of the viral and cellular membranes. It is also the target of several neutralizing antibodies, including those currently being used for disease prevention. Atomic-level structures of trimeric F proteins from related viruses in both pre-fusion and post-fusion conformations exist [5, 85, 86], allowing for a molecular understanding of interactions within the viral spike that is currently not available for HIV-1. We hope to apply many of the methods and techniques originally developed for creating HIV-1 immunogens to the design of an effective RSV F protein-based vaccine. These include the attachment of heterologous trimerization domains to the C-terminus of the RSV viral spike, to stabilize it in its prefusion conformation. Other efforts include the use of epitope-scaffold technologies to focus the immune response onto appropriate areas. Definition of beneficial immune responses and their mechanisms of protection against RSV may be necessary to prevent deleterious immunization effects.

39.8 Summary

The intramural program in the Vaccine Research Center of the National Institutes of Allergy and Infectious Diseases at NIH provides an excellent setting in which to combine basic discovery with translational efforts. Whether the confluence of structural information with protein design and immune analysis is sufficient to elicit broadly neutralizing antibodies will depend in part on our ability to optimize the immune response and also on the rationale of conformational mimicry, epitope accessibility, neutralization breadth, and target specificity. Our investigations have already provided insight into the parameters governing antibody elicitation and neutralization. Whether such basic science discoveries will provide benefits to public health depends on our ability to translate them from the laboratory to the clinic, a critical step facilitated

by close interactions with other laboratories at the center. True success with HIV-1 will ultimately depend on whether we succeed in creating immunogens that can elicit broadly neutralizing responses which can then be translated into vaccine regimens capable of substantially reducing the incidence of HIV-1 infection in humans. Other future and past pandemic pathogens such as Ebola virus and influenza A virus may also be surmounted with structure-assisted efforts.

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Chapter 40
How Do You Say "B-Cell Biology" In "Vaccinology": Translational Research In the NIAID

Susan K. Pierce

40.1 Introduction

B-cell antibody responses play a key role in protection from a variety of infectious diseases. It has long been appreciated that, to a large extent, protection relies on the ability of B cells to encode an immunological memory, namely, the ability to respond more quickly and robustly to reinfection with a pathogen. In fact, all vaccines are predicated on the ability to induce long-lasting immunological memory. For antibody responses, memory is encoded, in part, in long-lived, high-affinity memory B cells MBCs that can be rapidly activated by pathogen antigens to give rise to antibody-secreting cells and long-lived plasma cells that constitutively secrete antibodies maintaining a protective level of pathogen-specific antibodies [1]. Clearly, our ability to design potent, effective vaccines would profit from a detailed understanding of the mechanisms that underlie the activation of B cells in a non-immune, immunologically-naïve individual to yield memory B cells and long-lived plasma cells.

B cells express clonally distributed receptors (BCRs) for antigens on their surfaces. The binding of antigens to the BCRs initiates signaling cascades that, when accompanied by interactions with T cells and cells of the innate immune system and their secreted products, ultimately lead to B-cell proliferation and differentiation into memory B cells and long-lived plasma cells. Over the last several years, immunologists have learned a tremendous amount about the antigen-induced signals that are transduced through the BCR, which lead to B-cell activation [2,3]. Much of our knowledge of the biology of B-cell activation in recent years has come from studies of B cells in mice and, most often, in tissue culture *in vitro*. Mice are an attractive species for investigations of B cells, as abundant reagents are available for studies, including genetically modified mice and antibodies specific for an array of proteins involved in promoting and regulating BCR signaling. Although we've learned a tremendous amount about the molecular basis of B-cell activation by studying mice, we don't yet know how much of what we have learned is applicable to human disease. The results of studies of B cells *in vitro* in tissue culture tell us what can happen, but have no ability to predict what will happen *in vivo*. Disappointingly, as recently pointed out by Davis [4], mice have often proven to be poor models for clinical studies. At some point it is necessary to take what we are learning from our mouse studies *in vitro* and see if it can be applied to human disease. The translation from a basic B-cell biology lab to the human response to infectious disease requires a big plunge from the tidy world of mouse immunology to the messy world of human research. But, given the fact that mice may not be a successful clinical model, we have little choice. Here I describe what we are learning about the early events that follow the binding of foreign antigens to the BCR which ultimately lead to B-cell activation. I then briefly suggest how the knowledge we are gaining may be applicable to understanding certain aspects of human disease that can be studied *in vitro*, in particular, B-cell cancers and systemic autoimmune diseases. Lastly, I'll describe our recent move into understanding the human immune responses to malaria, an infectious disease for which antibodies can play a key protective role, but immunological memory is slow to develop, incomplete and short lived, leaving millions of children in Africa susceptible to severe disease and death [5].

40.2 What We Are Learning About the Antigen-Induced Initiation of B Cell Responses

The research in my laboratory is driven by the assumption that the more molecular details we have of the events that lead up to B-cell activation, the better able we will be to design vaccines to maximize B-cell activation, and to identify targets for therapies to block B-cell activation in B-cell cancers and in autoimmune disease. In recent years we have focused our studies on delineating the earliest molecular events that follow antigen binding to the BCR. To do so, we have applied cutting-edge, new live-cell imaging technologies that provide both temporal and spatial information concerning

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the BCR engaged by antigen over the time and length scales that are critical to decipher the earliest events in B-cell activation.

The BCR is a member of the multichain immune-receptor family and is composed of a ligand binding chain, a membrane form of Ig (mIg), and two associated membrane proteins: Igα and Igβ [6]. The cytoplasmic tail of the mIg is short and does not connect directly to the intracellular signaling apparatus. Signaling is through the Igα/β complex that contains within its cytoplasmic domains immunoreceptor tyrosine activation motifs (ITAMs). The long-standing observation is that when the BCRs are crosslinked by a multivalent antigen, the BCRs cluster and the tyrosine residues within the ITAMs become phosphorylated by the first kinase in the pathway, Lyn [7]. The question is, *How?* How does Lyn discriminate between the unligated BCR monomer and the antigen-bound BCRs? The thinking on this issue has been greatly influenced by two observations. The first is that B cells cannot be activated by monovalent antigens that are unable to crosslink the BCR [8,9]. Thus, physical clustering by the BCR by antigen appeared to be a requisite for the initiation of signaling. Second, crystal structures of the BCR Fab, with and without antigen bound, are very similar [10]. This suggests that antigen binding to one end of the mIg could not transduce conformational changes down the molecule to effect a change in the cytoplasmic domains that would trigger their phosphorylation by Lyn. These structural results were interpreted to mean that the mIg was an inert ligand-binding chain, the only function of which was to allow passively the clustering of the receptor by multivalent antigens. However, both of these key observations seemed inadequate to explain BCR-activation, as several new unexpected findings emerged. The first important observations came from intravital imaging that showed B cells engaging antigen on the surfaces of antigen-presenting cells (APCs), rather than from solution [11]. The dynamics of the interaction of BCRs binding antigen from solution in which the antigen could provide no constraints on the BCRs, other than its own intrinsic conformation, were likely to be different when compared to the dynamics of BCRs binding antigen on the surface of an opposing membrane of an APC, in which case the antigen could impose forces on the BCR emanating from the APC membrane. Indeed, we recently provided evidence that monovalent antigens on fluid-lipid bilayers were equally able to cluster BCRs and initiate signaling, compared to multivalent antigens [12]. This finding demonstrated that the physical crosslinking of the BCRs was not a requisite for signaling. Second, data began to accumulate that conformational changes in the cytoplasmic chains did accompany the binding of antigen to the BCRs [13], and its close relative, the TCRs [14,15]. Such findings were difficult to interpret based on the structure of Fabs or TCRs bound to their antigens and brought into sharp focus the need to understand the complete structures of the intact receptors in their native conformation—that is in the membranes of B and T cells. In the absence of evidence to the contrary, it had to be considered that conformational changes could be propagated along the BCR mIg chain to induce changes in the transmembrane or cytoplasmic domains.

Our observation that monovalent-membrane associated antigens clustered BCRs and activated B cells led us to investigate what domains of the BCR were necessary for BCR clustering. To do so we generated a series of mutant BCRs that were altered in their ectodomains, transmembrane domains and cytoplasmic domains. From the analyses of the ability of these various mutant BCRs to initiate signaling, we concluded that the Cμ4 domain of the mIg of the BCR was both necessary and sufficient to trigger signaling [12]. These findings led us to propose a ”conformation-induced oligomerization model” for BCR microclustering and signaling [16]. In this model, unligated BCRs in resting cells are in a conformation that does not allow oligomerization. Consequently, the random bumping of BCRs has no consequences. The binding of membrane-associated antigens, even monovalent antigens, exerts a force on the BCR, altering its conformation, to reveal an oligomerization interface on the Cμ4 domain. The encounter of two antigen-bound receptors now leads to oligomerization through the Cμ4 domain, clustering the BCRs into signaling active complexes.

The next immediate question was, *How does clustering the BCR trigger signaling?* To answer this, we looked carefully at the consequences of antigen binding to the BCR on the BCR's local membrane environment. Using high-resolution, live-cell imaging, we learned that BCR clustering perturbed the local lipid environment of the BCR cluster, resulting in a transient coalescence of ordered lipids around the BCR [17]. These lipids are the type of saturated lipids that form lipid rafts. The Lyn kinase is tethered to the membrane inner leaflet through its modification with saturated lipids through palmitoylation and myristoylation. We observed that Lyn associated with the BCR, first transiently, through lipid-protein interactions, and then, stably, through protein-protein interactions [17]. The stable association of Lyn with the BCR correlated both spatially and temporally with previously described, antigen-induced changes in the cytoplasmic domains of the clustered BCR [13]. Based on these findings, we suggested that BCRs oligomerize following antigen-induced conformational changes and that the BCR oligomer disturbs the local lipid environment, bringing Lyn into close molecular proximity to the BCRs to phosphorylate the BCRs cytoplasmic domains [18].

40.3 Translating Our Knowledge of the Molecular Events in BCR Activation to Solving Problems of BCR Hyperactivation

Our "conformation-induced oligomerization model" for BCR activation predicts that any alteration in the process of oligomerization or membrane perturbation would have the potential to promote constitutive BCR signaling and B-cell hyperactivation. One way to alter oligomerization is through mutations of the BCR. Our observation that the Cμ4 domain when expressed on the B-cell surface with Igα/β spontaneously clustered and signaled [12] is reminiscent of the activation of B cells by N-terminally truncated forms of the mIgM during neoplastic proliferation of B cells in heavy-chain disease [19]. Although the mechanism by which these mIgM truncations lead to proliferation has not been fully delineated, the expression of N-terminally truncated forms of mIgM in mice can drive B-cell development [20,21,22], presumably by spontaneously activating BCR signaling pathways. It will be of interest to determine if heavy-chain disease B cells or B-cell tumors that require the BCR for growth show BCR conformation-induced oligomerization.

It will also be important to learn if antigen-driven, conformation-induced BCR oligomerization is regulated by B-cell coreceptors and if alterations in the function of coreceptors results in hyper-oligomerization and signaling. The FcγRIIB is a potent B-cell inhibitory coreceptor that blocks B-cell activation in response to immune complexes (ICs) and, as such, plays a decisive role in regulating antibody responses [23]. The FcγRIIB regulates both the magnitude and persistence of antibody responses through effects on both B cells and plasma cells [24,25]. Deficiencies in FcγRIIB result in susceptibility to autoimmune diseases and, in certain genetic backgrounds, severe autoimmune disease and death [26]. We recently observed that in response to membrane associated ICs, the FcγRIIB blocks blocks the assembly of BCRs into signaling-active oligomers and the association of the BCR with ordered lipids. The ability of the FcγRIIB to block these early events is not a property of a loss of function mutant FcγRIIB associated with systemic autoimmune disease. These results are exciting, as they may provide new targets to modulate the function of the FcγRIIB in autoimmunity.

40.4 B-Cell Immunity in Malaria

Malaria is a disease that claims the lives of over a million African children, per year, and it is clear that an effective malaria vaccine would play a central role in preventing these deaths. As B-cell biologists, we were drawn to the study of the immune response to malaria for two reasons. First, it had been established from passive transfer of antibodies from adults living in malaria-endemic areas to children with acute malaria, that antibodies played a protective role in controlling the infection [27,28]. Second, epidemiological observations in malaria-endemic areas have long suggested deficiencies in the generation and maintenance of B-cell immune responses and memory to *Plasmodium falciparum* (*Pf*) in individuals chronically reinfected with the parasite [5]. The question is, *Why?* There are several mouse models for malaria infections and, although each captures some features of human malaria, none is a complete clinical model for the diseases in humans. Thus, although a tremendous amount has been learned about the cellular basis of the immune response in malaria infections in mice, there is a genuine need to carry out analogous studies in humans.

In 2003, we initiated studies in collaboration with the scientists in the Malaria Vaccine Development Branch (MVDB) in the NIAID, headed by Dr. Louis Miller, to describe the generation and maintenance of B-cell memory in individuals in the U.S., in response to vaccination with malaria-vaccine candidate proteins and in Mali, an endemic area of Africa, in response to natural infection and to vaccination. Through these studies we hoped to gain an understanding of a naïve immune system's ability to respond to malaria antigens and determine if chronic malaria infections influenced that process. We also wanted to learn if vaccines that were able to induce antibody responses in U.S. volunteers would be effective in individuals in malaria-endemic areas, the target population for the vaccines.

In collaboration with the MVDB, we carried out a longitudinal study to examine the MBC response to primary immunization with the candidate malaria vaccines, Apical Membrane Antigen-Combination 1 (AMA-C1) and Merozoite Surface Protein 1 (MSP1), both formulated on alum and mixed with CPG 7909, a TLR9 ligand [29]. We learned that the acquisition of MBCs is a dynamic process in which the vaccine-specific MBCs pool rapidly expands and then contracts. In vaccinees who received CPG, vaccine-specific MBCs appeared more rapidly, in greater numbers, and persisted for longer. The percentage of vaccine-specific MBCs present at the time of re-immunization predicted vaccine-specific antibody levels 14 days later and, at a steady state, there was a positive correlation between vaccine-specific MBCs and antibody levels. We also observed an antigen-independent decrease in the total MBC pool in circulation three days after each vaccination, possibly the result of adjuvant-induced trafficking of MBCs into tissues. These results established a baseline for MBC generation and provided evidence that the generation and maintenance of MBCs in humans is a dynamic process that is significantly enhanced by activating the innate immune system's TLR9.

We recently conducted a similar analysis of the response to the AMA1 vaccines in Malian adults in collaboration with

the MVDB and scientists in the Malaria Vaccine Training Center (MRTC) at the University of Mali in Bamako. In contrast to the potent effect of CpG on the generation of MBCs observed in U.S. volunteers, the response to CpG-containing vaccines in Malian adults was similar to the response to AMA1 alone. These results suggest a possible deficiency in TLR9-responsiveness in individuals in malaria-endemic areas, which is of interest enough to warrant further investigation.

To study the B-cell response to natural infection, in 2006, with our collaborators at the MRTC, we initiated a longitudinal study of 225 children and young adults in Mali, an area of intense, seasonal malaria transmission. We learned that classic malaria-specific MBCs do develop through natural exposure to *Pf* and the frequencies of these MBCs are similar to those in vaccinated U.S. volunteers but only slowly over years. Malaria infections did not appear to have significant effects on the total number of MBCs or on antigen-specific MBCs. However, we found that a functionally and phenotypically distinct population of hypo-responsive MBCs, recently reported to be expanded in HIV-infected individuals with high viral loads [30], is significantly expanded in *Pf*-exposed Malian adults and children as young as two years of age. The number of these atypical MBCs was higher in children with chronic asymptomatic *Pf* infections, compared to uninfected children, suggesting that the chronic presence of the parasite may drive the expansion of these distinct MBCs. This is the first description of an atypical MBC phenotype associated with malaria. Understanding the origin and function of these MBCs could be important in informing the design of malaria vaccines and lead to vaccines that go beyond the traditional empiric approach, and address *Pf*-specific modulation of the immune response.

40.5 Conclusions

The NIAID has provided the scientific environment that has allowed my laboratory to translate basic B-cell biology into research in human-clinical diseases that involve anomalies in B-cell function. The ability to apply our work effectively to human disease was critically dependent on the close interface between my basic B-cell biology laboratory and those NIH laboratories carrying out investigations into human diseases that involve B-cell immune dysfunction. My experience in the NIAID brings me to the conclusion that genuine progress in solving important problems in human disease cannot be easily achieved with either the basic biology labs or the clinical labs working in isolation. It is the close proximity and intellectual exchange between the basic and clinical investigators that will set the stage to combat the world's deadliest diseases.

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Chapter 41
Malaria Vaccine Development

Yimin Wu, Ruth Ellis, Kazutoyo Miura, David Narum, and Louis H. Miller

41.1 Introduction

Despite recent advances, malaria remains a major cause of morbidity and mortality in children in sub-Saharan Africa. An estimated 881,000 malaria deaths occurred in 2006, of which 91% were in Africa and 85% were in children under five years of age [1]. Morbidity and mortality caused by malaria also have significant direct and indirect costs on the economic development of endemic countries [2]. It is estimated that malaria accounts for 40% of public health expenditures, more than 30% of inpatient admissions, and approximately 50% of outpatient visits in some African countries [1]. The usefulness of interventions, such as insecticide treated bed nets; indoor residual spraying; intermittent preventive therapy, and artemisinin-combination therapy are limited by resistance to drugs and insecticides. In addition, such interventions require sustained and costly efforts as part of a highly functioning public health system, which is problematic in areas where malaria is most prevalent. Vaccines which reduce both mortality and morbidity due to *P. falciparum* infection remain badly needed tools in the fight against malaria. To achieve the goal of eliminating malaria entirely, malaria vaccines can close the gap left by other interventions.

Over time, people living in endemic areas develop natural immunity to *P. falciparum,* as a result of repeated infection. This acquired immunity supports the feasibility of developing a vaccine against malaria. The goal of the Malaria Vaccine Development Branch (MVDB) is to develop malarial vaccines that will reduce severe disease and death in children in Africa and eliminate malaria from parts of the world where there is low transmission of malaria. MVDB has produced and tested vaccines through Phase 2 clinical trials. Further development to Phase 3 trials leading to licensure will occur in collaboration with industry.

Malarial vaccines fall into three categories that mirror the life cycle of *Plasmodium* (Fig. 41.1).

41.1.1 Pre-Erythrocytic or Liver Stage

Sporozoites are inoculated by *Anopheles* mosquitoes that feed on humans. Sporozoites are present in the bloodstream very briefly, and then invade hepatocytes, where a single sporozoite can develop into up to 40,000 merozoites. The aim of vaccines targeting the liver stage is to prevent infection and reduce disease. Protection is both humoral (blocking sporozoite invasion of hepatocytes) and cellular (killing parasites in liver cells through interferon-γ induction of nitric oxide). Natural boosting may occur even if there is sterile immunity since sporozoites will continue to be injected while endemicity persists.

41.1.2 Erythrocytic or Blood Stage

When liver-stage parasites mature, thousands of merozoites are released to the blood stream. Each merozoite can invade an erythrocyte and develop into a mature, erythrocytic-stage schizont, which ruptures and releases 10–30 merozoites, each of which can invade another erythrocyte. This stage is associated with clinical manifestations and severe disease. As opposed to pre-erythrocytic vaccines, which aim to induce sterile immunity, the aim of blood-stage vaccines is to prevent clinical malaria, and in particular, severe disease. Acquired immunity against malaria is mediated in part by blood-stage, parasite-specific antibodies. Killing of parasites is predominantly antibody-mediated, either by preventing the extracellular merozoite from invading red blood cells—by binding to variant antigens on the erythrocyte surface; by inducing antibody-dependent cellular cytotoxicity (ADCC), or by some combination of these mechanisms. As with pre-erythrocytic vaccines, natural boosting is expected to occur as long as transmission persists.

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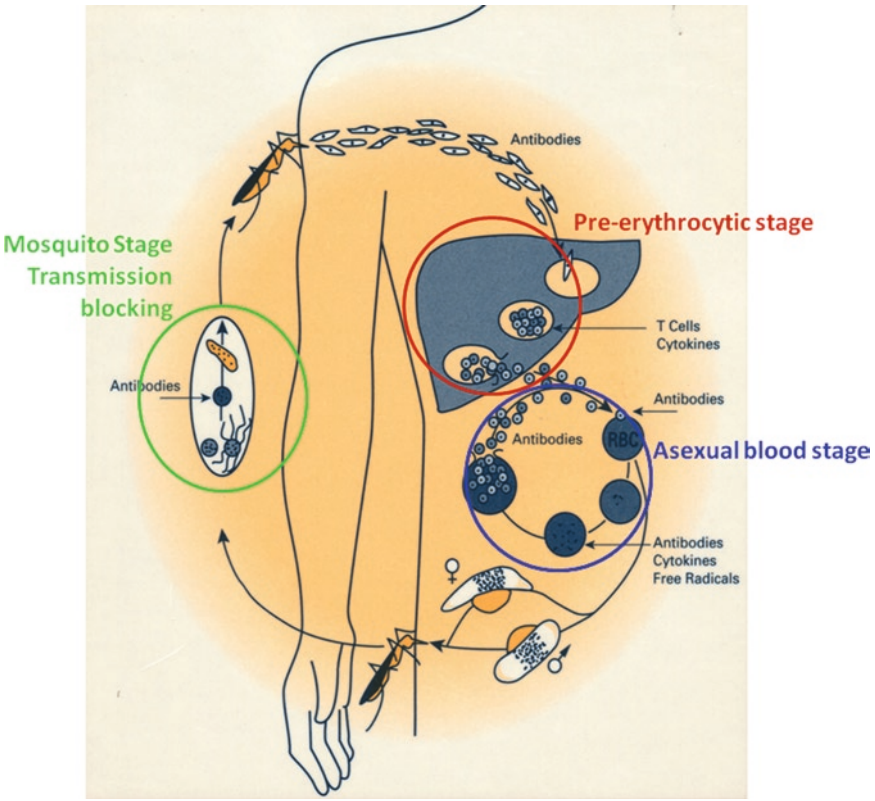

Fig. 41.1 Life cycle of malaria parasites showing types of vaccines. Malarial antigens from different stages of the life cycle may be included in one vaccine

41.1.3 Mosquito Stage

Parasites in an erythrocyte can also develop to sexual stages known as gametocytes. When a mosquito ingests blood meal containing gametocytes, the parasites further develop to male or female gametes in the mosquito midgut. Fertilization of male and female gametes leads to formation of zygotes, ookinetes, oocysts, and finally sporozoites. Because vaccines targeting antigens on zygotes and ookinetes in the mosquito midgut aim to prevent transmission of malaria in a community, they are known as transmission-blocking vaccines (TBV). TBV will induce antibodies in the vaccinated individual, and the antibodies taken up by the mosquito with the blood meal will act in the mosquito to block development of the parasite.

Ultimately, we envisage a vaccine that will combine antigens from multiple stages.

41.2 Lead Candidates for Vaccine Development

41.2.1 Blood-Stage Vaccines

Given the complexity of the *P. falciparum* parasite, it is unlikely that a single antigen can induce protection. Thus, we plan to start testing two lead blood-stage candidates, Apical Membrane Antigen 1 (AMA1) and Merozoite Surface Protein 1 (MSP1), as a combination vaccine. Later we may add more antigens if needed.

AMA1 has been shown in several studies to be a promising malaria vaccine candidate. Active or passive immunization of mice and monkeys with either purified or recombinant forms of AMA1 or an AMA1–specific mAb protected these animals against rodent and simian malaria, respectively [3]. Some of the most convincing evidence for the potential of AMA1 as a vaccine candidate has come from MVDB's study using the stringent *Aotus* model system [4]. Immunization of these New-World monkeys with recombinant *P. falciparum* AMA1-FVO expressed in *Pichia pastoris* provided significant protection against challenge with homologous *P. falciparum* parasites, in comparison to the control group. The level of protection in these experiments correlated with the anti-AMA1 titer.

AMA1 is the product of a single-copy gene and sequence analysis revealed that the protein consists of an *N*-terminal signal sequence, an ectodomain of 546 amino acids, a transmembrane domain, and a cytoplasmic tail of about 55 amino acids. The ectodomain appears as three structural subunits, identified as domains one to three. AMA1 is synthesized as an 83 kDa precursor in the micronemes of merozoites just prior to schizont rupture. At about the time of erythrocyte invasion, the *N*-terminus of the precursor form is processed

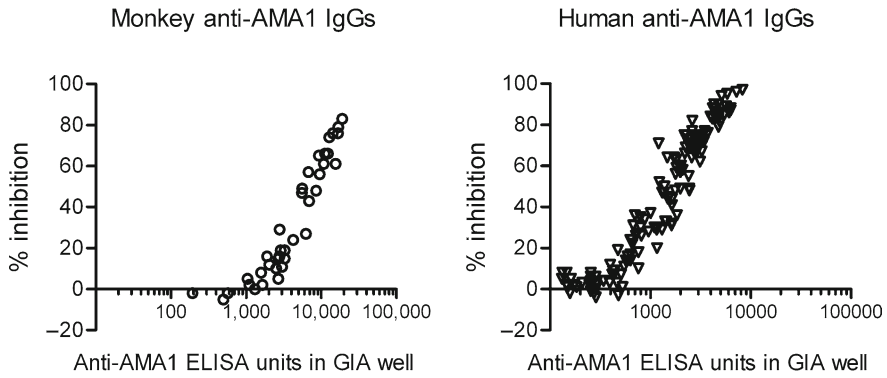

Fig. 41.2 Biological activity of rhesus monkey and human anti-AMA1 antibodies against *P. falciparum* 3D7 parasites judged by *in vitro* GIA. The anti-AMA1(3D7) ELISA units in the GIA well (x-axis) are plotted against % inhibition (y-axis) to *P. falciparum* 3D7 parasites

to a 66 kDa protein, which is then translocated to the merozoite cell surface. After analyzing the coding region of the AMA1 genes from 97 parasite clones from around the world and 61 isolates from Mali, Duan, et al [5] identified 150 haplotypes for domains one to three. A clustering algorithm grouped the 150 haplotypes into six populations that were independent of geographic location. Antibodies raised against AMA1 of *P. falciparum* strain 3D7, of haplotype population one, effectively inhibited the growth of parasite strains of the same haplotype population. Antibodies raised against AMA1 of[I am unable to fix this line-break]*P. falciparum* strain FVO effectively inhibited the growth of parasite strains from haplotype populations three and six. These data suggested that the inclusion of PfAMA1 sequences from each of the six populations may result in a vaccine that induces protective immunity against a broad range of malaria parasites.

Another leading blood-stage vaccine candidate is MSP1 [6] MSP1 is synthesized as a ~200 kDa polypeptide, and is processed, at or just prior to merozoite release from the red-blood cell, into four smaller fragments which form a non-covalently associated complex. The 42 kDa *C*-terminal fragment of MSP1, $MSP1_{42}$, is responsible for tethering the complex to the surface of the merozoite via a glycosylphosphatidylinositol (GPI) anchor. At the time of merozoite invasion of erythrocytes, $MSP1_{42}$ undergoes a secondary processing event and is cleaved into $MSP1_{33}$ and $MSP1_{19}$. The secondary processing of $MSP1_{42}$ is thought to be essential for parasite invasion of red-blood cells as inhibition of this cleavage inhibits invasion and methods that prevent invasion also block cleavage [7].

Antibodies to $MSP1_{19}$ and $MSP1_{42}$ are detected in immune sera from epidemiological studies and from animals vaccinated with $MSP1_{19}$ or $MSP1_{42}$. Although extensive epidemiological and animal data show that antibodies induced against $MSP1_{19}$ protect against malaria, $MSP1_{19}$ alone as a vaccine does not readily induce parasite-specific humoral responses because it lacks the necessary T-helper epitopes. For this reason, the larger molecule, $MSP1_{42}$, is a better choice for developing a successful vaccine strategy.

On the basis of sequence comparisons, MSP1 has been divided into blocks which are conserved, dimorphic or polymorphic. Alignments of published *P. falciparum* $MSP1_{42}$ gene sequences from different isolates revealed dimorphic forms of $MSP1_{33}$, FVO type or 3D7 type. The $MSP1_{19}$ fragment essentially contains four polymorphic amino acids. The $MSP1_{19}$-FVO contains the Q-KNG, whereas the E-T-SR form is present in $MSP1_{19}$-3D7. Thus, the FVO and 3D7 types of $MSP1_{42}$ cover both known dimorphisms in $MSP1_{33}$ and the majority of the point mutations in $MSP1_{19}$. The development and testing of a combination vaccine containing $MSP1_{42}$-FVO and $MSP1_{42}$-3D7 types could address the concerns of targeting a polymorphic protein in generating protective immune responses. This may overcome the concern that one form of $MSP1_{42}$ was ineffective in a previous study in Kenyan children [8].

41.2.2 Transmission-Blocking Vaccines (TBV)

The two malaria species which cause the most disease worldwide are *P. falciaprum* and *P. vivax*. The ideal transmission-blocking vaccine would be directed against both parasites. The leading vaccine candidate targeting the mosquito stage is a homologous protein identified in both species as Pfs25 and Pvs25 (or Pxs25), which is 25-kDa proteins expressed on the surface of zygotes and ookinetes of *P falciparum* and *P. vivax*, respectively[9]. The proteins are encoded by a single copy of homologous genes in their respective parasites, and are the dominant surface proteins of the free-living parasites (ookinetes) in the mosquito midgut. Animal-study data demonstrated that anti-Pfs25- and anti-Pvs25-specific antibodies have the ability to block parasite development in the mosquito in a membrane-feeding assay.

The second lead candidate of TBV is Pxs28, encoded by genes paralogous to the Pxs25 genes and expressed on the

surface of free-living ookinetes in mosquito midguts. Knockout experiments suggest that there is redundancy in the function since knockout of either the Pxs25 or the Pxs28 has little effect on parasite viability, but the double knockout almost completely blocks the ability of these parasites to develop. Thus a TBV combining Pxs25 and Pxs28 is expected to be more effective than the single-component vaccines.

Other candidates for TBV include Pxs48/45 and Pxs230 antigens that are expressed in gametocytes in human hosts, and on gametes and zygotes in mosquito stages. Because these antigens are expressed in the blood stage of the human host, vaccine-induced immunity may be boosted by natural malaria infection. Antibody-mediated killing in the mosquito midgut may be further enhanced by complement in the blood meal. However, production of these antigens has thus far proved to be challenging and no one has yet been able to express and purify the antigen in a scale suitable for vaccine development.

41.2.3 Pre-Erythrocytic Vaccines

Circumsporozoite Protein (CSP) is a leading candidate for pre-erythrocytic vaccines, and is a component of RTS,S, the only malaria vaccine to show consistent protection in field studies to date [10,11]. The protein is encoded by a single-copy gene and covers the entire surface of sporozoites. CSPs from *Plasmodium* spp. display common structural features, including an *N*-terminal signal peptide, a *C*-terminal glycosyl phosphatidylinositol (GPI) anchor that links the protein to the sporozoite surface, and a central domain composed mostly of amino acid repeats. The repeat region is immunodominant and is the target for neutralizing antibodies against sporozoites. Flanking the central repeats, all CSPs contain highly conserved domains designated as Region I and Region II. Region I contains a pentapeptide, KLKQP, and is involved in attachment of sporozoites to mosquito salivary gland and liver tissue. Region II is also called a thrombospondin (TSP)-like domain, containing four cysteines and CD4 and CD8 epitopes, and is involved in adhesion to and invasion of mosquito salivary gland and human liver tissues. The CD4 and CD8 epitopes in the *C*-terminal region of the CSP are polymorphic.

41.3 Production of Recombinant Malarial Proteins for Human Clinical Trials

MVDB's current approach for development and manufacturing of a bulk vaccine product is to follow a process known as Quality by Design [12]. Quality by Design aims to build the quality attributes or characteristics of a recombinant protein into a product, from the beginning of development. This approach requires appropriate analytical techniques to evaluate the recombinant protein. With this approach, MVDB has successfully produced twelve unique cGMP manufactured recombinant proteins (Table 41.1).

MVDB's development efforts, in principle, are divided into four parts: molecular design and cloning of the expression construct; fermentation; recovery, and purification. A fifth critical component is development of analytical methods for product characterization throughout the development effort.

41.3.1 Molecular Design

MVDB primarily uses two expression systems: *Escherichia coli* and *Pichia pastoris* (Fig. 41.4). *E. coli* expression system uses BL21(DE3) cells and pET expression vectors with a kanamycin resistance gene for selection (Novagen). The T7*lac* promoter system is used with IPTG for induction of recombinant protein expression which is targeted to form inclusion bodies. *P. pastoris* is a methylotrophic yeast that may metabolize methanol as its sole carbon source, using the enzyme alcohol oxidase. The alcohol oxidase gene promoter is a strong inducible promoter, which is used for control of heterologous-gene expression following integration with the *P. pastoris* expression vectors pPIC9K or pPICzαA (Invitrogen).

Historically, the *E. coli* and *P. pastoris* expression systems were difficult to use for expressing malarial proteins. In the case of *E. coli,* which is a prokaryote, the critical disulfide-bond structure of many malarial proteins, such as the EGF-like domains of $MSP1_{42}$ and Pfs25, was not properly formed. The development of refolding strategies effectively removed this obstacle for $MSP1_{42}$ [13]. However, the development of refolding conditions for some other malarial proteins has still not been possible. In particular, Pfs48/45 and Pfs230 have been difficult to produce in the correct conformation.

Another area of difficulty has been due to the unique A+T bias in the genome of *P. falciparum*. The design and synthesis of malarial genes with a modified codon bias, using codons optimized for *E. coli* or yeast, significantly improved the level of successful production of recombinant malarial proteins. Gene design also enables codon optimization, such that the most frequently used codon for maximum protein expression of the selected transcriptome may be used. Other features of gene design comprise the inclusion or exclusion of specific restriction enzyme

Table 41.1 Pilot production of bulk antigens or drug substances on clinical path

		WRAIR cGMP Pilot Production of bulk antigen			Clinical Path for Bulk Products	
Bulk Product	Strain ID	Lot #	Year Mfr	Final yield (mg/L broth)[1]	Pre-clinical	Clinical
ScPvs25H	Sal I	0865	May 01	7.9		√
		1286	Sept 05	3.4		
PpPfs25H	3D7	0913	Aug 01	4.3		√
		1082	Apr 03	17.4		
		1228	Oct 04	5.6		
		1338	Apr 06	15.6		
		1390[3]	May 07	46.6		
PpAMA1	3D7	0941	Dec 01	29.6		√
		1442	Dec 07	18.6		
	FVO	0932	Nov 01	49.9		√
		1406	Aug 07	20.8		
$EcMSP1_{42}$	FVO	0997	Jun 02	29.2		√
		1101	Jul 03	34.0		
		1161	Jan 04	32.0		
		1274	Jul 05	32.0		
		1359	Nov 06	48.2		
		1498	Jul 08	46.1		
		1512	Oct 08	36.0		
	3D7	0984	May 02	25.0		√
		1125	Sept 03	45.5		
		1152[4]	Dec 03	102.2		
		1527[4]	Dec 08	69.4		
$EcMSP1_{42}$	FUP	1183	Apr 04	87.8	√	
PpAMA1	L32	1195	Jun 04	12.0	√	
PpPvs25H	Sal I	1213	Aug 04	7.2	√	
PpPvs28	Sal I	1306[3]	Nov 05	46.6	√	
PpPfs28-LN	3D7	1350	Sept 06	8.6	√	
EcEPA[2]		1365	Jan 07	58.0	√	

[1]Final yields of bulk recombinant protein are based on 60L working volume of WRAIR bioreactor.

[2]EcEPA, *E. coli* derived ExoProtein A is a carrier protein for the purpose of preparing protein-protein conjugate vaccines.

[3]Produced in a *P. pastoris* host which over expresses Protein Disulfide Isomerase (PDI) (14).

[4]Increased yield due to process improvement without detectable change in product quality

sites; control of recombinant protein boundaries; the removal of putative N-linked glycosylation sites for yeast expression, and modification of potential Shine-Dalgarno sequences for expression in *E. coli*. After a gene is designed, synthesized and cloned into an expression vector, appropriate cells are transformed and evaluated for expression of the recombinant protein by fermentation. Once production-cell lines are identified, master and production cell banks are prepared following cGMP requirements (Fig. 41.4).

41.3.2 Fermentation

Fermentation development involves the identification of the best parameters for producing a high-quality recombinant protein in sufficient quantity in a bioreactor. Each expression system uses a limited number of standard or platform processes, which include defined media preparation, and defines procedures for cell growth prior to induction of the recombinant malarial protein. The types of fermentation conditions evaluated for each expression system are restricted due to the stage of our clinical development program which is directed toward Phase 1 and 2 human clinical trials. In the case of *P. pastoris,* temperature, pH and methanol feed rate may be evaluated during induction, and for *E. coli,* temperature, duration of induction (hours), and concentration of inductant may be evaluated. In each situation it is desirable to understand the best fermentation parameters to determine the optimal conditions for fermentation, such that performance problems identified during pilot production will be resolved prior to a costly cGMP production run.

41.3.3 Recovery

The recovery or harvest procedures used by MVDB are generally standard or platform methods designed to function well at our cGMP pilot-production facility. During the performance of these platform procedures, the processes are analyzed with respect to process efficiency and stability of the quality characteristics of the drug substance, using analytical procedures developed for each product (see details below). *E. coli* production is generally harvested by continuous centrifugation (Fig. 41.4). The biomass or cell paste is then frozen and stored until purification. Production in *P. pastoris* is through secretion of the recombinant protein in to the fermentation media, therefore the recovery is performed by a serial process of tangential-flow filtration through use of hollow fiber microfiltration (dialysis) and diafiltration/ultrafiltration (concentration/dialysis) cartridges (Fig. 41.4). The intermediate product of fermentation is frozen and stored while being evaluated for its quantity and quality, prior to proceeding to purification.

41.3.4 Purification

Even though there is a standard approach for purification of *E. coli*- and *P. pastoris*- expressed recombinant proteins

using standard scalable column chromatography, purification is an area of development that is the most product-specific and requires the building of several smaller column procedures into one batch production record. There are generally three to five procedural steps that are required for each batch production (Fig. 41.4). The procedures include protein capture, refolding (if expressed in *E. coli*), and other column-purification steps to remove host-cell proteins, endotoxin and DNA prior to sterile filtration and storage. Purification and polishing strategies commonly include ion-exchange column chromatography, hydrophobic-interaction column chromatography and size-exclusion column chromatography [14,13]. Each column procedure is constantly monitored by appropriate analytical methods for assessment of the quality and quantity of the recombinant protein being produced.

41.3.5 Analytical Assessment

In order for a development project to be successful there must be qualified analytical methods. These methods are required to determine the concentration of the expressed product throughout the entire development project and provide information on the integrity, purity and stability of the recombinant protein [12]. It is important to investigate the biophysical nature of the recombinant protein while in solution; understanding this condition is critical for development [13].

In summary, as indicated in Table 41.1, following a quality-by-design approach provides the opportunity to develop robust, scalable and stage-appropriate processes for the successful technology transfer and pilo-scale production of recombinant malarial proteins.

41.4 Development of Immunological Assays to Evaluate Vaccine Efficacy and Understand Protective Correlates

41.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Although it was shown that transfusion of immunoglobulin G (IgG) from malaria-immune individuals reduces parasitemia in malaria patients [17], there is no widely accepted *in vitro* assay relevant to protection for blood-stage malaria. There is a consensus that measuring antibody titer to a target antigen by ELISA has importance for vaccine development; therefore, we have developed and standardized the procedure of ELISA [18]. We have utilized the standard ELISA for all of our pre-clinical and clinical trials. In addition, to perform ELISA with larger numbers of samples (e.g., for phase 2 trials), MVDB has investigated high-throughput ELISA using automated liquid handling equipment and has successfully utilized the high-throughput ELISA in our laboratory.

41.4.2 Growth Inhibition Assay (GIA)

For blood-stage malaria vaccines, MVDB has developed and standardized a Growth Inhibition Assay (GIA) to evaluate vaccine efficacy in pre-clinical and clinical trials. In collaboration with Dr. Carole Long's laboratory at Laboratory of Malaria and Vector Research /NIAID, we have demonstrated that (i) the biological activity of an antibody is a function of antibody titer in animal models and in Phase 1 trials in malaria-naïve populations (Fig. 41.2) [19,20]; (ii) antibody titer and biological activity correlates with protection in

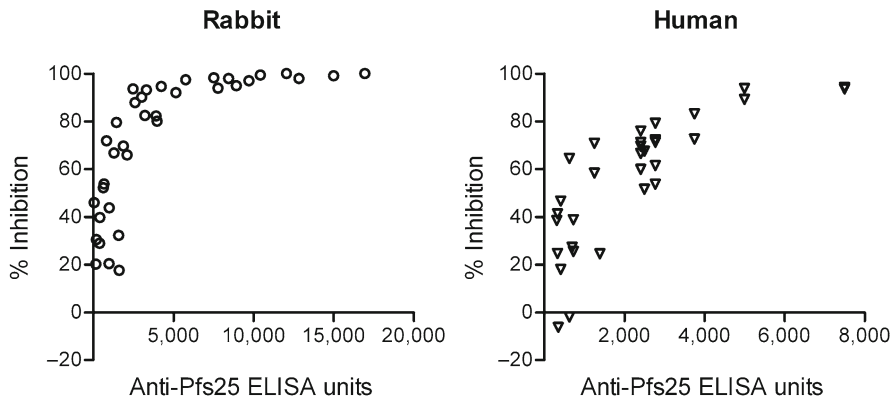

Fig. 41.3 Biological activity of rabbit anti-Pfs25 antibodies (left) and humany anti-Pfs25 antibodies (right), judged by membrane feeding assay. The anti-Pfs25 ELISA units (x-axis) are plotted against % inhibition of oocysts (y-axis)

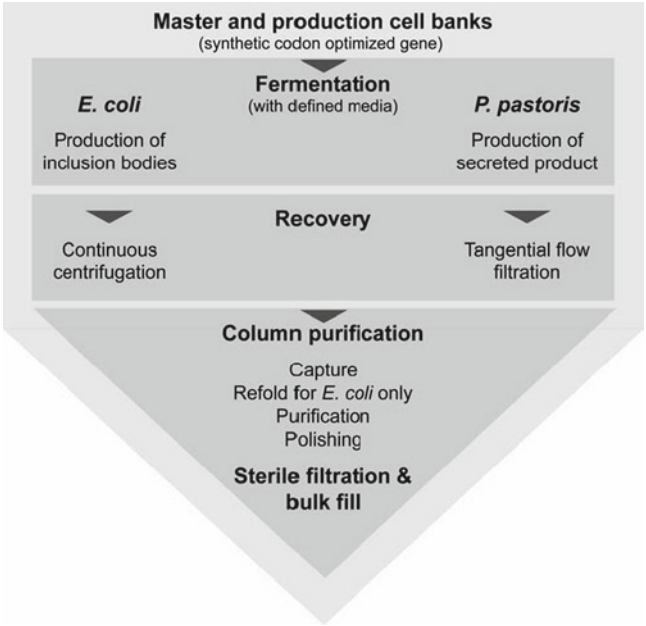

Fig. 41.4 General scheme for antigen production processes

Aotus-monkey challenge models with AMA1 and/or MSP1 [21,22]; (iii) both AMA1-specific antibodies induced by vaccination (in U.S. adults) and by natural infection (in Malian children) have comparable biological activity in GIA when the ELISA titer is normalized, and (iv) malaria- specific IgGs in the Mali IgGs reduced the biological activity of the AMA1 antibodies [23].

41.4.3 Membrane Feeding Assays (MFA)

For transmission blocking vaccines, an *ex vivo* mosquito Membrane Feeding Assay (MFA) is thought to predict efficacy of a TBV in the field. MVDB is one of a few laboratories that perform MFA in a standardized way. By using the assay, we have shown that the biological activity of antibodies against Pfs25 and Pvs25 is a function of antibody titer, both in pre-clinical and clinical trials (Fig. 41.3) [24,25].

41.4.4 Sporozoite-Challenge Assays (SCA)

The sporozoite-challenge model has been used for evaluating protective efficacies of liver-stage vaccines in Phase 2a clinical trials. For preclinical evaluation, animal-challenge models are not available, due to the host specificity of *Plasmodium* species. We are employing, as well as developing, animal-challenge models, using transgenic parasites that express *P. falciparum* CSP on their sporozoite surface. A *P. berghei* parasite (rodent malaria) expressing a human PfCSP transgene may be used to evaluate the *P. falciparum* CSP-based vaccines in mice [26]. We are using this model to evaluate P. *falciparum* CSP vaccines to investigate the correlation between protection and humoral and cellular immune responses. We are also developing a *P. knowlesi* parasite (monkey malaria) expressing a PfCSP transgene, which may be used to evaluate *P. falciparum* CSP-based vaccines in the rhesus model. A construct for the gene replacement will be used to generate a transgenic *P. knowlesi* parasite expressing PfCSP on its sporozoite surface. The transgenic parasites may be used to challenge monkeys immunized with test vaccines and protective efficacy can be assessed by measuring the prepatent duration or parasitemia in the animals.

41.5 Formulation: Strategy to Increase Vaccine Immunogenicity and to Ensure Vaccine Safety

Many potentially protective malaria antigens are weak immunogens. Protein antigens injected in saline typically produced weak and transitory antibody responses, while those injected in effective adjuvants produced strong and sustained responses. Various novel adjuvants have been tested to optimize the formulation for increased immunogenicity of malaria antigens. To date, these adjuvants have not shown significant enhancement of immunogenicity of the antigens under our development. Aluminum adjuvants are the only ones currently approved for human use in the United States. The aluminum hydroxide gel, Alhydrogel (Brenntag, Denmark), is our primary vaccine platform due to its extensive safety record when used with other recombinant proteins in all age groups. Montanide ISA 720 and Montanide ISA 51 formulations were also tested by MVDB , but they were eliminated from our clinical program due to reactogenicities. Recently we have demonstrated that conjugating malaria antigens to carrier proteins significantly increased immunogenicity and longevity of antibody responses to the TBV antigen Pfs25 [27,29]. Limited enhancement of antibody responses has also been observed against AMA1 conjugates [30]. Optimizing these conjugated formulations will require analyzing the conjugates and the binding behavior and stability of antigen post-formulation.

41.5.1 Alhydrogel+CPG

Despite substantial immune responses induced by Alhydrogel formulations in animals, Alhydrogel formulations induced minimal responses in humans with transmission-blocking

antigens, and only moderate responses with blood-stage antigens. We have partnered with Coley Pharmaceutical Group, a Pfizer Company (Ottawa, Ontario, Canada) to test CPG 7909, a synthetic oligodeoxynucleotides known specifically to stimulate the Toll-Like Receptor 9 (TLR9), with malarial antigens on Alhydrogel. The combination leads to a Th1-type response in humans and may facilitate T-cell–dependent killing of malaria-infected red blood cells. Alhydrogel+CPG 7909 as a combined adjuvant platform has provided some of the highest antibody levels to our malarial antigens, observed in animal models and in humans, in both US and Malian adults [31]. Recently, MVDB has started testing CPG 10104, another synthetic oligodeoxynucleotide that differs from CPG 7909 by only one nucleotide. Comparative studies demonstrated similar immune responses in animals induced by CPG 7909 and CPG 10104. Antibodies induced in rabbits by AMA1-C1/Alhydrogel+CPG 10104 formulation are also capable of inhibiting *in vitro* growth of malaria parasites in a manner comparable to the antibodies induced by AMA1-C1/Alhydrogel +CPG 7909. These data paved a way to switch the use from CPG 7909 to CPG 10104 in the Alhydrogel+CPG platform. If the formulation proves to be safe in toxicology studies, we will proceed to a Phase 1 human trial to test AMA1- and MSP1-based vaccines formulated with Alhydrogel+CPG 10104. This will be the first use in humans of CPG 10104, a product which Coley/Pfizer has reserved for use as an adjuvant in infectious-disease vaccines.

41.5.2 Water-in-Oil Emulsions

Montanide® ISA 720 (ISA720) and Montanide® ISA 51 (ISA51), manufactured by SEPPIC Inc., are two experimental adjuvants, formulated as water-in-oil emulsions. ISA51 is a mix of mineral oil and a surfactant from the mannide monnooleate family, whereas ISA720 is a mix of non-mineral oil of vegetable origin with the same mannide monnooleate surfactant. Both formulations have been reported to induce strong cellular as well as humoral responses, and have been tested extensively in clinical trials. The formulations are generally well-tolerated. The most common adverse reactions include local pain or discomfort, tenderness, and swelling. Induration and sterile abscess at the injection sites have also been reported, though at a lower rate. Based on strong evidence in animal studies that ISA 51 and ISA 720 can significantly enhance immunogenicity of malaria antigens, the adjuvants have also been used as a platform formulation in malaria-vaccine development. Extensive efforts were invested to develop formulation processes and to test the formulation stability. We found all malaria antigens including Pfs25, Pvs25, AMA1, and MSP1 were subject to proteolytic degradation after formulated with ISA720 and ISA51 and stored over time. We also found that including glycine in the formulation can significantly improve the stability of the antigen [32]. Two human trials were conducted to test Pfs25/ISA51, Pvs25/ISA51, and AMA1-C1/ISA720, as described in Section VI, Clinical Development and Evalution.

41.5.2.1 Conjugation as a Platform Technology for Increase Immunogenicity of Malaria Antigens

One of the conventional strategies to improve immunogenicity of a poor immunogen is by conjugating the immunogen with carrier proteins. The approach has led to the licensure of several polysaccharide-conjugate vaccines including Prevnar; Meningitec; HibTITER (Wyeth); Meninjugate

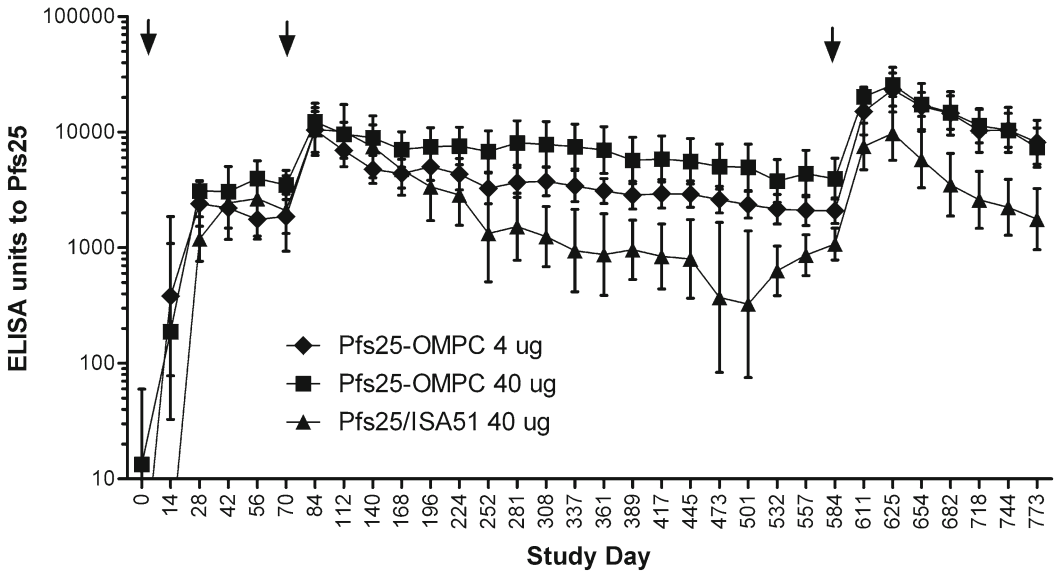

Fig. 41.5 Pfs25H-OMPC induced sustained antibody responses in rhesus monkeys

(Chiron); ActHIB (Aventis); Hiberix (GSK), and PedvaxHIB (Merck). Our first direct evidence that conjugation may help to increase immunogenicity of the malaria antigens came from studies with Pfs25, a transmission-blocking vaccine candidate. In collaboration with Merck Research Laboratories, Pfs25 was conjugated to the Outer-Membrane Protein Complex (OMPC) from *Neisseria meningitides* serogroup B, to recombinant, non-toxic exoprotein A (rEPA), or to itself. All three conjugation products were shown to be significantly more immunogenic than the unconjugated Pfs25 (33,34]. The most remarkable finding was in rhesus monkeys, where the Pfs25-OMPC conjugate induced high antibody levels that were sustained over 16 months—longer than the group given the Pfs25/ISA 51 formulation, the formulation with known "depot" effect. Moreover, the antibody responses were further boosted by unconjugated Pfs25 (Fig. 41.5).

Two groups of monkeys were given two immunizations of four or 40 μg of Pfs25H conjugated to OMPC (diamond or solid square, respectively) on Days zero and 70. A third group of monkeys as given 40 μg Pfs25H/ISA 51 (solid triangles) on Days zero and 70. On Day 596, a dose of 40 μg nonconjugated Pfs25H adsorbed on Merck Aluminun Adjuvant was given to all three groups of monkeys. The antibody levels were assayed by ELISA, and the data are expressed as the geometric mean */÷ geometric standard error. In order to carry on conjugation studies independently, we developed a program to use rEPA, recombinant ExoProtein A from *Pseudomonas aeruginosa* as a carrier. rEPA is a modified form of *P. aeruginosa* exotoxin A, which is non-toxic due to deletion of a critical amino acid for enzymatic activities. rEPA has been used in conjugating Salmonella Typhi Vi polysaccharide vaccine that was shown to be safe in thousands of infants in Vietnam. We produced sufficient quantities of research-grade rEPA and cGMP bulk rEPA substance to develop and produce a cGMP-conjugate vaccine. Pfs25; Pvs25; Pfs28; Pvs28; AMA1-FVO, and MSP1 were conjugated to the rEPA carrier. Conjugation increased, in various degrees, the immunogenicity of these antigens. Conjugation of Pfs25 to itself also induced significantly increased antibody responses. The conjugation process did not alter the functional epitopes of these antigens, as confirmed by *in vitro* growth-inhibition assays and *ex vivo* membrane-feeding assays.

41.6 Clinical Development and Evaluation

41.6.1 Blood-Stage Vaccine Development

The goal for a blood-stage vaccine is to prevent severe disease in young children and infants. The clinical-development program for blood-stage vaccines can be broken down into three components: (i) Phase 1 trials in healthy, malaria-naïve adults to demonstrate safety and immunogenicity; (ii) Phase 1 trials in semi-immune adults in a malaria-endemic area to show safety, followed by age de-escalation into young children in a malaria-endemic area to show safety and immunogenicity, and (iii) Phase 2b proof-of-concept trials in young children and infants in malaria-endemic areas to demonstrate impact on surrogate endpoints for severe disease. Due to lack of definitive protective correlates, the go/no go criteria for blood-stage vaccines are based on the demonstration of safety and potential efficacy, the surrogate of which is immunogenicity as measured by antibody titers. Growth Inhibition Activity (GIA) is a functional measure for biological activity of the antibodies. However, it is not on the go/no go decision path because its relationship to protection is not known in humans. In Phase 1 trials of adults from endemic areas, safety/reactogenicity is the main criterion for going forward, since antibody responses in these malaria-experienced individuals are not likely to be predictive of responses in young children or infants. Vaccine candidates that are safe and immunogenic based on these criteria will be taken to Phase 2b proof-of-concept trials. MVDB has conducted clinical trials using a total of seven different blood-stage vaccine formulations. AMA1-Combination 1 (AMA1-C1), containing allelic proteins from the FVO and 3D7 strains of *P. falciparum*, and formulated on Alhydrogel, has been tested in Phase 1 studies in malaria-naïve adults (Johns Hopkins University Center for Immunization Research (JHU/CIR)) and semi-immune adults and children (Malaria Research and Training Center, Mali), and was evaluated in a Phase 2b study in children in Mali during 2006–2007. The vaccine was moderately immunogenic, with the highest antibody responses seen in Malian adults, who are primed by extensive prior exposure to malaria infection. No impact on parasite density or clinical malaria was seen in the Phase 2 study in Malian children of two-three years. A Phase 1 study in malaria-naïve adults (University of Rochester) showed that antibody responses were enhanced 10-14-fold when the novel adjuvant CPG 7909 was added to AMA1-C1/Alhydrogel (Fig. 41.6). A slightly modified formulation has also been evaluated in US adults at JHU/CIR, and has been taken forward to a Phase 1 trial in Malian adults. The vaccine administered with CPG 7909 induced *in vitro* growth-inhibition levels in homologous parasites as high as 96%. No significant safety issues have been identified in any of these trials, although concerns about autoimmunity with the CpG ODN class of adjuvants means that clinical development must proceed with caution, particularly as trials move forward to children.

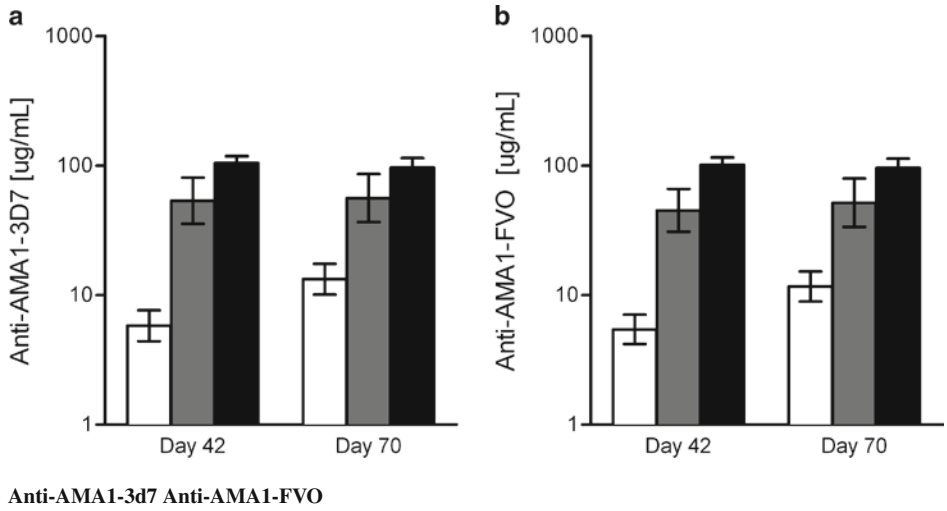

Fig. 41.6 Immunological Responses to AMA1-3D7 and -FVO in US Recipients of the AMA1-C1/Alhydrogel® + CPG 7909 Vaccine. Anti-AMA1 antibody responses: Day 42 and 70 AMA1 antibody responses in recipients of the 20 or 80 μg AMA1-C1/Alhydrogel ± CPG 7909 vaccines. Antibody responses against AMA1-3D7 (**a**) and AMA1-FVO (**b**) measured by ELISA in sera collected on Day 42 (14 days postvaccination 2) and Day 70 (14 days postvaccination 3). ELISA results are shown as the geometric mean of AMA1-specific antibody (μg/mL) with standard error. The bars represents 80 μg AMA1 (white bar, $n = 25$), 20 μg AMA1 + CPG (grey bar, $n = 12$), and 80 μg AMA1 + CPG (black bar, $n = 29$)

41.7 Anti-AMA1-3D7 Anti-AMA1-FVO

AMA1-C1, formulated in the water-in-oil emulsion ISA720, has also been tested in malaria-naïve adults (Queensland Institute for Medical Research, Brisbane, Australia). The vaccine was found to be immunogenic and was generally well tolerated, although some severe local reactions occurred. In addition, while the vaccine was stable and potent through all vaccinations, longer term stability was poor. Further development has been stopped due to the loss of stability and the significant local reactogenicity. FVO and 3D7 allelic forms of $MSP1_{42}$, separately formulated on Alhydrogel, were tested in a Phase 1 trial in malaria-naïve adults (Quintiles, Lenexa, Kansas), and were found to be poorly immunogenic [35]. $MSP1_{42}$-C1, containing a combination of these proteins, was evaluated in malaria-naïve adults at JHU/CIR, with and without CPG 7909. As with AMA1, the addition of CPG 7909 markedly enhanced antibody responses (Fig. 41.7). Some *in vitro* growth inhibition was detected, although levels were not as high as with AMA1.

Even with the addition of CPG 7909, there is a high degree of variability in antibody responses in humans to AMA1 and $MSP1_{42}$. It is also thought to be likely that an effective immune response will require antibody to more than one antigen. For these reasons, MVDB is planning to test combination vaccines such as BSAM-2, consisting of AMA1-C1 and $MSP1_{42}$-C1 in a 1:1 ratio. The polymorphism of AMA1 and $MSP1_{42}$ may be overcome by inclusion of

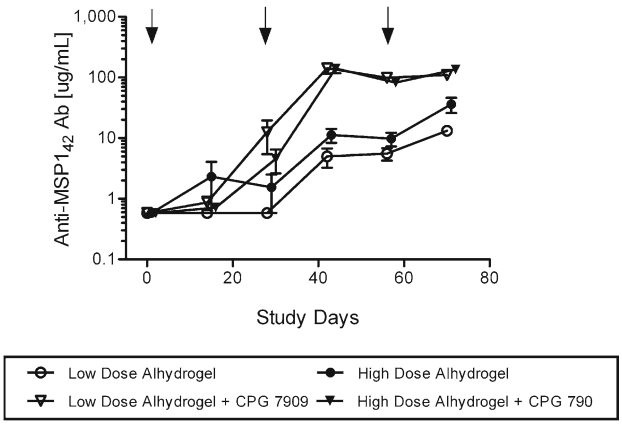

Fig. 41.7 Anti-$MSP1_{42}$ antibody responses following vaccination with $MSP1_{42}$ C1/Alhydrogel® with and without CPG 7909. Volunteers were vaccinated on Study Days 0, 28, and 56 (as indicated with arrows) with 40 μg or 160 μg dose of the $MSP1_{42}$-C1/Alhydrogel® with or without the addition of 500 μg CPG 7909. The points represent the group arithmetic means and standard error. ELISA units to $MSP1_{42}$-FVO are expressed relative to a $MSP1_{42}$ standard plasma pool. ELISA units to $MSP1_{42}$-3D7 (not shown) follow the same pattern as the anti$MSP1_{42}$-FVO. Arrows indicate the days of immunization

multiple alleles of population types. Thus, AMA1-C2, consisting of 3D7, FVO, and L32 alleles of AMA1 in 1:1:1 ratio ,was tested in animals. A preclinical toxicology study is underway testing the two combination vaccines formulated onto Alhydrogel with CPG 10104, a human TLR9 stimulator similar to CPG 7909. Phase 1 human trials are planned if the vaccines are demonstrated to be safe in the toxicology study.

41.7.1 Transmission-Blocking Vaccine Development

Transmission-blocking vaccines (TBVs) are conceptually quite different from blood-stage or pre-erythrocytic vaccines in that they would offer no direct benefit to the individual vaccinated; rather, they would reduce morbidity and mortality by reducing transmission within a population, hence reducing disease in that population. A TBV would act by inducing antibodies that block parasite development in the mosquito midgut. This would interrupt the transmission of malaria to susceptible individuals in the population. The clinical-development program for TBVs is different in that efficacy for this type of vaccine is not determined in an individual but in a population. Also, an *ex vivo* membrane-feeding assay (MFA), which can predict efficacy of a TBV in the field, is available. Initial Phase 1 trials in healthy, malaria-naïve adults to demonstrate safety and immunogenicity, which leads to activity in the MFA, will be performed. Successively larger Phase 1 trials for safety in the target populations in an endemic area will then be required for scale-up prior to large proof-of-concept trials in the field. These proof-ofconcept trials to show population efficacy (decreased transmission) will require larger numbers of individuals randomized in large groups that are separated geographically (e.g., villages). A TBV will depend on herd immunity. Studies have suggested that in order actually to decrease disease, the entomologic inoculation rate (EIR) would need to be lowered to less than 10 infective mosquito bites per year. This is not likely to be achievable in areas of high transmission. In areas of moderate to low transmission (EIR<20), a vaccine that decreased transmission in a community by threefold when 80% of the community is vaccinated would be potentially beneficial. Modeling of immune responses in several malaria-vaccine trials suggests that a single component vaccine would need to induce a group mean antibody level >50 times the IC50 (the amount of antibody which gives 50% reduction of oocysts number per mosquito) of that component in the membrane-feeding assay to achieve this goal. The go/no go criteria for advancing the two-component vaccine to the Phase 2b trials is as follows:

- Vaccine should be well tolerated in the Phase 1 study in U.S. naïve volunteers
- The group mean antibody level in the malaria-endemic area induced by each of the two components reaches >8 times the IC50 in the MFA
- The immune responses to the two components are independent, i.e., there are very few poor responders to both components. MVDB has conducted two Phase 1 trials testing Pfs25 and Pvs25 TBVs. The first human trial tested Pvs25 formulated with Alhydrogel. The trial enrolled three cohorts, 10 volunteers in each, receiving three doses of 5 μg, 20 μg, or 80 μg Pvs25/Alhydrogel [24]. The vaccine was well tolerated and there were no safety concerns identified. Transmission-blocking antibodies were induced among the vaccinees, though the activities were insufficient to be an effective TBV. The second trial tested a more immunogenic formulation, Pvs25 formulated with Montanide® ISA 51, and Pfs25 formulated with Montanide® ISA 51 [15]. The trial was terminated before completion due to unexpected reactogenicity, including two cases of erythema nodosum, which had not previously been reported in association with these antigens or adjuvant. However, all five volunteers who received two vaccinations of 5 μg Pfs25, the lowest dose group, developed detectable Pfs25-specific antibodies, which blocked parasite growth in mosquitoes in a dose-dependent manner in MFA (Figure 41.3 in Section III). With the goal of developing a potent and safe formulation for TBVs, we demonstrated that conjugating Pfs25 to OMPC or rEPA or to Pfs25 (self conjugation) significantly increased the immunogenicity of Pfs25. In collaboration with Drs. John Robbins and Rachel Schneerson of National Institute of Child Health and Human Development (NICHD), we are planning a Phase 1 trial to test Pfs25-Pfs25 conjugates formulated onto Alhydrogel.

41.7.2 Pre-Erythrocytic Vaccine

The pre-erythrocytic or liver-stage vaccine would block sporozoite invasion of liver cells or eliminate sporozoite-infected liver cells, thus preventing or reducing parasitemia. The clinical development program for pre-erythrocytic vaccines is similar to that of blood-stage vaccines, except that there is an established parasite-challenge model to measure protective efficacy for pre-erythrocytic vaccines in malaria-unexposed adults. The clinical development program for the vaccine is broken down to (i) Phase 1 trials in healthy, malaria-naïve adults to demonstrate safety and immunogenicity; (ii) Phase 2a challenge trial in healthy, malaria-naïve adults. If this trial confirms that there is an acceptable level

of protection (>30%) from challenge, then (iii) Phase 1 age de-escalation trials in an endemic area, followed by Phase 2b proof-of-concept trials in the target population. Only one malaria vaccine has shown consistent, if partial, protection in field trials. This vaccine is RTS,S, a recombinant CSP (corresponding to aa 207–395 of the CSP from *P. falciparum* strain 3D7) fused to hepatitis-B surface antigen (HBsAg). MVDB added CSP to our product-development plan in October 2006. Our approach is to produce a full-length recombinant protein, excluding only the *N*-terminal signal and *C*-terminal GPI anchor-signal sequences. The rationale for this approach is that the *N*-terminal segment of the CSP, including the conserved Region I, may also induce antibody-dependent protective immunity. RTS,S contains half of the natural CSP repeats without NVDP sequences, whereas our rCSP will include a complete repeat region. Moreover, the recombinant CSP will be chemically conjugated to a recombinant *P. aeruginosa* rEPA to enhance the immunogenicity of the CSP. Recombinant CSP may also be conjugated to Pfs25. This approach may enhance immunogenicity and longevity of responses, and produce a vaccine that both protects against infection and disease, and prevents transmission. Such a vaccine would be an invaluable tool for malaria eradication. As described in Section IV, animal models are currently being developed to guide selection of formulations to be taken forward to clinical trials.

41.7.3 Development of Clinical Trial Capacity in Endemic Regions

Conducting Phase 1 and 2 field trials requires an enormous amount of preparation, particularly when they are to be performed in resource-limited settings such as those where malaria is endemic. Preparations for these trials have been ongoing for the past few years. The primary vaccine trial sites were developed in collaboration with the Malaria Research and Training Center (MRTC) at the University of Bamako in Mali, resulting from the MVDB's long-standing partnership with researchers at this institution. Preparation for large-scale Phase 2b field trials in Mali comprising 300–400 children per trial has involved several components: (i) assembling a highly trained and qualified team of Malian investigators and support staff; (ii) investing in physical infrastructure; (iii) upgrading of local Malian laboratory facilities, both research and clinical, and (iv) conducting preparatory studies to establish the baseline epidemiology of malaria in the area and population to be studied. These preparations have been divided into two phases for both logistical and budgetary reasons: (i) the establishment of the capacity to perform Phase 1 studies, and (ii) the capability to perform larger Phase 2 studies. A vaccine testing center in the Malian village of Donéguébougou has been developed, is fully operational, and has successfully completed two Phase 1 trials. A second, larger vaccine testing site in Bancoumana, Mali is also fully developed and was recently the site of the first Phase 1/2 blood-stage vaccine trial in Malian children.

41.8 Future Directions

Search for Novel Malaria-Vaccine Candidate Antigens. MVDB is investigating the potential of recently identified malaria proteins shown to be either involved in merozoite invasion or, based on cellular location, thought to be involved. Good quality and characterized recombinat proteins are produced and tested for their capacity to induce growth-inhibitory antibodies with similar levels of activity as AMA1-specific antibodies. In addition, immunological investigations are planned using invasive merozoites. Possible candidates for TBV are also being studied including HAP2 (16].

Search for Safe and Potency Adjuvants Suitable for Malaria Vaccines. Siginificant progress has been made in selecting, producing, and testing recombinant malaria antigens and their formulations for development of malaria vaccines. We still havea long way to go before a safe and highly effective vaccine will be ready for licensure. Because malaria antigens are in general poorly immunogenic, one of the strategies to increase immunogenicity of the vaccines is through formulation with potent and safe adjuvants. Only a limited number of such adjuvants is available, such as GLA, a monophosphate lipid (MPL)-like adjuvant, developed by the Infectious Disease Research Institute, USA. Preclinical studies are underway to test these adjuvants. Conjugation of antigens to carriers or to themselves to form multimeric molecules may also be an effective strategy to improve immunogenicity. A prime/boost strategy, where vaccinees are primed with a viral-vector vaccine followed by a booster dose of recombinant protein vaccine, has been shown to enhance cellular responses. This might be critical for CSP-based vaccines. MVDB is collaborating with the US Naval Medical Research Center, Crucell, and Division of Microbiology and Infectious Disesases, NIAID, to test prime/boost strategy of blood-stage vaccines and the CSP-based vaccines.

Search for Protective Correlates for Malaria Vaccines. While there is a sporozoite-challenge model for liver-stage vaccines and a membrane-feeding assay for transmission-blocking vaccines, the lack of protective correlates is an obstacle to the development of blood-stage malaria vaccines. Development of a blood-stage challenge model in monkeys or humans might lead to identification of

protective correlates. With partial funding from Malaria Vaccine Initiative, MVDB has planned a study in *Aotus* monkeys where animals will be immunized with a combination of six blood-stage vaccine candidate antigens, including AMA1; $MSP1_{42}$; MSP2; MSP3;, MSP4, and the Region II of the EBA175, in collaboration with La Trobe University, Australia; Monach University, Australia; Pasteur Institute, France, and Division of Microbiology and Infectious Diseases, NIAID, USA. The monkeys will then be challenged with a blood-stage parasite, and the protection against parasitemia and anemia will be evaluated in conjunction with humoral and cellular-immune responses. MVDB is also collaborating with Dr. Adrian Hill of Oxford University to conduct a blood-stage challenge study in human volunteers. In this planned study, the volunteers will be immunized with AMA1C1/Alhydrogel+CPG 7909, followed by a challenge with a well characterized blood-stage parasite stock. The main goal of this study is to evaluate *in vitro* humoral and cellular responses in correlation with *in vivo* reduction in parasite-multiplication rates.

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Chapter 42
The Development of Live-Attenuated Vaccines for Pandemic Influenza

Catherine J. Luke and Kanta Subbarao

42.1 Introduction

The emergence of highly pathogenic H5N1 avian influenza A viruses in humans since 1997 has raised concerns that we may be facing the next influenza pandemic. Since 2003, there have been over 400 confirmed cases of human infection with highly pathogenic H5N1 avian influenza in 15 countries worldwide, with a case fatality rate of 63% (http://www.who.int.csr/disease/avian_influenza/country/cases_table_2008_12_09/en/index.html, accessed March 6 2009). In addition, since 1999, sporadic human infections with avian influenza viruses of the H7, H9 and H10 subtypes have occurred, resulting in disease of varying severity, from mild upper-respiratory infection to severe pneumonia and death [1–8]. Although the majority of avian influenza (AI) infections in humans have been caused by H5N1 AI viruses, the next pandemic could be caused by an influenza A virus of any of the 16 hemagglutinin (HA) subtypes known to infect birds. Influenza A viruses also infect a wide variety of mammalian species in nature, presenting another source from which humans could acquire infection.

Vaccines represent a critical strategy for the control of seasonal influenza in humans, and are a significant part of pandemic-preparedness efforts. It is imperative that both existing and novel approaches be explored for the development of vaccines for pandemic influenza. The currently licensed vaccines for seasonal influenza in the United States are the trivalent inactivated vaccine (TIV) and a live-attenuated influenza vaccine (LAIV), FluMist®, licensed in 2003. The technology used for TIV has been largely successful for the control of seasonal influenza epidemics, and manufacturing infrastructure and regulatory procedures for licensure of these vaccines are well established. It has been demonstrated that inactivated H5N1 influenza vaccines are suboptimally immunogenic, compared to those for human influenza viruses, requiring larger amounts of HA antigen than in the seasonal influenza vaccines, and requiring two doses of vaccine to achieve levels of antibody that are predicted to be protective [9,10]. Immunogenicity of inactivated H5N1 influenza vaccines has been improved by the use of adjuvants [9,11–13], or by the use of whole virion vaccines, rather than split-virion vaccines [14], although there is concern that the former may be more reactogenic. Experience with seasonal influenza suggests that the LAIV approach may offer advantages over inactivated vaccines: such vaccines may require fewer doses, and may induce more rapid and broader immune responses than inactivated vaccines.

In response to the need to prepare for an influenza pandemic, the Laboratory of Infectious Diseases (LID) of the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), under a Cooperative Research and Development Agreement (CRADA) with MedImmune, is pursuing a program to generate and evaluate live-attenuated vaccines against influenza viruses with pandemic potential. LID has several decades of experience in pre-clinical and clinical testing of LAIV, and MedImmune has the manufacturing and regulatory infrastructure for their development. This article describes the cold-adapted LAIV platform on which this program is based, and summarizes the pre-clinical characterization of the candidate vaccine viruses that have been developed to date for protection against infection with influenza viruses with pandemic potential.

42.2 Live-Attenuated Influenza Vaccines

The HA and neuraminidase (NA) proteins of influenza viruses are the targets of the protective immune response. Antibody directed against HA can prevent infection, therefore the principle of currently licensed vaccines is to induce antibody to this surface glycoprotein. FluMist®, the first LAIV to be licensed in the United States, is a trivalent vaccine, containing attenuated influenza A H1N1 and H3N2 and influenza B viruses that bear the HA and neuraminidase (NA) genes of the circulating influenza viruses for a particular season, and the internal protein genes of a cold-adapted (*ca*) vaccine donor strain – A/Ann Arbor/6/60 *ca* (H2N2) for the influenza A components, and B/Ann Arbor/1/66 *ca* for the influenza B

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component. Although FluMist® was licensed in 2003, the research that resulted in the development of this product began in the 1960s. Dr. Hunein Maassab, at the University of Michigan, developed the A/Ann Arbor/6/60 *ca* virus by passaging the A/Ann Arbor/6/60 (H2N2) influenza virus in primary chicken-kidney cells at sequentially lower temperatures [15–19]. This process is summarized in Fig. 42.1. The resulting A/AA/6/60 *ca* (AA *ca*) virus was able to replicate efficiently at 25 °C, but was restricted in replication at 39 °C. In animal models such as ferrets, this temperature-sensitive (*ts*) phenotype is characterized by efficient replication of the virus in the upper-respiratory tract of animals, but undetectable or highly restricted replication in the lower-respiratory tract. It was subsequently demonstrated that the cold-adaptation process had resulted in the introduction of mutations in several internal-protein gene segments of the virus, conferring the *ts* and attenuation (*att*) phenotypes [20,21].

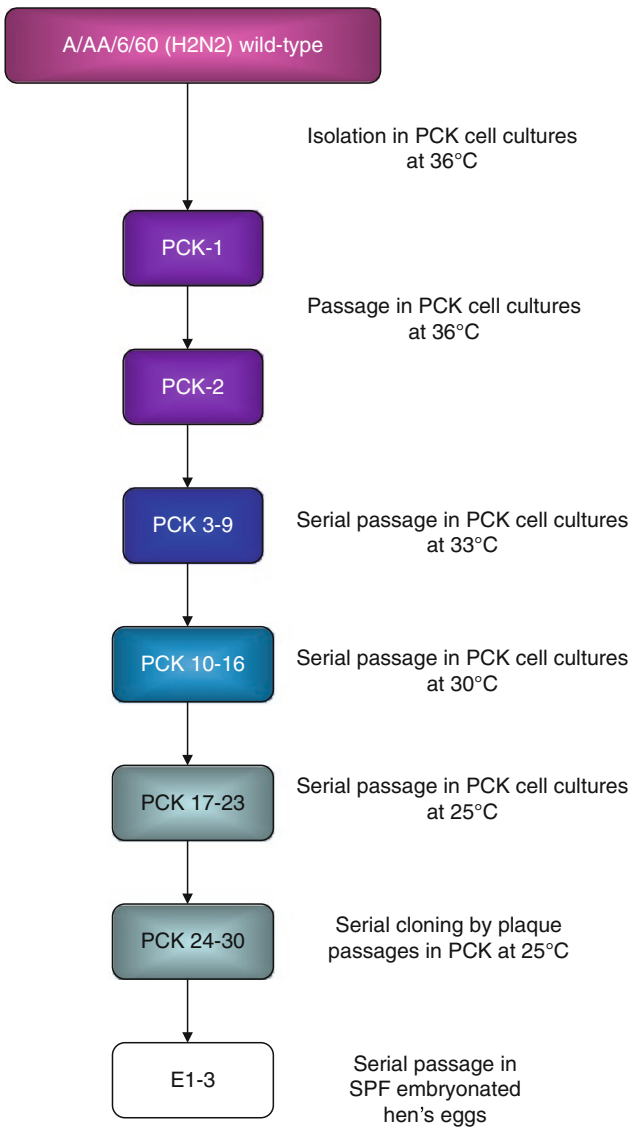

Fig. 42.1 Flow chart to show the steps in the derivation of the A/Ann Arbor/6/60 (H2N2) cold-adapted virus that is the donor of the internal protein genes for cold-adapted live attenuated influenza A vaccines developed at LID. The A/Ann Arbor/6/60 virus was sequentially passaged at lower temperatures in primary chick kidney (PCK) cells, resulting in a virus that could replicate efficiently at 25ºC, was temperature sensitive, and attenuated [18]

In the following two decades, extensive studies were conducted by NIAID alone and, later, in collaboration with Wyeth Vaccines and MedImmune, on the development of *ca* LAIV based on the AA *ca* virus. Vaccine viruses were generated that bore the HA and NA proteins of human H1N1 or H3N2 influenza viruses, and the six internal protein gene segments of the AA *ca* virus. Monovalent and bivalent *ca* LAIV generated thusly were tested extensively in humans for safety and efficacy [reviewed in [22]]. Trivalent *ca* LAIV have been evaluated in over 40,000 individuals, including 18,000 children under the age of five years [23]. In all cases, these vaccines have been shown to be generally well tolerated, non-transmissible and phenotypically and genetically stable. In clinical trials the *ca* vaccine viruses were significantly restricted in replication compared to the wild-type (*wt*) H1N1 and H3N2 parent viruses, achieving a mean peak titer of $10^{0.7}$ to $10^{2.6}$ $TCID_{50}$/mL, compared to 10^{4} to 10^{6} $TCID_{50}$/mL for the *wt* parent virus. This resulted in less common and less severe febrile or upper-respiratory tract illness than was seen with *wt* virus infection [24–30]. The vaccine viruses retained a low level of reactogenicity in infants and young children, but this was limited to the upper-respiratory tract [25,31–33], and the *ca* LAIV were found to be safe in high-risk subjects, including HIV-infected individuals, the elderly with underlying cardiopulmonary disease and cystic fibrosis patients [34–37]. It was also found that a large number of the *ca* H1N1 and H3N2 reassortant vaccine viruses had a similar infectivity for humans, that was lower than that of the *wt* viruses [38]. The LAIV were phenotypically stable. The observed phenotypic stability of the *ca* influenza viruses that have been studied in humans likely reflects the fact that several mutations on multiple gene segments are responsible for the attenuation of the AA *ca* virus. It has also been demonstrated that the *ca* vaccine viruses are genetically stable, both throughout the manufacturing process and in vaccine recipients [39–42]. The *ca* reassortant viruses have been shown repeatedly to be poorly transmissible in adults and in seronegative infants and children [28,30,43–46]. The lack of symptoms associated with infection in both the lower- and upper-respiratory tract of vaccinees, the low level of peak replication, and the lower human-infectious dose of these vaccine viruses contribute to their poor transmissibility.

In 2003, the United States Food and Drug Administration (FDA) approved the licensure of FluMist® for use in healthy individuals aged five to 49 years. By 2007, this was expanded to include children aged between two and five years

(http://www.medimmune.com/products/flumist/index.asp). FluMist® is marketed by MedImmune.

42.3 Live-Attenuated Vaccines for Pandemic Influenza

The goals of the LID pandemic influenza vaccine program are to generate a library of live-attenuated influenza viruses that bear the HA and NA of influenza viruses of all the different subtypes; then, to test these candidate vaccine viruses in pre-clinical studies and in clinical trials in humans to determine the safety, infectivity and immunogenicity of the vaccine viruses. Seed viruses of all known HA subtypes, with the corresponding NA found in nature, will be generated. Priority has been given to those subtypes that have already caused infection in humans, namely H5, H7 and H9. The vaccine viruses are generated by plasmid-based reverse genetics – the general strategy is shown in Fig. 42.2. This process allows the genetic engineering of the HA or NA to remove any virulence motifs that may be present; for example, the multi-basic amino acid cleavage site in the HA of highly pathogenic H5 and H7 influenza viruses that is associated with virulence in birds [47].

Candidate viruses for vaccine development are selected based on several criteria, including the ability to induce a high-titer antibody response that is broadly cross-reactive with other influenza viruses of the same subtype; year of isolation – generally, the better candidates will be those viruses that have circulated most recently in avian or animal species; and the ability to replicate in available animal models, primarily mice and ferrets [48,49]. To date, candidate vaccine viruses of four subtypes have been generated and tested in pre-clinical studies to confirm their attenuation phenotype and to evaluate their immunogenicity and protective efficacy in animal models. The pre-clinical findings are described in the following sections.

42.4 H5N1 Influenza

Human infections with highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype were first reported in 1997 in Hong Kong, when 18 people were infected and six people died [50,51]. Avian species in live-bird markets and farms in Hong Kong infected with similar H5N1 viruses were the source of the human infections. In an effort to end the outbreak, 1.5 million poultry in the Hong Kong Special Administrative Region was slaughtered – an action which many experts believe averted an influenza pandemic. In early 1998, new cleaning and stocking practices were introduced in the Hong Kong live-bird markets and surveillance of birds for influenza viruses in these markets continues.

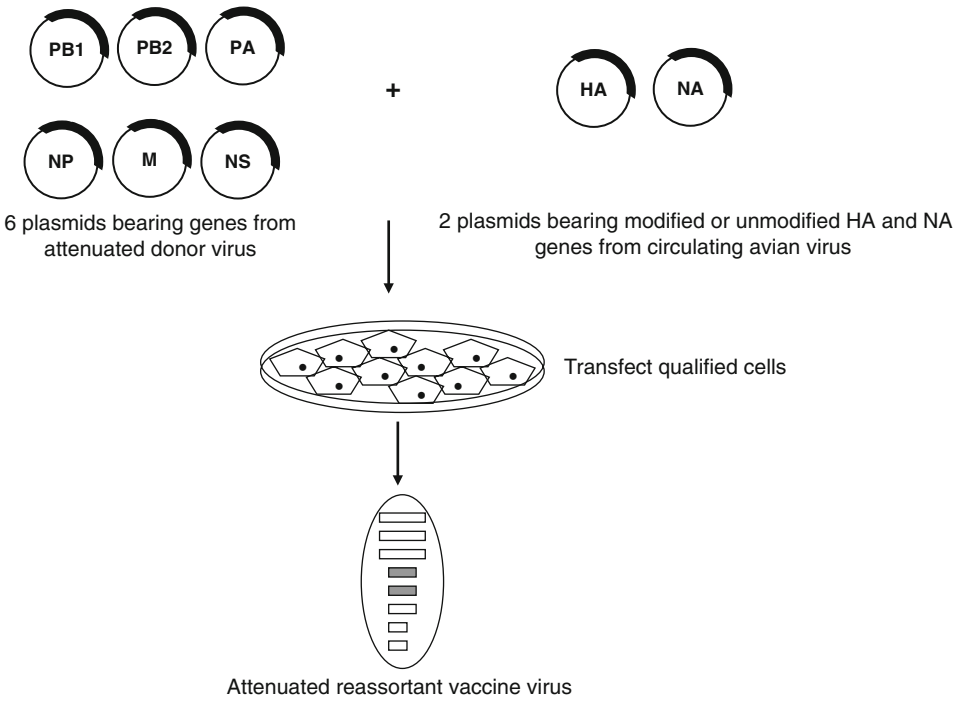

Fig. 42.2 The eight-plasmid reverse genetics system to generate recombinant live attenuated pandemic influenza vaccines. Six plasmids encoding the internal genes of the attenuated donor virus are mixed with two plasmids encoding the circulating avian virus HA and NA genes (which may or may not have been modified to remove virulence motifs). Qualified cells are transfected with the plasmids and the attenuated reassortant virus is isolated. Adapted from [75]

A small cluster of human infections with highly pathogenic H5N1 influenza virus was reported in 2003 in a family that had recently traveled from Hong Kong to southern China. The father and young daughter of the family died, while the young son recovered from the infection [52].

In January of 2004, a cluster of severe respiratory illnesses in people in Hanoi, Viet Nam was reported, and concurrently, large poultry die-offs were reported in the region [53]. These cases marked the beginning of the HPAI H5N1 panzootic in avian species that continues to the present, and the first of several-hundred cases of H5N1 infection in humans, in 15 countries around the world. The emergence of HPAI H5N1 infection in humans prompted unprecedented pandemic-preparedness activities worldwide.

42.5 Live-Attenuated H5N1 Influenza Vaccines

Three candidate vaccine viruses of the H5N1 subtype were generated by LID, in collaboration with MedImmune and two were evaluated in pre-clinical studies [54]. These viruses derive their HA and NA from H5N1 HPAI viruses that were isolated from humans in Hong Kong in 1997, Hong Kong in 2003 and Viet Nam in 2004, and the internal protein genes were derived from the AA *ca* virus. The HA gene was modified to remove a virulence motif. All three of the candidate vaccines were avirulent in chickens, and were restricted in replication in the upper -and lower-respiratory tract of mice. In addition, the H5N1 *ca* viruses did not replicate in the lungs of ferrets [54]. These findings confirmed the *att* phenotype of these candidate vaccine viruses. Repeated intranasal administration of the H5N1 *ca* viruses in ferrets did not result in systemic toxicity [55]. Two doses of the vaccine viruses elicited immune responses in mice that resulted in complete protection against pulmonary replication of homologous and heterologous *wt* H5N1 viruses [54]. The pre-clinical studies supported the evaluation of these vaccines in phase 1 clinical studies in humans. The H5N1 *ca* vaccines based on the A/Viet Nam/1203/2004 and A/Hong Kong/213/2003 viruses were evaluated for safety, infectivity and immunogenicity in healthy adults. Data from these studies are pending.

42.6 H7N3 Influenza

Like H5 influenza viruses, influenza viruses of the H7 subtype can be of either low- or high-pathogenicity phenotypes. Sporadic human infections with both low pathogenicity and highly pathogenic H7 influenza viruses have been reported, and the resulting illnesses have typically been mild, presenting as mild influenza-like illness and/or conjunctivitis [3,5,6,8,56–62]. Most human cases of H7 infection have been associated with outbreaks of H7 influenza in birds and have resulted from direct transmission of the virus from infected poultry to humans. In 2003, during an outbreak of highly pathogenic H7N7 avian influenza in domestic poultry in the Netherlands, 89 human cases of infection were reported, including one fatal case in a veterinarian who had direct contact with infected birds [6]. Since then, there have been several reports of H7 influenza infection in humans, confirmed by either isolation of virus or evidence of seroconversion [5,8,59]; http://www.euro.who.int/flu/situation/20070526_1; http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm).

42.7 Live-Attenuated H7N3 Influenza Vaccine

In collaboration with MedImmune, LID developed an H7N3 *ca* vaccine candidate. This vaccine virus derived its HA and NA from a low pathogenicity H7N3 avian influenza virus, isolated from a chicken, that was the precursor of a highly pathogenic H7N3 virus that caused an outbreak of highly pathogenic avian influenza in chickens in British Columbia in 2004 [5,] in which two human cases occurred in poultry workers [8]. The H7N3 *ca* vaccine virus was restricted in replication in mice and was attenuated in ferrets, replicating only in the upper-respiratory tract. In addition, the vaccine virus did not replicate or cause disease in chickens. Immunization of mice with a single dose of the H7N3 *ca* vaccine virus protected against pulmonary replication of an homologous highly pathogenic H7N3 virus and other H7 influenza viruses of the North American lineage, but two doses of vaccine virus were required to protect mice against pulmonary replication of genetically and antigenically divergent H7 viruses of the Eurasian lineage [63]. In ferrets, although some protection against replication of homologous and heterologous H7 influenza viruses from both the North American and Eurasian lineages was observed following a single dose of the H7N3 *ca* vaccine, two doses of vaccine improved the level and breadth of protection. On the basis of these data, the H7N3 *ca* vaccine was evaluated in a phase 1 clinical trial in healthy adults, during 2007. The data from this study are pending.

42.8 H9N2 Influenza

Influenza viruses of the H9N2 subtype are known to be prevalent in southern China and surveillance studies conducted in Hong Kong in 1997 determined that H9N2 influenza viruses co-circulated with H5N1 influenza viruses in the live bird markets [64], and that the internal protein genes of the H9N2 and H5N1 viruses were closely related [65]. H9N2

influenza viruses have also been isolated from domestic swine [66,67]. Sporadic human cases of H9N2 influenza have been reported, and in all of these cases, illness has been mild. Human infections with H9N2 influenza were reported in five individuals in Guangdong Province, China, in 1998 [4]; two children in Hong Kong in 1999 [7]; a child in Hong Kong in 2003 [1]; a nine-month old child in Hong Kong in 2007 (http://www.cdc.gov/flu/avian/gen-info/avian-flu-humans.htm, accessed January 22nd 2009), and a two-month old child in Hong Kong in December 2008 (http://www.cidrap.umn.edu/cidrap/content/influenza/avianflu/news/dec3008influenza.html, accessed January 22nd 2009). Unlike the H5 and H7 subtypes, AI viruses of the H9N2 subtype are not highly pathogenic in birds. Nonetheless, the prevalence of these viruses in birds and their ability to infect humans and cause disease suggests that H9 influenza viruses have pandemic potential.

42.9 Live-Attenuated H9N2 Influenza Vaccine

A candidate live-attenuated H9N2 influenza vaccine was developed in a collaboration between the Influenza Branch, Centers for Disease Control and LID. The vaccine virus bears the HA and NA from the A/chicken/Hong Kong/G9/1997 (H9N2) AI virus and the internal protein genes from the AA *ca* virus. Unlike the other vaccine viruses generated by LID and MedImmune, the H9N2 *ca* vaccine virus was derived by classical reassortment rather than plasmid-based reverse genetics. In this process, embryonated hens' eggs are co-infected with the two parent viruses, and during virus replication, gene segments can reassort. Progeny viruses with the desired genotype are selected using specific antiserum against the AA *ca* virus. The H9N2 *ca* vaccine virus was restricted in replication in mice; was avirulent in chickens, and was immunogenic in mice, and it protected them against subsequent challenge with homologous and heterologous H9N2 influenza viruses [68]. The H9N2 *ca* vaccine virus was also highly restricted in replication in ferrets (unpublished observations). Phase 1 safety, infectivity and immunogenicity studies in humans were conducted and the vaccine virus was safe and well tolerated, and, although highly restricted in replication in healthy adults, two doses were immunogenic in 100% of seronegative subjects, either by HAI or microneutralization assay [69].

42.10 H6N1 Influenza

Avian influenza viruses of the H6 subtype are among the most frequently detected influenza A viruses in surveillance studies in birds [70–73]. Recent studies of AI viruses in migratory birds revealed that H6 viruses have the broadest host range of any AI subtype. Phylogenetic studies suggest that an H6N1 influenza virus may have been the donor of the NA and internal-protein genes of the highly pathogenic H5N1 viruses that infected humans in Hong Kong in 1997 [71]. H6 influenza viruses, along with H5 and H9 viruses, continue to circulate in birds in southern China and have become established in minor poultry, such as quail and pheasant [70]. The prevalence of H6 AI viruses in a wide range of wild and domestic avian species and their propensity for reassortment have raised concerns regarding the pandemic potential of these viruses, because the potential for transmission to humans or reassortment with circulating human influenza viruses exists.

42.11 Live-Attenuated H6N1 Influenza Vaccine

Based on serum antibody cross-reactivity studies on 14 different H6 influenza viruses from North American and Eurasian lineages [48], LID and MedImmune have generated three candidate H6N1 *ca* vaccine viruses; A/teal/HK/W312/97 *ca* (H6N1); A/mallard/Alberta/89/85 *ca* (H6N2), and A/duck/HK/182/77 *ca* (H6N9) [74]. Pre-clinical studies determined that these H6 *ca* viruses were attenuated in mice and ferrets, and that a single dose of each of the H6 *ca* vaccine viruses was immunogenic in mice and ferrets, and provided complete protection against homologous challenge with the corresponding *wt* H6 influenza virus. The A/teal/HK/W312/97 *ca* vaccine virus provided the broadest cross-protection against challenge with antigenically distinct H6 influenza viruses, and this vaccine was evaluated for safety, infectivity and immunogenicity in a late-2008 phase 1 clinical trial in healthy adults. Data from this trial are pending.

42.12 Practical Considerations for Clinical Testing and Use of LAIV For Pandemic Influenza

There is a theoretical risk that reassortment between a pandemic vaccine virus and a *wt* human influenza virus could occur, resulting in the generation of an influenza virus that has a novel HA that can be efficiently transmitted from person-to-person. This theoretical risk has implications for both the clinical testing and use of LAIV for pandemic influenza viruses. Although the data generated to date on the poor transmissibility of seasonal influenza LAIV and the few studies we have conducted with pandemic LAIV candidates

suggest that the risk of person-to-person spread is extremely low, clinical trials with our pandemic LAIV candidates are performed in an inpatient setting only during the months of the year when influenza is not circulating in the community. In accordance with recommendations of the FDA, our clinical trial design includes careful monitoring of amount and duration of shedding of vaccine virus by study subjects; contact precautions for subjects and study staff; monitoring of subjects and staff for possible illness and transmission of vaccine virus, and provision to treat subjects and staff who develop symptoms suggestive of influenza illness with antiviral drugs, pending laboratory confirmation of infection [69]. The use of attenuated influenza virus vaccines for pandemic influenza would only occur upon the recommendation of public health authorities after the onset of a pandemic.

In summary, vaccines will be a critical part of our response to an influenza pandemic. LAIV offer several potential advantages, including induction of serum and mucosal antibody responses and T-cell responses, and of broader cross-protection against antigenically diverse influenza viruses in naïve populations than inactivated vaccines; higher yield of vaccine doses in embryonated eggs compared to inactivated vaccines and the possibility that a single dose of LAIV may be sufficient to elicit a protective antibody response. Therefore, this approach to vaccination against influenza viruses with pandemic potential merits careful evaluation.

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Chapter 43
Targeting the Messenger: Vector-Based Vaccines to Control *Leishmania* Infection and Transmission

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43.1 Background

Leishmaniasis is a vector-borne disease caused by the *Leishmania* parasite and transmitted by the bite of *Phlebotomine* sand flies. Approximately 350 million people are at risk for this neglected disease with an annual incidence of two million cases and a loss of 2,357,000 disability-adjusted life years [1,2]. Leishmaniasis has multiple clinical manifestations ranging from cutaneous, mucocutaneous and diffuse, to visceral leishmaniasis; the latter being fatal if untreated [1]. To add to the complexity of this disease, different Leishmania species are responsible for the different disease manifestations and the different Leishmania parasites are transmitted by different sand fly species.

43.2 Molecular Interactions Between Sand-Fly Gut Proteins and Leishmania Parasites

Leishmania undergoes a complex life cycle within the digestive tract of competent vectors, beginning with the ingestion of blood containing amastigotes and terminating with the transmission of infective metacyclic promastigotes to a mammalian host [3]. Attachment of parasites to the midgut lining of the sand fly is crucial to the successful completion of this life cycle, serving to prevent excretion of developing promastigotes along with the blood meal remnants. *Phlebotomine* vectors of leishmaniasis are diverse and, in the case of some sand-fly species, will only permit the complete development of the species of *Leishmania* they transmit in nature. LPG, the major glycol-conjugate on the surface of *Leishmania* promastigotes, has been implicated as the ligand-mediating midgut attachment [3]. The phosphoglycan moieties of all LPG share a common backbone, composed of repeating disaccharide units consisting of PO_4-6Gal(β1–4)Manα1, where the C-3 position of the Gal residue can either be unchanged (*L. donovani*, Sudan), partially substituted with glucose side chains (Indian *L. donovani*, *L. mexicana*, and *L. chagasi*), or completely substituted with side-chain sugars, which terminate primarily in galactose (*L. major*) or glucose and arabinose (*L. tropica*) [3]. The involvement of LPG side-chain sugars in the species-specific attachment of Leishmania parasites to their vectors has been most clearly demonstrated for *L. major* and *P. papatasi* [3]. The nature of the LPG receptor in sand flies, although of obvious interest, remained undefined for many years. For this, we studied the transcripts present in the midgut of different sand flies, with the objective of identifying and characterizing potential receptors for *Leishmania* parasites.

43.2.1 Identification of the Most Abundant Midgut Transcripts of Phlebotomus Papatasi, the Vector of Leishmania Major

Transcriptome analysis of *P. papatasi* midgut cDNA library resulted in the identification of the most abundant midgut-specific molecules from this sand fly [4], including those with putative roles in digestion and peritrophic matrix formation. Midgut transcripts that are expressed only after a blood meal were microvilli-associated like proteins (*PpMVP1*, *PpMVP2* and *PpMVP3*); a peritrophin (*PpPer1*); a trypsin homologue (*PpTryp4*); a chymotrypsin homologue (*PpChym2*) and two unknown proteins. Importantly, the transcripts *PpChym2*; *PpMVP1*; *PpMVP2; PpPer1* and *PpPer2* were considerably downregulated when the sand fly was given a blood meal in the presence of the parasite *L. major*. The suggested upregulation of specific transcripts in a blood-fed cDNA library was validated by real-time PCR, suggesting that our customized bioinformatic analysis is a powerful and accurate tool for analyzing expression profiles from different cDNA libraries. These results suggest that the *Leishmania* parasite is modulating key proteins, including enzymes, in the gut of the sand fly and that these proteins are attractive targets for transmission-blocking vaccines.

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43.2.2 Identification, Isolation and Expression of a Galactose-Binding Protein (PpGalec) from a Phlebotomus Papatasi Midgut-cDNA Library

Transcriptome analysis of the *P. papatasi* midgut cDNA library also resulted in the identification of a cDNA coding for a protein homologous to a galactose-binding protein named PpGalec [4]. Because the branching sugar of the outer structure of *Leishmania* is composed of galactoses, we hypothesized that PpGalec is the receptor for *Leishmania major. PpGalec* is a protein of 35.4 kDa that lacks a signal peptide and a transmembrane domain. *PpGalec* was expressed as a recombinant protein, refolded and purified by reverse-phase HPLC, and used both to prepare antibodies and for parasite-binding assays.

43.2.3 Specific Binding of Procyclic, Leishmania-Major Promastigotes to Recombinant PpGalec

We demonstrated by flow cytometry that *L. major* procyclics bind rPpGalec with a significant four-fold increase, compared to control samples [5]. Promastigotes of two other *Leishmania* species, *L. donovani* and *L. tropica,* did not bind to rPpGalec. Importantly, *L. major* metacyclics, the stage that does not bind to the midguit epithelium, and that replace many of their side-chain terminal sugars with arabinose, did not bind rPpGalec, providing further evidence of the significance of PpGalec *in vivo*.

43.2.4 Anti-Ppgalec Antibodies Inhibit Midgut-Parasite Binding and Development in Vivo

In order to test the importance of PpGalec in parasite development, P. papatasi sand flies were fed on blood meals containing *L. major* amastigotes reconstituted with serum from PpGalec-immunized mice or control-vaccinated mice. Six days post infection, when the blood meal was lost, there was an 86% decrease in the number of parasites in the midgut of sand flies that fed on anti-PpGalec, compared with controls. Importantly, antibodies to PpGalec also prevented the development of mature infections in the anterior midgut, by decreasing by 89% at day 14 the number of metacyclic promastigotes capable of initiating infection in the vertebrate host [5]. These studies demonstrate the feasibility of using midgut receptors for parasite ligands as target antigens for transmission-blocking vaccines (Fig. 43.1).

43.2.5 Identification of the Most Abundant Midgut Transcripts of Lutzomyia Longipalpis, the Vector of Leishmania Infantum Chagasi

We also studied the midgut transcriptome of the most important vector of *L. infantum chagasi* in Latin America, the sand fly *Lutzomyia longipalpis*. For this we sequenced five cDNA libraries with a total of 10,000 sequences from the midgut tissue of this sand fly, including midgut tissue after a sugar meal, a blood meal and a blood meal in the presence of *L. infantum chagasi* [6]. This represents the largest set of sequence data reported from a specific sand-fly tissue. Proteases were the most abundant transcripts and included trypsin-like proteases; chymotrypsins; carboxypeptidases, and an astacin-like metalloprotease. Other abundant transcripts were microvillar-associate proteins; glutathione s-transferase (GST); catalase; copper-zinc superoxide dismutase (SOD) and peroxiredoxin (PRX) [6].

Comparative analysis of the transcripts from the sugar-fed and blood-fed cDNA libraries resulted in the identification of transcripts differentially expressed during blood feeding. Upregulated transcripts were four distinct microvillar-like proteins; two peritrophin-like proteins; a trypsin-like protein; two chymotrypsin-like proteins and an unknown protein. Downregulated transcripts by blood feeding were a microvillar-like protein (LuloMVP3), a trypsin like protein (Lltryp2) and an astacin-like metalloprotease. Importantly, we identified molecules that were differentially expressed due to the presence of *Leishmania* in the gut of the sand fly. The downregulated transcripts were the four microvillar-like proteins, a chymotrypsin (LuloChym1A) and a carboxypeptidase (LuloCpepA1), among others. Upregulated transcripts due to the prescence of Leishmania were a peritrophin-like protein (LuloPer1), a trypsin-like protein (Lltryp2) and an unknown protein [6]. These data are reinforced by the similar trend observed in our studies of *P. papatasi* and *L. major,* and suggest that *Leishmania* parasites alter the expression of sand-fly midgut transcripts and these vector molecules may be relevant for the survival and establishment of the parasite in the insect midgut.

43.3 Conclusion

This project has contributed significantly to the understanding of the molecular repertoire of sand-fly midgut proteins; the interactions between *Leishmania* outer-surface molecules and a sand-fly midgut protein, as well as the relevance of these interactions to parasite attachment and development and the potential use of these molecules as transmission-blocking vaccines. This project also demonstrated for the

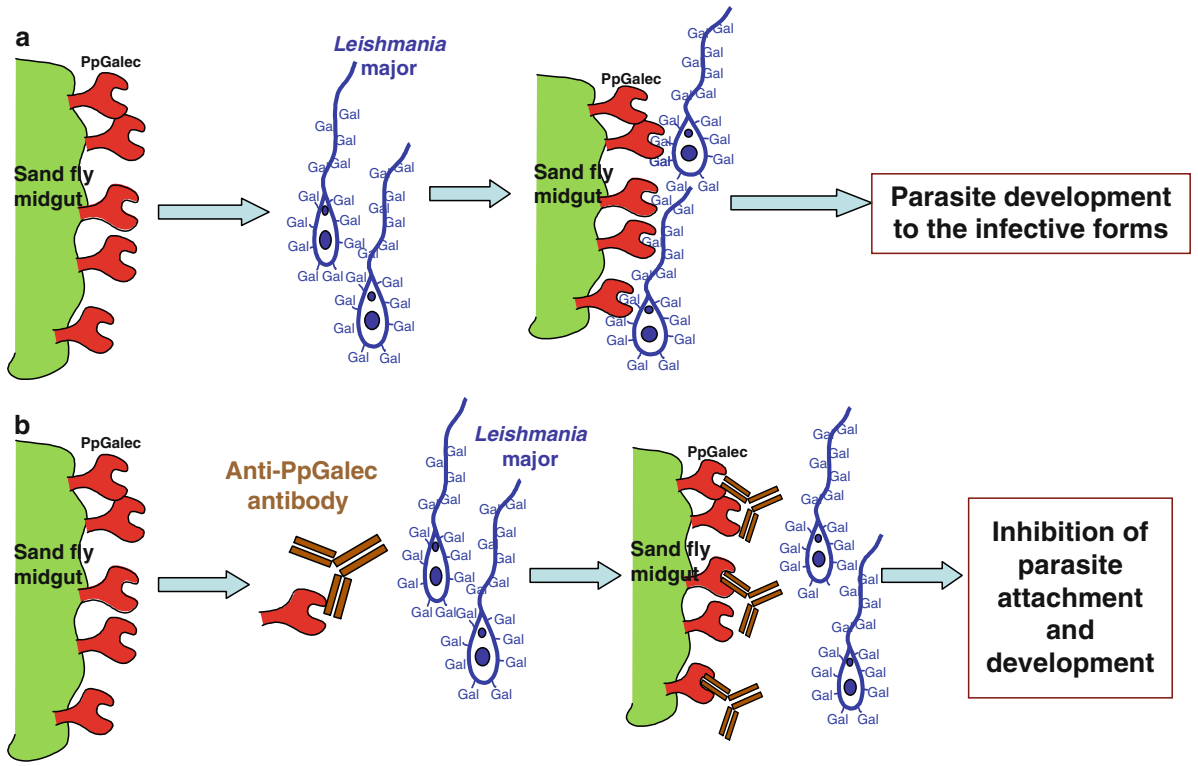

Fig. 43.1 Schematics of the Interaction Between Ppgalec in the Sand-Fly Midgut and the Galactose Residues of *Leishmania major* LPG Structure. (**a**) *Leishmania* parasite binds specifically to Ppgalec. This attachment is necessary for survival and development of the parasite to the infective metacyclic form. (**b**) Principle of the transmission-blocking vaccine: blocking Ppgalec, the attachment site for the *Leishmania major* parasite, inhibits binding and, consequently, development of the parasite to the infective form in the gut of the sand fly

first time the effect of *Leishmania* parasites on the transcript profile of sand-fly midgut proteins at very early time-points during the blood-digestion process, and that these midgut proteins represent important targets to blocking parasite development in the gut of the vector.

43.4 Impact of Immune Responses to Vector-Salivary Proteins in Pathogen Transmission

After the *Leishmania* parasite develops to an infective form inside the gut of the sand fly, it is then injected, together with saliva, into a mammalian host during blood feeding. Components present in sand-fly saliva contain potent anti-hemostatic and immunomodulatory activities [7], and are able to enhance *Leishmania* infection [8]. Conversely, immune responses to either sand-fly salivary-gland homogenate or to the bites by sand fly protect animals against *Leishmania* infection [9]. The protective effect of insect saliva is not exclusive to sand flies and leishmaniasis. Animals pre-exposed to tick saliva by bites were protected from *Borrelia* infection [10]. Vaccination with a tick salivary protein conferred protection to mice from the fatal outcome of encephalitis virus [11]. Immunization with the saliva of the aquatic insect *Naucoris* genus protected mice against *Mycobacterium ulcerans* infection [12]. However, there is limited information regarding the nature of the protective-vector salivary molecules and mechanism of protection. Until recently, only two sand-fly salivary proteins, maxadilan from *Lu. longipalpis* and PpSP15 from *P. papatasi,* were identified as protective proteins against leishmaniasis [13]. It is proposed that immunity to maxadilan results in the neutralization of the exacerbation of *L. major* infection caused by this molecule [13], while the protection conferred by PpSP15 immunization indicates that cellular immunity as a form of a delayed-type hypersensitivity response (DTH) to this molecule is sufficient for protection [14].

43.4.1 Isolation of Secreted Salivary Sand-Fly Proteins that Can Work as Vaccines to Protect from Leishmaniasis by Using Transcriptomic Analysis of Sand-Fly Salivary Gland cDNA Libraries

To understand sand fly-mammalian host interactions and the importance of salivary proteins on these interactions, we need to know first the repertoire and the properties of the salivary molecules injected by the sand fly to the mammalian host.

For this we combined transcriptomic and proteomics approaches to identify the most abundant secreted proteins from the saliva of different sand flies [15], including the sand fly *Lu. longipalpis,* the vector of *L. infantum chagasi* in Latin America [16]. With this approach, we identified 35 transcripts coding for secreted proteins indicating the low complexity of proteins in the salivary gland of *Lu longipalpis*. The identified molecules included a novel RGD-containing peptide; three members of the yellow-related family of proteins; the small peptide maxadilan; a PpSP15-related protein; six members of a novel c-type lectin family of proteins; an antigen 5-related protein; a D7-related protein; a *Cimex* apyrase-like protein; a protein homologous to a silk protein with amino acid repeats resembling extracellular matrix proteins; a 5'-nucleotidase; a peptidase; a palmitoyl-hydrolase; an endonuclease; nine novel peptides and four different groups of novel proteins. It is important to note that sixteen of these proteins appear to be unique to sand flies and were not found in any of the existing sequenced genomes [16]. These salivary proteins represent the initial screen of attractive targets for the development of vector-based vaccines to control *Leishmania* infection.

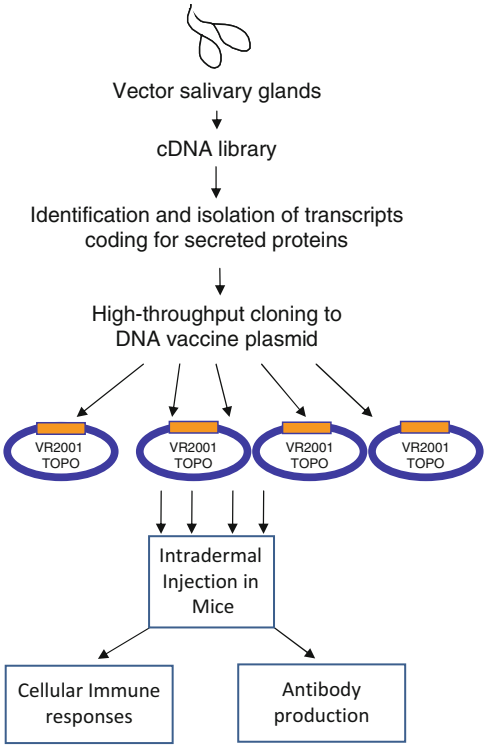

Fig. 43.2 From Transcriptome to Immunome: Schematic of the approach used to isolate vector salivary transcripts and to construct DNA plasmids to test immune responses in animals to search for vector salivary vaccine candidates

43.4.2 Identification of Proteins Inducing Cellular Immunity from a Phlebotomus-Salivary Gland cDNA Library

Our next objective was to identify the salivary proteins that can produce a DTH response in animals, since cellular immune response to sand-fly saliva as a form of DTH is associated with protection against *Leishmania* infection [9,14]. For this we developed a functional transcriptomic approach based on high-throughput DNA-plasmid construction and DNA immunization to screen the different proteins from the sand fly salivary gland cDNA libraries (Fig. 43.2). Using molecules from *Phlebotomus ariasi* salivary gland cDNA library, we identified three DTH-inducing salivary proteins in mice [17]. The DTH response was specific to these proteins and consisted of infiltration of mononuclear cells in varying proportions at 24 and 48 hours, post-challenge. Additionally, we developed a reverse-antigen screening approach (RAS) which consists of the use of mice previously exposed to sand-fly saliva and the challenge of these animals with the intradermal injection of selected sand fly DNA plasmids, to induce a specific-recall cellular-immune response as a form of DTH [17]. High-throughput screening combined with RAS represents a novel method for rapid screening of vector-salivary molecules to search for vaccine candidates which may be related to host protection against vector-borne diseases that are controlled by a cellular-immune response. We believe this approach will accelerate vaccine development by improving the rationale for choosing candidates in large animal models.

43.4.3 Immunity to Distinct Sand-Fly Salivary Proteins Primes the Anti-Leishmania Immune Response towards Protection or Exacerbation of Disease

After the identification of DTH-inducing salivary proteins from sand flies, we tested the hypothesis that DTH-inducing molecules will protect against *L. major* infection. For this we tested two DTH-inducing salivary proteins from the sand fly *P. papatasi,* PpSp15 and PpSp44. DNA immunization with these salivary proteins resulted in contrasting outcomes of infection upon challenge with *L. major* parasites. PpSP15-immunized mice protected animals against cutaneous leishmaniasis caused by *L. major,* while immunization with PpSP44 aggravated the infection. This suggested that immunization with these two different molecules could alter the course of anti-*Leishmania* immunity. Furthermore, two weeks post-infection, $CD4^+$ T cells isolated from PpSP15-immunized mice produced four times more IFN-γ after stimulation with

killed *Leishmania* than $CD4^+$ T cells from PpSP44-immunized mice. IL-4 producing cells were three-fold higher in PpSP44-mice. This specific anti-*Leishmania* response correlated with an early anti-saliva immunity, where just two hours after challenge with sand-fly saliva and *L. major*, the expression profile of PpSP15-mice showed over three-fold higher IFN-γ and IL-12-Rβ2 and 20-fold lower IL-4 expression, relative to PpSP44-mice, suggesting that salivary proteins differentially prime anti-*Leishmania* immunity. In this work, we demonstrated that not all DTH-inducing *P. papatasi* sand-fly salivary molecules are universally protective against *L. major* infection, and that immunization with two distinct DTH-inducing salivary proteins produced opposite immune profiles that correlated with resistance or susceptibility to *Leishmania* infection. This stresses the importance of the proper selection of vector-based vaccine candidates.

43.4.4 Immunity to a Salivary Protein of a Sand-Fly Vector Protects Against the Fatal Outcome of Visceral Leishmaniasis in a Hamster Model

We then tested if immunity to a sand-fly salivary protein that produces a DTH response can protect against visceral leishmanasis (VL) in hamsters. Hamsters co-inoculated with 100,000 *L. infantum chagasi* parasites and *Lu. longipalpis* saliva developed classical signs of VL, resulting in a fatal outcome five to six months post-infection. Hamster immunized with LJM19, a novel 11-kDa protein from the sand fly *Lu. longipalpis*, protected hamsters against the fatal outcome of VL, compared to control immunized hamsters. LJM19-immunized hamsters maintained low parasite numbers in liver and spleen. This correlated with a high IFN-γ/TGF-β ratio and iNOS expression in the spleen and liver of the protected animals, and not in control-immunized animals. This systemic and protective immunity correlated with the early appearance of anti-saliva immunity, where a DTH response with high expression of IFN-γ was observed in the skin of LJM19-immunized hamsters 48 hours after exposure to uninfected sand-fly bites. This is the first account on the immunity to a defined salivary protein conferring powerful protection against the fatal outcome of a parasitic disease. This reinforces the concept of using components of arthropod saliva in vaccine strategies against vector-borne diseases**.**

43.5 Conclusion

This project has contributed significantly to the understanding of the molecular repertoire of salivary proteins in sand flies and the identification of potential salivary vaccine candidates by bioinformatic analysis and functional transcriptomic approaches. We also learned the impact of the immunity to specific salivary proteins in protection from cutaneous and visceral leishmaniasis and the knowledge that a DTH response alone to a sand-fly salivary protein is not sufficient to predict protection. Finally, this project contributed significantly to the understanding of the importance of the early and protective (Th1) immune response to salivary proteins in dictating outcome of parasite infection and how this information has the potential to be exploited in large animals, including dogs and humans.

In summary, the Vector Molecular Biology Unit, NIAID, focuses on the molecular aspects of vector-salivary and midgut proteins, with emphasis on understanding vector/host and vector/parasite interactions, specifically sand fly/*Leishmania*/mammalian host interactions. Unit research combines basic approaches, together with veterinary and clinical research, thereby broadening our understanding of the relationship between immune responses to vector-salivary proteins in animal and human reservoirs and disease outcome, and between the *Leishmania* parasite and sand fly midgut proteins, ultimately to develop a vector-based vaccine against leishmaniasis, a neglected disease.

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