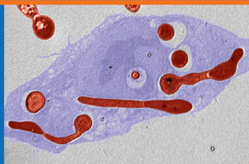
Gordon D. Brown
Mihai G. Netea
Editors

Immunology of Fungal Infections



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Immunology of Fungal Infections

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PREFACE

The history of mankind has been shaped by infections, more than by war and famine together. At the same time, however, the development of society has had an equally important effect on human diseases. The emergence of agriculture, urban societies and high population densities has been proven to be crucial for the spread of pathogens, and thus human action is currently the single most important driver of infectious epidemiology. Even today, where once major killers such as poliomyelitis have been eradicated, new pathogens are appearing as result of human activity.

One such group of pathogens are the fungi, whose emergence is mainly due to modern medical practices. Fungal microorganisms, from yeasts colonizing the skin or mucosa, to molds from soil or water, are usually harmless in the context of normal host responses. However, the success of chemotherapy, as well as the AIDS pandemia, has led to immune deficiencies in a significant segment of the patient population, and the extensive use of intravenous catheters has provided a way of access for microorganisms which otherwise would find difficult to infect the host. As a result, a yeast such as *Candida* is now on the 4th place on the list of the most frequent sepsis agents, whereas infection with the mold *Aspergillus* is increasing in incidence and it is one of the most feared complications in patients with hematological malignancies. Fungal infections have thus become an important factor of morbidity and mortality, and represent an increasing burden on the medical system. An effective treatment of these infections is an absolute necessity.

We are at a cross-road in our efforts to tackle infections in general, and fungal infections in particular. While the last decennia have brought important progress in the development of more effective and safe antifungal agents, an important percentage of patients still succumb to these infections. The failure of therapy has more to do with the ineffectiveness of host defense mechanisms, than to the absence of effective antifungal agents. Therefore, combining classical antibiotic treatment with adjunctive immunotherapy would seem the logical step forward in the management of fungal infection. Until now, this goal was elusive due to the lack of proper knowledge of the immune system and its interaction with infectious microorganisms.

However, this is changing rapidly, and research done in the last 20 years has enabled us for the first time to design ways of boosting the immune system in an effective way. Discoveries such us the description of the receptors recognizing fungi, an increasing understanding of the host defense mechanisms and cell types important for host defense, as well as the ways through which fungi escape immune surveillance, are important milestones in the way towards understanding host defense to these pathogens. In the present book on the "Immunology of fungal infections" we want to provide an overview of these recent advances, and a guide for understanding the immunology to fungal infections. By asking leading experts

to present the cutting edge information in the field, as well by sharing their views on the challenges for tomorrow, we intend to provide a key source of information on the pathways through which fungi interacts with the host.

In order to respond to these aims, two approaches have been pursued: the first sections of the books present chapters on the major components of the antifungal defense (cells, soluble factors, pathogen recognition receptors), while sections in the second part of the book are devoted to the immune response to specific fungal pathogens. Special chapters deal with immune evasion mechanisms employed by the fungi, as well as with the current status of immunotherapy of fungal infections. By providing the scientific community with a comprehensive overview of the most essential aspects of fungal immunology, we believe that an important need is being addressed, and that this book will represent an principal source of information for everybody interested in this topic.

Gordon Brown and Mihai Netea

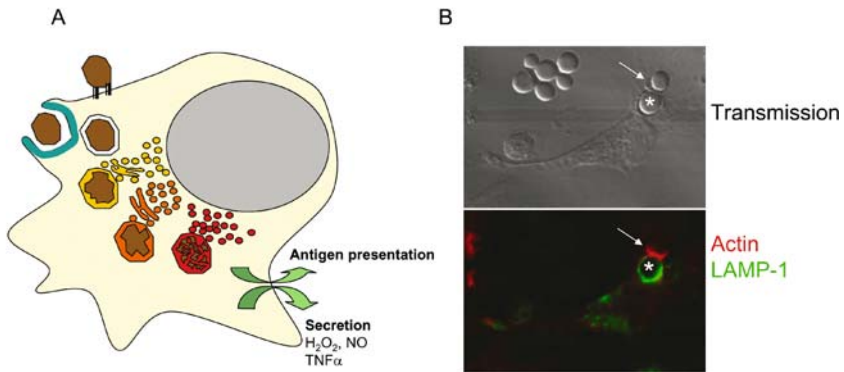


Figure 1.2. Phagocytosis A) Schematic representation of phagocytosis. Engaging of phagocytic receptors leads to particle binding and signalling for cytoskeletal rearrangements. This leads to the formation of a so called phagocytic cup that results in membrane engulfment of the particle. Once a phagosome is formed it matures by fusion and fission with early endosomes, late endosomes and lysosomes, sequentially. During this process the pH of the phagosome is lowered from 6 to around 4.5. Phagocytic signalling cascades also stimulate secretion of H_2O_2, NO and TNFα. B) *Candida albicans* phagocytosis by RAW264.7 macrophages. The cells were stained for actin with TRITC phalloidin (red) and for endosomes and lysosomes with LAMP-1 (green). The first yeast is taken up in a phagosome where the lysosomes have fused (asterisk). The second yeast is in the process of being phagocytosed and there is a clear phagocytic cup present (arrow)

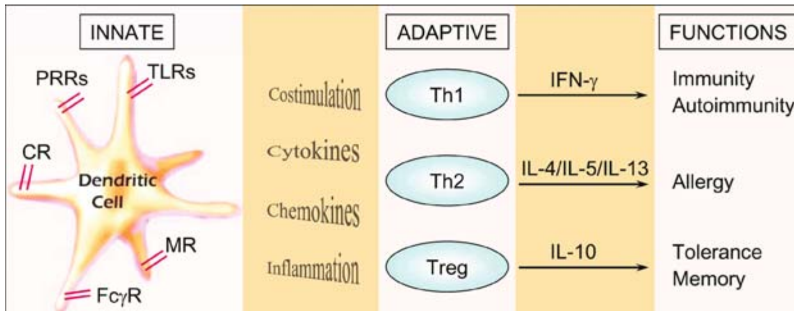


Figure 2.1. Th1/Th2/Treg polarization by dendritic cells in fungal infections. Essential to the successful removal of fungal pathogens is the early recognition of fungi by the innate immune system. Dendritic cells express numerous pathogen recognition receptors which enable them to sense distinct microbial stimuli, and they process this information and elicit distinct functional responses that induce different T-cell responses. DCs that produce IL-12 p70 stimulate protective Th1 responses. Those that produce IL-4 may yield allergic Th2 responses, and those that produce IL-10 may induce Treg implicated in tolerance and memory to fungi. PRRs, pattern recognition receptors; TLRs, Toll-like receptors, Th, helper T cells, Treg, regulatory T cells

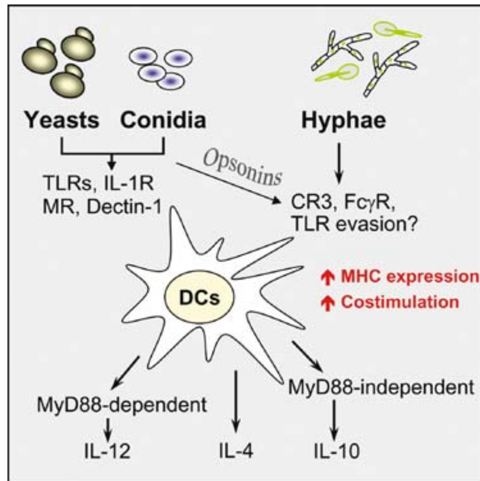


Figure 2.3. The exploitation of distinct recognition receptors in dendritic cells by the different fungal morphotypes. Dendritic cells sense fungi in a morphotype-dependent manner. The engagement of distinct receptors on dendritic cells translated into downstream signaling events that differentially affect cytokine production. The exploitation of a specific receptor invariably leads to the occurrence of a specific type of T helper cell reactivity. Fungal opsonins may subvert the receptor exploitation by fungal morphotypes. TLRs, Toll-like receptors; IL-1R, IL-1 receptor; MR, mannose receptors; CR3, complement receptor 3; FcγR, receptor for the Fc portion of immunoglobulins; MyD88, Drosophila myeloid differentiation primary response gene 88

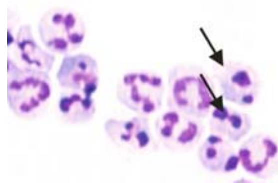


Figure 3.1. Neutrophils in culture. Healthy viable neutrophils exhibit the classical polymorphonuclear phenotype. As they age, they undergo constitutive apoptosis, with shrinking of the nucleus (pyknosis). Two apoptotic neutrophils, showing nuclear condensation, are marked by arrows

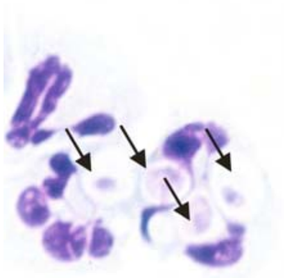


Figure 3.2. Neutrophils engulfing fungal particles. This photomicrograph shows neutrophils that have taken up particles of zymosan (derived from yeast cell walls) into phagosomes (arrowed) for destruction. The ability of the neutrophil to engulf multiple foreign particles in an attempt to neutralise infection is shown in the cell on the right

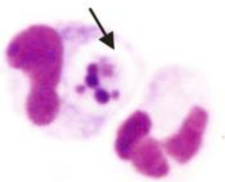


Figure 3.3. Removal of aptoptotic neutrophils. In this smear, leukocytes were isolated from the joint of a patient with rheumatoid arthritis. The two cells shown here are monocytes, one of which has engulfed an apoptotic neutrophil (arrowed), for removal in an injury-limiting fashion that is associated with downregulation of proinflammatory macrophage function

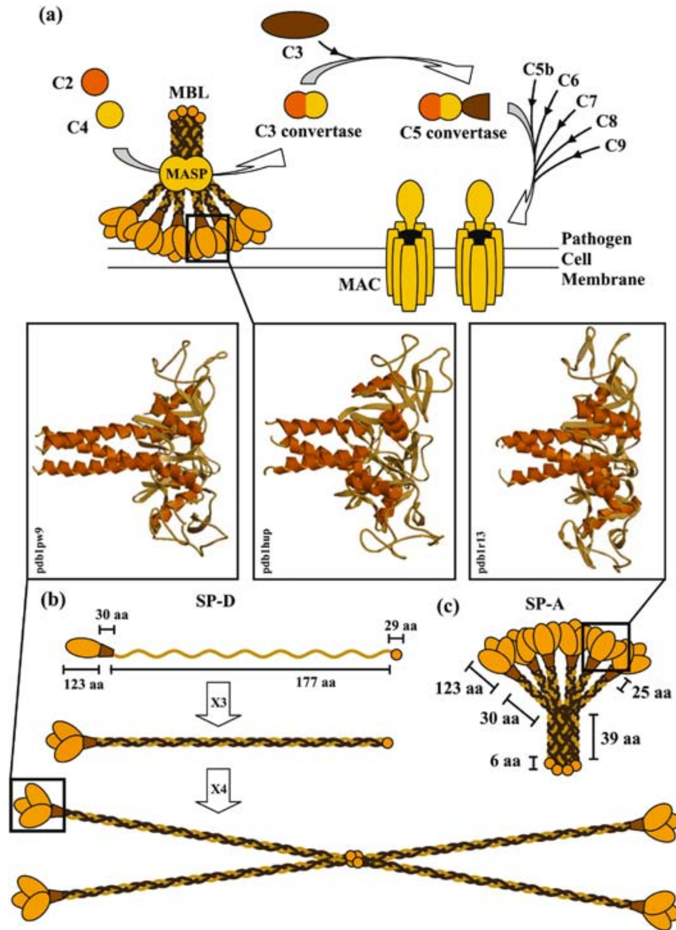


Figure 7.1. Structural organisation of human collectins. The basic polypeptide structure found in all the collectins is organised into four regions: a cysteine-containing N-terminus, a triple-helical collagen-like region composed of repeating Gly-X-Y triplets, followed by an α-helical coiled-coil neck region, and a globular CRD. This polypeptide chain undergoes trimerisation via the neck and collagen-like regions to form a trimeric structural subunit (b). Six of these trimeric subunits then undergo further assembly to yield hexameric structures in case of MBL and SP-A (a, c), although dimers, trimers, tetramers and pentamers are also found. The hexameric forms of MBL and SP-A resemble complement protein C1q in their overall organisation (C1q is only found as a hexamer of a structural subunit which is composed of three different polypeptide chains). SP-D has a tetrameric structure with four of the homotrimeric structural subunits linked via their N-terminal regions, but trimers, dimers and monomers also exist (a). Ribbon diagrams (inset) of the X-ray crystal structures of trimeric neck and CRDs of MBL, SP-A and SP-D show their predominantly β-sheet jellyroll three-dimensional structure. The primary ligand-binding sites (one per CRD) are located at the CRD surface opposite the neck region. The SP-A and SP-D illustrations are approximately to scale. MBL binding to the microbial surface via the CRDs activates the MASPs. MASP-2 cleaves C4 and C2 to generate C3 convertase (C4b2a), which cleaves C3. This leads to the complement lytic pathway, culminating in the formation of membrane attack complex (MAC) and pathogen killing (a)

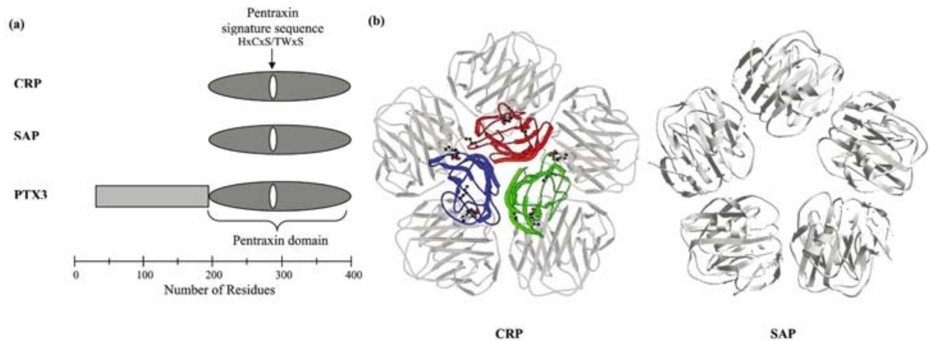


Figure 7.2. (a) Organisation of short and long pentraxins. Pentraxins are characterised by the presence in their carboxy-terminal of a 200 amino acids pentraxin domain, with an 8 amino acid long conserved pentraxin signature (HxCxS/TWxS, where x is any amino acid). The human CRP and SAP genes are located on chromosome 1q23 and are organised in two exons, the second exon encoding for the pentraxin domain. The long pentraxin, human PTX3 gene, localised on human chromosome 3 band q25, is organised in three exons separated by two introns, the third exon codes for the pentraxin domain. The mature SAP protomer is 204 amino acid long (25,462 Da) and has a pentameric structure in the presence of physiological levels of calcium (127,310 Da). In the absence of calcium, SAP consists of both pentameric and decameric forms. Each SAP protomer is glycosylated with a single N-linked biantennary oligosaccharide at Asn^{32}. Human CRP is composed of five identical nonglycosylated protomers. The PTX3 protein (40,165 Da) consists of a C-terminal 203 amino acids pentraxin-like domain (containing an N-linked glycosylation site in the C-terminal domain at Asn^{220}) and an additional N-terminal region (178 aa) unrelated to other known proteins. PTX3 protomers can assemble as decamers and higher oligomers upto 900 kDa. (b) Crystal structures of CRP and SAP. Each CRP protomer has a characteristic lectin fold composed of two layered ß sheets with a flattened jellyroll topology; five protomers are noncovalently associated to form a pentamer (115,135 Da)(Shrive et al. 1994). Ligand bound CRP or SAP can bind to C1q and activate the classical complement pathway (Nauta et al. 2003), which may be one of the mechanisms involved in enhanced phagocytosis of pathogens by phagocytic cells. The interaction between one pentameric molecule of CRP and the heterotrimeric globular domain of C1q has been shown (Kishore et al. 2004a, 2004b). The three chains of C1q (in color) are docked within the CRP pentameric structure. SAP is composed of 5 or 10 identical subunits noncovalently associated in pentameric rings interacting face to face (Emsley et al. 1994). Human SAP has a tertiary fold, which resembles that of the legume lectins like Concanavalin A. SAP protomers have a flattened ß-jelly roll topology with a single long helix folded on the top of the ß-sheet. The five subunits are arranged in a ring around a hole and are held together by hydrogen bonds and salt bridges. The decamer is stabilised by ionic interactions between the two pentamers. Each SAP subunit can bind two calcium ions, and residues involved in calcium binding are conserved. Based on molecular modelling, the PTX3 pentraxin domain has a simlar structural fold to SAP, since most of the ß-strands and the α-helical regions are conserved

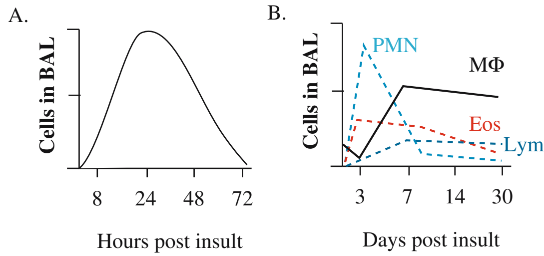


Figure 9.2. (A) The time course of total cell infiltration in the acute response to Aspergillus fumigatus occurring in the allergic airway. (B) Each leukocyte and macrophage population typically follows a distinct time course of recruitment during the chronic allergic response

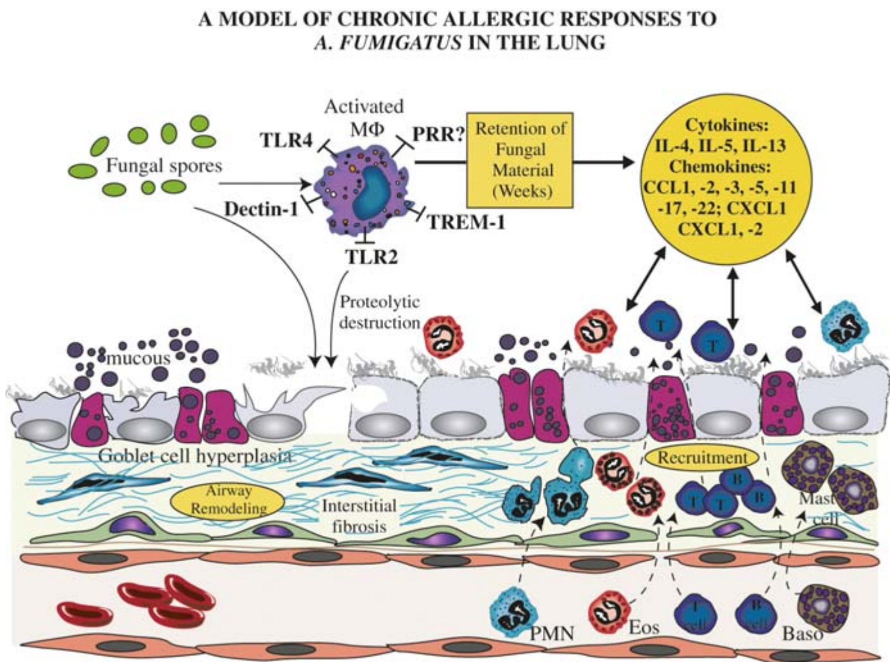


Figure 9.3. An outline of the chronic responses occurring in the allergic airway following challenge with *Aspergillus fumigatus*, featuring a central role of macrophage activation and cytokine and chemokine production

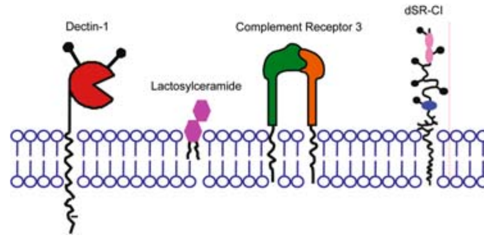


Figure 12.2. Pattern recognition receptors for β-glucans, including Dectin-1, CR3 and lactosylceramide. Also shown is SR-CI, a Drosophila scavenger receptor shown to recognise β-glucans, as the mammalian scavenger receptor(s) which recognise these carbohydrates has not been identified

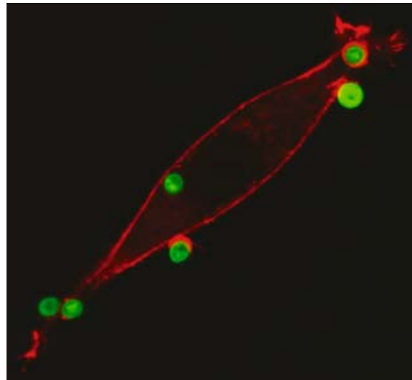


Figure 12.3. Dectin-1 can mediate the phagocytosis of zymosan and the binding and phagocytosis of yeast particles in transfected cells. Shown are Dectin-1 transfected NIH3T3 fibroblasts binding and internalising fluorescently labelled zymosan (green) via actin (red)-based phagocytic cups. Reproduced with permission from (Brown and Gordon, 2001)

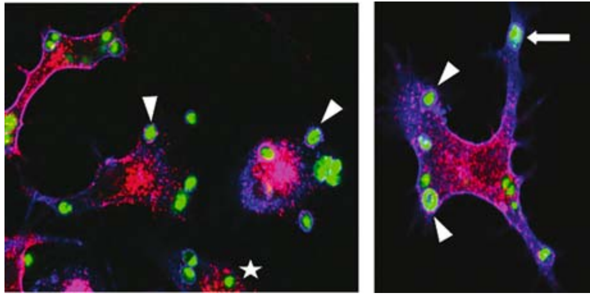


Figure 13.2. Uptake of zymosan (heat-inactivated yeast cell wall preparation derived from *Saccharomyces cerevisiae*) by immature DCs. FITC-labelled zymosan particles (green) were added to immature DCs and taken up. Cells were fixed and stained with anti-DC-SIGN- (blue) and anti-MR-antibodies (red). Most phagocytic vesicles contain both, MR and DC-SIGN (white arrow heads), while few contain exclusively MR (white arrow) or DC-SIGN (white star)

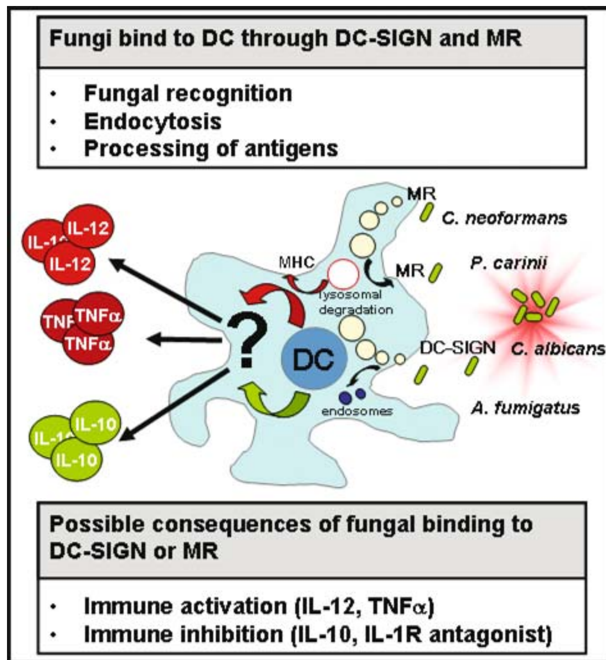
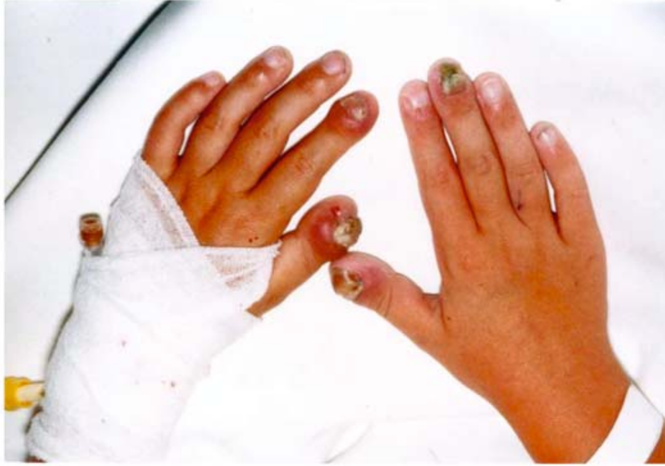


Figure 13.3. Fungi bind to DCs through DC-SIGN and MR and induce immunity or immune evasion. Binding to fungi induces DC-SIGN- and MR-mediated phagocytosis. After ligand binding MR is routed to the late endosomes while DC-SIGN ends up in early endosomes. It is intriguing that depending on the fungus, its form, and other unknown factors, fungal binding to MR or DC-SIGN may induce immune activation, while under different conditions fungi use the same receptors to evade the host immune system. The underlying molecular mechanisms are not yet known

A



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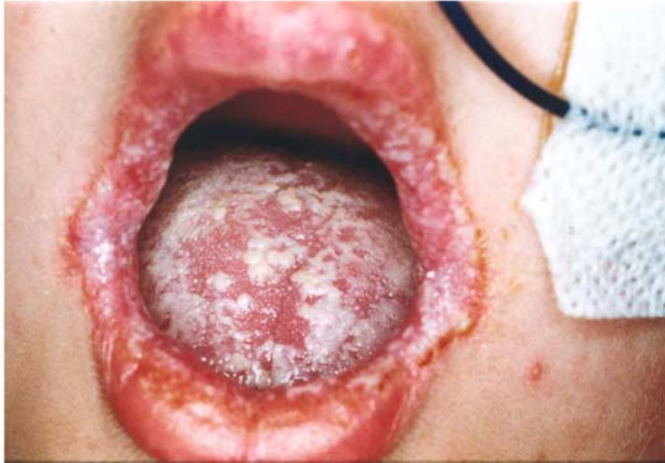

Figure 16.1. Severe, debilitating CMC of the nails (A) and mouth (B) in a six-year old girl. Reprinted with permission from Dr Mario Abinun and Professor Andrew J Cant

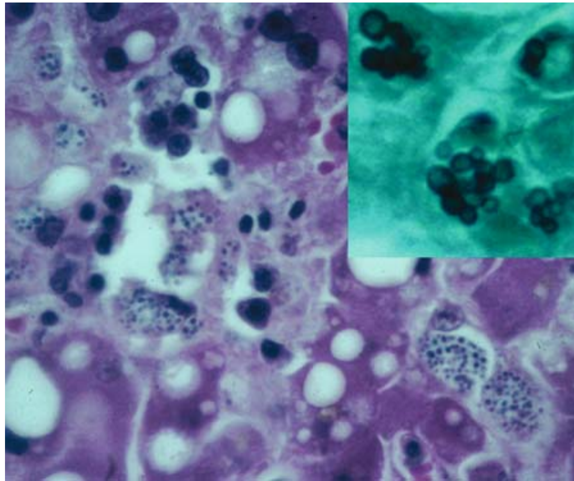

Figure 18.2. H. capsulatum in tissue. Micrographs of a liver depicting swollen and vacuolated hepatocytes and sinusoidal Kuppfer cells filled with *H. capsulatum* yeast cells. The liver section has been stained with Haematoxylin and eosin with the inset showing Gomori's methenamine silver staining. Original magnification, X400

SECTION 1

CELLS

CHAPTER 1

MACROPHAGES

SIGRID E.M. HEINSBROEK AND SIAMON GORDON

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Abstract: Macrophages are important for both tissue homeostasis and immunity. A great variety of macrophage subpopulations exist that are specialised in different functions eg, osteoclasts that remodel bone, inflammatory macrophages that orchestrate the immune response. As immune regulators macrophages recognise, internalise, degrade and present antigens. Different levels of macrophage activation can be distinguished and this influences the type of immune stimulators secreted by macrophages. Different pathogens have developed ways to evade the macrophage or influence macrophage function to their advantage. This chapter introduces the complexity of macrophage interaction with pathogens and fungal pathogens in particular

This chapter describes the function of macrophages in the immune system with emphasis on macrophage cell biology including phagocytosis of microbes and phagosome maturation. Different strategies employed by pathogens to evade macrophage killing will be discussed, including mechanisms used by some fungi.

1. DEVELOPMENT

Macrophages develop from granulocyte/monocyte precursors in the bone marrow. These precursor cells can also develop into neutrophils, dendritic cells (DC), Langerhans cells and osteoclasts depending on stimulation with growth factors. Precursor development into monocytes is mediated by macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3. Monocytes leave the bone marrow, enter the bloodstream and after approximately one day migrate into tissues where they differentiate into macrophages (Gordon, 2001). Macrophages contribute to homeostasis by clearance of apoptotic/senescent cells, tissue remodelling and repair after inflammation. Macrophages also play distinct roles in immunity; resident and recruited macrophages are highly efficient phagocytes that clear pathogens and dying cells

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and can present antigen to primed T-cells. Furthermore, macrophages can secrete cytokines that affect the migration and activation of other immune cells, and reactive metabolites (reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI)) and other products that contribute to microbial killing and tissue injury (Gordon, 2001).

2. MACROPHAGE SUBPOPULATIONS

Development into tissue macrophages is mediated by factors specific to the local environment (Gordon, 1999). This renders tissue macrophages very heterogeneous, reflecting the specialisation of function adopted in different anatomical locations (Fig. 1) (Gordon and Taylor, 2005). Osteoclasts, for example, are able to remodel bone (Quinn and Gillespie, 2005). Macrophages from the lamina propria on the other hand are continuously in contact with symbiotic gut bacteria and these cells

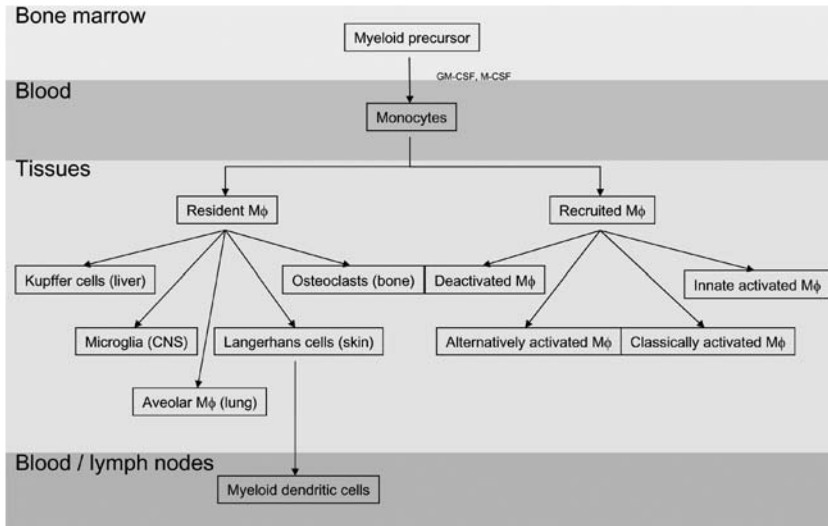

Figure 1. Macrophage heterogeneity. Myeloid precursors develop into circulating monocytes under stimulation of different factors including GM-CSF and G-CSF. Monocytes are recruited into tissues or inflammatory sites where they can differentiate into macrophages. Tissue macrophages fulfil different functions for instance Kupffer cells in the liver are involved in clearance of cells and complexes from blood, osteoclasts play an important role in bone remodelling and Langerhans cells capture epidermal antigens. The microenvironment plays an important part in the development of monocytes into these specialised cells. Macrophages recruited to inflammatory sites can be activated in different ways dependent on the environment. Upon recognition of microbes, macrophages undergo innate activation which leads to stimulation of antigen presentation and production of reactive oxygen species, nitric oxide and IFN-α/β. Macrophages become classically activated by priming with IFN-γ and a subsequent microbial trigger and alternative activation is mediated by IL-4 and IL-13. Uptake of apoptotic cells mediates deactivation of macrophages.

have a high phagocytic ability, but are less effective in producing pro-inflammatory cytokines (Smythies et al., 2005). Alveolar macrophages play a role in clearing microbes, viruses and other particles from the lung, which is an environment poor in opsonins. These cells express high levels of a range of pattern recognition receptors including scavenger receptors (McCusker and Hoidal, 1989; Palecanda et al., 1999; Taylor et al., 2002). Both local proliferation and recruitment of new precursors contribute to renewal and maintenance of the different macrophage populations (Gordon and Taylor, 2005).

3. MACROPHAGE ACTIVATION

Inflammatory monocytes are recruited and differentiate into macrophages at the site of inflammation (Van Furth, Diesselhoff-den Dulk, and Mattie, 1973). Different *in vitro* activation states have been described (Fig. 1) (Gordon, 1999). Classical activation is associated with high microbial activity, pro-inflammatory cytokine production and cellular immunity and is induced by interferon-γ (IFN-γ), and enhanced by microbial stimulation such as lipopolysaccharide (LPS). Alternative activation is stimulated by IL-4 and IL-13, which is associated with humoral immunity and tissue repair. Deactivation, which promotes an anti-inflammatory response, is induced by IL-10, transforming growth factor β (TGFβ) or ligation of inhibitory receptors (Gordon, 1999; Gordon and Taylor, 2005). It is unclear if distict activation states exist *in vivo* or whether macrophages exhibit a broad spectrum of phenotypes. It is likely that in the majority of situations the inflammatory environment will lead to exposure of macrophages to multiple stimuli with complex phenotypic consequences (Gordon and Taylor, 2005).

Inflammatory macrophages differ from resident tissue macrophages in the surface expression of different receptors. Resident macrophages in humans express LPS receptor (CD14), FcγIII receptor and high levels of chemokine receptor CX3CR1, but don't have FcγI receptor (CD64). On the other hand, inflammatory macrophages have higher CD14 expression and express CD64 besides expressing lower levels of CX3CR1 and losing CD16 expression (Gordon and Taylor, 2005). The use of human macrophages in research is limited by difficulty to access different macrophage populations in living individuals. Most research is done with the use of monocyte derived macrophages from blood which are matured into macrophages by co culture with M-CSF. Recruited macrophages can be obtained by subjecting volunteers to abrasion of a small skin area which is covered with filter paper. This is kept sterile and moist overnight after which the recruited cells can be harvested from the filter paper (Willment et al., 2005).

A frequently used model for human macrophages are macrophages from mice. These cells have very similar properties, however slight differences in receptor expression do exist; for instance resident macrophages in mice do not express MHC class II and CD14 (Gordon and Taylor, 2005). Different primary mouse macrophage populations can be studied, the most common include bone marrow derived

macrophages and peritoneal macrophages. Bone marrow derived macrophages develop by culturing bone marrow cells in M-CSF for 5–7 days. Resident peritoneal macrophages can be collected by peritoneal lavage. Elicited peritoneal macrophages are often obtained by intraperitoneal injection of small particles like bio-gel polyacrylamide beads or thioglycollate broth four days before collection. These primed elicited macrophages are terminally differentiated and proliferate partly, in the presence of M-CSF. Elicited peritoneal macrophages are different from resident macrophages, for instance Dectin-1 expression is higher on elicited macrophages while SIGN-R1, F4/80 and CD11b expression is higher on resident macrophages (Taylor et al., 2004).

Macrophages express a broad range of receptors whose expression levels vary depending on the macrophage population and activation state. The receptors are involved in a variety of macrophage functions and include responses to growth factors, cytokines, chemokines and other inflammatory mediators. Receptors are involved in migration, adhesion and antigen presentation.

4. ADHESION AND MIGRATION

Migration from blood into tissues involves a sequence of interactions between monocytes and endothelial cells, these include monocyte rolling, adhesion, polarisation and migration through the endothelium. Rolling is mediated by selectins expressed on the surface of monocytes. At this stage the monocytes are able to sense chemo-attractants such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF) or leukotriene B_4 (LTB_4), which leads to activation of β1- and β2-integrins, resulting in firm adhesion (Imhof and Aurrand-Lions, 2004). Some chemokines can be presented to the monocytes by endothelial cells. Transmembrane heparan sulphate proteoglycans expressed by endothelial cells bind chemokines in a way that leaves the binding site for monocyte chemokine receptors exposed. Initially, chemokine recognition leads to signalling cascades that activate a cytoskeletal protein called talin and a member of the RAS family of GTPases, that subsequently activate β2 integrins. Activated β2 integrins change their conformation leading to binding to ICAM1 molecules expressed by endothelial cells, resulting in strong adhesion (Kim, Carman, and Springer, 2003).

The monocytes, now adherent to the endothelium, need to migrate through the vascular barrier. This requires polarisation of the cell which is driven by integrins and the cytoskeleton. Lamellipodia which are actin-dependent flat protrusions from the cell, are formed at the anterior end. Constant vesicle transport takes place that moves lipid membrane from the golgi apparatus to the lamellipodia so that these can continue to extend; this transport is controlled by PKC-ζ. At the rear of the cell a structure forms called the uropod, which is enriched in adhesion molecules (Imhof and Aurrand-Lions, 2004). Different types of junctions have been described that maintain integrity of the endothelium and in order to migrate between adjacent

endothelial cells these junctions need to be breached. Some of the proteins involved in the formation of such junctions also play a role in monocyte migration by binding to integrins, these proteins include junctional adhesion molecules (JAMs), platelet/endothelial cell-adhesion molecule 1(PECAM1, CD31) and CD99 (Imhof and Aurrand-Lions, 2004).

5. MICROBIAL RECOGNITION BY MACROPHAGES

To eliminate pathogens macrophages, like other cells from the immune system, need to be able to distinguish self from non-self. The non-opsonic recognition of microbes is mediated by pattern recognition receptors (PRR), which recognise highly conserved molecules expressed by microbes referred to as pathogen associated molecular patterns (PAMPs) (Janeway, 1989). PAMPs are generally important for survival of the microbes and include LPS which is part of the cell wall of Gram negative bacteria, lipoteichoic acid (LTA) as part of Gram positive bacteria, mannans and β-glucans that are mainly found in the fungal cell wall and also in plants and some bacteria. Other receptors are able to sense double stranded RNA or foreign DNA of microbes (Janeway and Medzhitov, 2002).

The range of PRRs encompasses soluble serum factors, membrane bound (surface) receptors and intracellular proteins. Their functions include phagocytosis, opsonisation, activation of pro-inflammatory signalling, activation of complement and co-agglutination cascades and induction of apoptosis (Janeway and Medzhitov, 2002). Serum proteins like mannan binding lectin (MBL), C-reactive protein (CRP) and serum amyloid protein serve as opsonins for microbes. They are secreted by the liver and bind a broad range of pathogens, which leads to activation of the complement pathway and enhance phagocytosis. These soluble PRR will be discussed further in section 2 of this book. Several PRR are expressed on the surface of phagocytes (Table 1); their microbial recognition can be very specific, like Dectin-1 which binds to exposed β-glucans, or very broad like the scavenger receptors. Recognition by these receptors can lead to binding to phagocytes, phagocytosis and stimulation of a pro-inflammatory response. Toll-like receptors (TLR) form another family of membrane bound PRR. A broad array of microbes can be detected by TLRs and recognition leads to pro-inflammatory signalling. Cells also have cytosolic PRR, these include the nucleotide-binding oligomerisation domains (NODs) that recognise bacterial cell wall components, leading to apoptosis and secretion of pro-inflammatory cytokines.

Besides recognising foreign objects some PRR are also known to recognise host derived ligands. For instance, Dectin-1 has an unidentified ligand on T cells (Ariizumi et al., 2000), DC-SIGN (DC-specific ICAM-3 grabbing non-integrin) recognises intercellular adhesion molecule (ICAM)-2 and ICAM-3 (Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000b) and MR binds endogenous glycoproteins such as myeloperoxidase and lysosomal hydrolases (Shepherd and Hoidal, 1990).

Table 1. Pattern recognition receptors expressed at the macrophage surface

Facility	Member(s)	Selected microbial ligands
Classic C-type lectins	Mannose receptor	*C. albicans, P. carinii, M. tuberculosis, K. pneumoniae, Leishmania donovani*, HIV-1, zymosan
	DC-SIGN	HIV, Ebola virus, *Leishmania* spp.
Non-classic C-type lectins	Dectin-1	β-glucans, zymosan, *S. cerevisiae, C. albicans*
Leucine-rich repeats containing proteins	CD14	*E. coli*, LPS, LTA, peptidoglycan
	Toll-like receptors	LPS, LTA, zymosan, bacterial lipoproteins, peptidoglycan, viral proteins, flagellin, bacterial DNA
Scavenger receptors	SR-A (I and II), LOX-1, MARCO	*E. coli, S. aureus, L. monocytogenes, M. tuberculosis, Enterococcus faecalis, N. meningitidis*, LPS, LTA, bacterial DNA
Integrins	CR3, CR4	Complement coated microbes, LPS, LPG, *C. albicans, M. tuberculosis, C. neoformans*

6. TOLL LIKE RECEPTORS

The Toll receptor, previously shown to be important in development, was first found to be involved in immune defence against fungi in *Drosophila* (Lemaitre et al., 1996). Soon after mammalian homologues were characterised, and to date the TLR family is known to contain at least 11 members. Monocytes and macrophages express mRNA for most TLRs except perhaps TLR3 (Muzio et al., 2000). TLR1, TLR2 and TLR4 can be found on the surface of cells while TLR7, TLR8 and TLR9 are expressed on intracellular membrane compartments, including endosomes and endoplasmic reticulum (Ahmad-Nejad et al., 2002; Heil et al., 2003; Latz et al., 2004; Matsumoto et al., 2003). It has been proposed that intracellular compartments may be the main site for TLR recognition of microbial components (Takeda and Akira, 2005) including TLR2 which is also expressed on the surface (Underhill et al., 1999).

Microbial recognition by TLR leads to homo- or hetero- dimerisation of TLR. Upon ligation TLRs activate MyD88 -dependent and -independent signalling pathways (Akira, 2003), triggering expression of different genes that can induce many processes, including cytokine production (eg. TNFα and IL-12), nitric oxide production, actin re-organisation (West et al., 2004), phagocytosis (Doyle et al., 2004), phagosome maturation (Blander and Medzhitov, 2004; Doyle et al., 2004; Shiratsuchi et al., 2004) and induction of apoptosis. TLRs collaborate with other

pattern recognition receptors, to produce a response specific to the microbe (Underhill, 2003). The role for TLR in recognition of fungi will be discussed in chapter 11.

7. PHAGOCYTOSIS

Endocytosis is the process of taking up components of the extracellular environment, this includes pinocytosis which is the uptake of soluble molecules and small particles like viruses, a process which can be clathrin-dependent, but is independent of actin. Uptake of larger ($> 0.5\,\mu$m) particles is mediated via an actin-dependent process, called phagocytosis (Fig. 2) (Aderem and Underhill, 1999). General features of phagocytosis include receptor ligation, which results in receptor clustering, to mediate particle binding and downstream signalling. This leads to actin-based membrane motility that forms the membrane around the particle resulting in a phagosome. Actin depolymerises once the phagosome is formed, which enables the phagosome to mature by a series of fusion and fission events with endosomes and later lysosomes, forming a phagolysosome (Aderem and Underhill, 1999). During maturation of the phagosome, the pH drops and oxygen-dependent and -independent killing mechanisms are activated.

Macrophages are professional phagocytes that express a series of phagocytic receptors. The variety of receptors expressed by macrophages greatly increases the range of particles that can be phagocytosed and also provides cytosolic

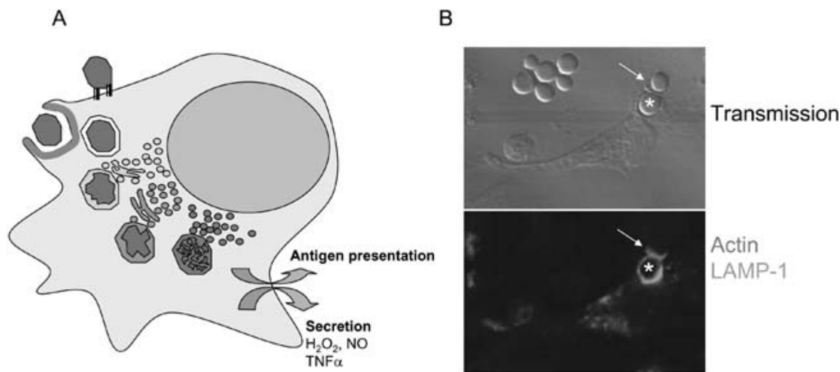

Figure 2. Phagocytosis. A) Schematic representation of phagocytosis. Engaging of phagocytic receptors leads to particle binding and signalling for cytoskeletal rearrangements. This leads to the formation of a so called phagocytic cup that results in membrane engulfment of the particle. Once a phagosome is formed it matures by fusion and fission with early endosomes, late endosomes and lysosomes, sequentially. During this process the pH of the phagosome is lowered from 6 to around 4.5. Phagocytic signalling cascades also stimulate secretion of H_2O_2, NO and TNFα. B) *Candida albicans* phagocytosis by RAW264.7 macrophages. The cells were stained for actin with TRITC phalloidin (red) and for endosomes and lysosomes with LAMP-1 (green). The first yeast is taken up in a phagosome where the lysosomes have fused (asterisk). The second yeast is in the process of being phagocytosed and there is a clear phagocytic cup present (arrow) (See Color Section.)

Table 2. Examples of macrophage phagocytic receptors (modified from (Greenberg, 1995))

Opsonin-dependent receptors	Opsonin-independent receptors
$Fc_{\gamma}R$ I, IIA, IIIA	Complement receptor 3 (CR3)
Complement receptors 1 and 3 (CR1, CR3, CR4)	Macrophage mannose receptor
IgA receptor ($Fc_{\alpha}R$)	β1 integrins
High affinity IgE receptor ($Fc_{\varepsilon}RI$)	Dectin-1
Low affinity IgE receptor (CD23, $Fc_{\varepsilon}RII$)	SIGN-R1/DC-SIGN family
Vitronectin receptor ($\alpha_v\beta 3$)	Macrophage scavenger receptors

signalling that couples uptake to effector responses. Macrophages are able to recognise particles with more than one receptor, leading to cross-talk and synergy of the downstream signalling. This is a complex process that enables macrophages to produce a response appropriate to the ingested particle (Aderem and Underhill, 1999; Stuart and Ezekowitz, 2005). Several macrophage receptors have been suggested to mediate binding and ingestion of particles, either via opsonin-dependent or -independent recognition (Table 2), FcγR and CR3 mediated phagocytosis will be discussed below.

7.1. FcγR Mediated Phagocytosis

Fc receptors (FcRs) are receptors that recognise the Fc portion of immunoglobulins. They belong to the immunoglobulin-superfamily of proteins and consist of an α chain associated with a signaling chain, namely the β, ζ or γ (Greenberg, 1999). There are two classes of Fc receptors, one is involved in effector functions while the other transports Ig across epithelial surfaces (Ravetch, 1997). The FcγR can be divided into receptors that activate effector functions, containing immunoreceptor tyrosine-based activation motifs (ITAM) and receptors that inhibit these functions, which have an immunoreceptor tyrosine-based inhibition motif (ITIM) (Ravetch and Bolland, 2001). FcRs that mediate phagocytosis fall within the activation class and include FcγRI (CD64), FcγRIIA (CD32) and FcγRIIIA (CD16) (Garcia-Garcia and Rosales, 2002). Macrophages express these three phagocytic FcγR and also express FcγIIB which negatively regulates phagocytosis via its ITIM motif (Hunter et al., 1998).

FcγR are some of the best understood phagocytic receptors. IgG coated particles cause clustering of FcγR which leads to phosphorylation of tyrosine within the ITAM motif by Src tyrosine kinases (Fitzer-Attas et al., 2000; Suzuki et al., 2000). The phosphorylated ITAMs recruit a range of proteins including Syk kinase (Swanson and Hoppe, 2004; Turner et al., 2000). Syk is essential for the downstream signalling of FcγR phagocytosis (Indik et al., 1995). Macrophages from Syk-deficient mice can polymerise actin into a phagocytic cup, but are unable to complete internalisation of antibody opsonised particles, however they are able to phagocytose latex beads and yeast (Crowley et al., 1997). Blocking phosphatidylinositol 3 kinase

(PI3K) blocks FcγR internalisation at the same stage (Araki, Johnson, and Swanson, 1996; Crowley et al., 1997). PI3K phosphorylation by Syk activates PI3K which leads to phosphorylation at the D-3 position of the inositol ring of phosphatidylinositides (PI). This leads to the production of several signalling molecules that influence processes like phagosome formation, phagosome maturation and NADPH oxidase assembly and activation (Swanson and Hoppe, 2004).

Cdc42 and Rac1 from the Rho family of GTPases play important roles in actin assembly for phagocytosis via FcγR. GTPases are generally in their inactive guanosine 5′-diphosphate (GDP)-bound form in the cytosol and become activated in their guanosine 5′-triphosphate (GTP)-bound form when they also become membrane bound. Guanine nucleotide exchange factors (GEF) mediate the transition from GDP to GTP and different GEF proteins are activated by PI(3,4,5)P3, a product of PI3K (Swanson and Hoppe, 2004). Activation of FcγR mediated phagocytosis is blocked by overexpression of dominant negative forms of either Cdc42 or Rac1 (Cox et al., 1997). Cdc42 and Rac1 have distinct activation patterns and contribute to phagocytosis in different ways. Cdc42 is thought to play a role in actin polymerisation for pseudopod extension (Chimini and Chavrier, 2000). Consistent with this it was shown that Cdc42 localises to the tips of advancing pseudopodia (Hoppe and Swanson, 2004). Furthermore, artificial clustering of Cdc42 near cell bound particles induced actin polymerisation, but not phagocytosis (Castellano et al., 1999). On the other hand, Rac1 is thought to play a role in phagosome closure since artificial clustering of Rac1 induces particle uptake (Castellano, Montcourrier, and Chavrier, 2000; Swanson and Hoppe, 2004). Members of the Wiskott-Aldrich Syndrome Protein (WASP) family function downstream of the Rho GTPases (Chimini and Chavrier, 2000). WASP proteins regulate the actin cytoskeleton through activation of the Arp2/3 complex (Castellano, Chavrier, and Caron, 2001). Rac1 also activates NADPH oxidase activity (Bokoch and Diebold, 2002) which is antagonised by Cdc42 (Diebold et al., 2004).

Another GTPase involved in FcγR mediated phagocytosis is Adenosine 5′-diphosphate-ribosylation factor 6 (ARF6). Macrophages expressing defective ARF6 are unable to phagocytose antibody-opsonised particles (Zhang et al., 1998). Although ARF6 plays a role in actin assembly (Radhakrishna et al., 1999), it is not required for actin polymerisation in FcγR mediated phagocytosis. However, it is important for membrane delivery to the phagosome and may also play a role in NADPH oxidase activation (Niedergang et al., 2003; Swanson and Hoppe, 2004).

Intracellular membrane sources are needed to complete phagocytosis (Greenberg and Grinstein, 2002). Membrane for the new phagosome has been proposed to come from the plasma membrane, endosomes and ER (Bajno et al., 2000; Cox et al., 2000; Garin et al., 2001; Hackam et al., 1998; Muller, Steinman, and Cohn, 1980). During invasion of trypanosomes, lysosomes have also been shown to be a source of membrane (Tardieux et al., 1992). Endosomes are most likely a primary source for this membrane as is shown by toxins that inactivate vesicle-associated membrane protein 3 (VAMP3), a SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) protein found in recycling endosomes that

plays a role in vesicle docking and fusion (Jahn, Lang, and Sudhof, 2003), and which blocks phagocytosis (Braun et al., 2004). Furthermore, fusion of VAMP3-containing vesicles precedes phagosome closure (Bajno et al., 2000). However, Allen et al. showed that antibody-opsonised bead phagocytosis was not impaired in macrophages from VAMP3-deficient mice (Allen, Yang, and Pessin, 2002). Expression of inactive Rab11, a GTPase involved in trafficking and sorting of recycling endosomes, impaired phagocytosis via FcγR (Cox et al., 2000).

Following actin polymerisation, membrane delivery and pseudopod extension the final stage of phagosome formation is phagosome closure. Myosins and PI3K amongst other signalling proteins have been shown to be involved in this last stage of phagosome formation (Swanson et al., 1999). Besides signalling for phagocytosis, FcγR can also stimulate the production of reactive oxygen intermediates and arachidonic acid metabolites and induce the secretion of TNFα, IL-1β, IL-6, chemokines and growth factors (van de Winkel and Anderson, 1991).

7.2. CR3 Mediated Phagocytosis

CR3 is also called Mac-1, Mo-1, αmβ2 or CD11b/CD18 integrin. Besides its ability to phagocytose C3bi-opsonised particles it recognises other endogenous ligands including ECM proteins, collagen, fibrinogen and ICAM-1 and -2 (Plow and Zhang, 1997). CR3 functions also as an adhesion receptor and mediates leukocyte migration. Moreover, CR3 is described to be a pattern recognition receptor able to recognise many ligands including: LPS, lipophosphoglycan (LPG), β-glucans, zymosan and *C. albicans* (Ehlers, 2000; Forsyth and Mathews, 1996; Forsyth, Plow, and Zhang, 1998; Ross, Cain, and Lachmann, 1985; Thornton et al., 1996). CR3 is a member of the β2 integrins that share the CD18 (β2) subunit. These β2 integrins are exclusively expressed by leukocytes. The CD11b subunit contains a C-terminal lectin site, a calcium binding site, an (inserted) I- domain and a small signalling domain (Ross, 2000). The lectin site and the I-domain are both involved in ligand recognition. The I-domain has overlapping, but not identical sites for binding many protein ligands, including C3bi. The lectin site has been shown to bind β-glucan, zymosan and N-acetyl-D-glucosamine (Ross, Cain, and Lachmann, 1985; Thornton et al., 1996). The interaction of *C. albicans* with CR3 is suggested to be mainly mediated by the I-domain, but this recognition is modulated by the lectin site (Forsyth, Plow, and Zhang, 1998).

Unlike FcγR, CR3 needs additional stimuli for the internalisation of particles upon recognition (Pommier et al., 1983; Wright and Silverstein, 1983). These stimuli include PKC activators such as PMA (phorbol 12-myristate 13-acetate), cytokines like TNFα or GM-CSF, microbial products like LPS, ligation of co-receptors such as FcγR, or attachment to a laminin- or fibronectin- coated substratum (Aderem and Underhill, 1999; Underhill and Ozinsky, 2002). Two mechanisms have been described to mediate activation of CR3. First, ligation of co-receptors like FcγR or selectins leads to cytoskeletal rearrangements that release CR3 from its cytoskeletal

constraints leading to CR3 clustering and activation (Jones et al., 1998; Jongstra-Bilen, Harrison, and Grinstein, 2003). The second mechanism involves ligation of G-protein-coupled receptors and is independent of actin reorganisation (Jones et al., 1998; Newton, 1998).

The phagocytic mechanisms of CR3 are different from FcγR mediated phagocytosis. Antibody-opsonised particles are engulfed by lamellipodia that project from the cell surface and tightly cover the particle interacting sequentially with IgG molecules distributed over the particle before it is drawn into the cells; this process is known as the 'zipper' mechanism (Kaplan, 1977; Silverstein, 1995). Particles phagocytosed via CR3 appear to sink into the phagocyte without apparent involvement of membrane extensions (Kaplan, 1977). The membrane is also less tightly apposed with point-like contacts with the particle, separating regions of looser membrane (Allen and Aderem, 1996). These contact areas are rich in cytoskeletal proteins like F-actin and Arp2/3 while these proteins are uniformly distributed on or near the phagosome surface in FcγR mediated phagocytosis (Allen and Aderem, 1996; May et al., 2000). Furthermore, CR3 mediated phagocytosis does not require tyrosine kinase activity, but does need intact microtubules (Allen and Aderem, 1996). Differences in phagocytic mechanisms could be explained by the differential involvement of Rho GTPases since these GTPases stimulate different actin structures (Hall, 1998). Unlike FcγR, CR3 mediated phagocytosis depends on Rho GTPase, but is independent of Cdc42 and Rac1 (Caron and Hall, 1998). However, Le Cabec et al., have also suggested that CR3 can mediate both types of phagocytosis depending on the ligand (Le Cabec et al., 2002).

A third difference between FcγR and CR3 mediated phagocytosis is that CR3 mediated phagocytosis does not automatically induce an oxidative burst and release of arachidonic acid (Aderem et al., 1985; Wright and Silverstein, 1983). This may lead to the use of CR3 as a portal of entry by pathogens. However, as suggested by Ehlers (Ehlers, 2000) the response to an infectious challenge needs to be in proportion to the threat in order to prevent unnecessary tissue damage by overactive macrophages and neutrophils. Therefore, cell activation needs additional signals besides CR3 ligation such as; receptor clustering, which indicates a high ligand density; receptor activation by costimulation by cytokines, chemokines or microbial ligands; or cooperation with other receptors, which indicates the presence of more than one foreign ligand (Ehlers, 2000).

8. PHAGOSOME MATURATION

Phagosome maturation occurs by phagosome fusion and fission with endosomes and later lysosomes, leading to the formation of a phagolysosome (Desjardins et al., 1994). The rate of phagolysosome fusion is likely to depend on the nature of the ingested particle. Both microtubules and the actin cytoskeleton are involved in phagosome maturation. Rab proteins and SNARE proteins are specific to the different organelles of the endocytic pathway and mediate the fusion of these vesicles. The endosomal compartment is a dynamic network of

tubular and vesicular membrane structures that can be divided into early and late endosomes. Early endosomes have a pH arround 6, contain small amounts of proteases and can be distinquished using specific markers like early endosomal antigen 1 (EEA1) and Rab5 while late endosomes have a slightly higher pH and contain more hydrolytic enzymes. Specific markers for late endosomes include mannose-6-phosphate receptor, Rab7 and Rab9. Lysosomes are vesicles that contain the majority of lipases and hydrolases and have a pH below 5. Lysosomal markers like lysosomal associated proteins (LAMPs) and cathepsin D can also be found in late endosomes. A method used to specifically label lysosomes includes a pulse of fluid-phase markers (e.g. fluorochrome-conjugated dextrans) followed by a long chase that localises the endocytosed marker to the lysosomes (Vieira, Botelho, and Grinstein, 2002).

As the phagosome matures it acquires markers that are specific for the organelles it is interacting with, the assembly of an ATPase complex mediates acidification and the pH lowers from neutral to around 4.5. The acidic environment affects pathogen growth, stimulates NADPH oxidase assembly and creates an optimal environment for hydrolytic enzyme activity. The phagosomal pH is also suggested to play a role in phagosome maturation (Vieira, Botelho, and Grinstein, 2002). Other regulators of phagosome maturation include calcium and different phosphoinositides. Phosphoinositides (PI) is the collective name for phosphorylated derivatives of phosphatidylinositol. PIs are membrane bound and comprise less than 10% of the total cellular phospholipids. However, these lipids are important signalling molecules in receptor-mediated signal transduction, actin remodelling and membrane trafficking (Downes, Gray, and Lucocq, 2005; Matteis, 2004). A total of eight different PIs can be produced by different combinations of phosphate groups arranged around the inositol ring. Organelle specific PI kinases and PI phosphatases mediate rapid subcellular distribution of specific PIs. This leads to recruitment, binding and activation of effector proteins that mediate downstream signalling.

The NADPH oxidase assembly at the phagosome results in the production of reactive oxygen species that are believed to mediate killing. The complex consists of six subunits that include membrane bound phagocytic oxidase subunits: gp91*phox*, p22*phox* which form flavocytochrome b upon activation, a Rho guanosine triphosphatase (GTPase), usually Rac1 or Rac2, and cytosolic subunits p40*phox*, p47*phox*, and p67*phox* that are recruited and assemble into the full complex. Upon activation, the NADPH oxidase catalyses the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2{}^- + H^+$$

The superoxide anions play a prominent role in oxygen dependent microbial killing; moreover O_2^- can be dismutated to hydrogen peroxide (H_2O_2), either spontaneously or by the antioxidant enzyme superoxide dismutase, and H_2O_2 may subsequently be converted into a variety of active oxygen species, including hydrogen peroxide and hydroxyl radicals. Another important enzyme involved in microbicidal activity is inducible nitric oxide synthase (iNOS) which catalyses the production of NO

from arginine, oxygen and NADPH. Ca^{2+} signalling is involved in activation of this enzyme. Subsequent conversion into nitric oxide radicals leads to the production of other microbicidal agents like peroxynitrite, NO_2 radicals, NO_2Cl and N_2O_3.

Recently Reeves et al. has shown that NADPH oxidase also mediates killing in a different way. They show that the ions pumped into the phagosome by the oxidase create a charge across the membrane. This charge is corrected by an influx of K^+ ions. The high pH and the high level of ions lead to activation of proteases that kill the microbes (Segal, 2005). Other killing mechanisms include enzymes like different proteases and lysozyme, antimicrobial peptides like defensins and metabolic competitors like lactoferrin that binds iron.

9. ANTIGEN PRESENTATION

As a professional antigen presenting cell the macrophage is able to load degraded material from the endocytic pathway onto major histocompatibility complex II molecules for presentation to primed T-cells leading to T-cell activation.

MHC II molecules consist of two noncovalently associated, transmembrane subunits. A third component called the nonpolymorphic invariant chain acts as a chaperone, blocking the antigen binding site and is added during production of the MHCII molecules in the ER. The MHCII molecule is sorted to endosomes where the invariant chain is degraded exposing the binding groove for antigen loading. Antigen loaded MHCII travels to the cell surface where, in combination with costimulatory molecules such as CD40, it is able to activate helper-T cells. In turn, the activated T-cells produce IFN-γ which activates the macrophages, including their antigen presentation pathways. Another molecule that presents antigens from the endocytic pathway is CD1, which presents lipid and glycolipid antigens.

10. SECRETORY RESPONSE

After recognition of microbes, macrophages are able to secrete products like NO, H_2O_2, eicosanoids, cytokines, complement proteins and antimicrobial enzymes like lysozyme. Activated macrophages secrete pro-inflammatory cytokines like IL-1, IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1), IFNα/β and TNF-α. IL-8 and MCP-1 mediate recruitment of inflammatory cells. IL-1 and IL-6 can circulate and play a role in regulating host defence mechanism including, fever, hepatocyte acute phase protein synthesis and catabolism of muscle, fat and connective tissue (Gordon, 1999). IL-1, IFNα/β and TNFα regulate activation of leukocytes and have an autocrine effect on macrophages. Macrophages can also secrete anti inflammatory cytokines, like IL-10 and TGFβ, that suppress inflammatory and immune responses, including T-cell proliferation.

IL-1β and IL-18 production can be activated by a complex called the inflammasome, this intracellular complex consists of NALP (NACHT-, LRR- and pyrin domain (PYD) containing proteins) and caspases. Inflammasome activation can be triggered by hypotonic stress or by recognition of muramyl dipeptide (Martinon

and Tschopp, 2005). Muramyl dipeptide is a degradation product of peptidoglycan, a major component of bacterial cell walls.

11. APOPTOSIS

Cell death occurs either through necrosis or through programmed cell death. Necrosis is caused by cell damage due to environmental stress which leads to uncontrolled release of cellular content resulting in immunological activation. Programmed cell death requires active signals through cascades that control different stages of cell death and prevent uncontrolled leakage out of the cell. Programmed cell death has been suggested to include apoptosis, pyroptosis, oncosis, and autophagy (Fink and Cookson, 2005), and will be described further below.

Apoptosis is programmed cell death that is mediated by the activation of certain caspase signalling cascades. The group of caspases involved in apoptosis can be divided into initiator caspases and effector caspases. The apoptosis initiator caspases include caspase -2, -8, -9 and -10 and are activated by binding of adaptor molecules. Downstream signalling leads to activation of the effector caspases which include caspase -3, -6 and -7. Effector caspase activation is mediated by proteolytic cleavage leading to formation of active heterotetramers that can cleave selected target proteins. Apoptosis can be triggered by either stimulation of certain receptors, including TNF receptors and Fas, leading to caspase 8 activation, or by mitochondrial release of cytochrome c leading to activation of caspase 9 (Fink and Cookson, 2005).

Downstream signalling leads to the classic signs of apoptosis. Caspase proteolytic cleavage of an inhibitor of CAD (caspase activated DNase) results in activation of this enzyme that mediates DNA cleavage, producing DNA fragments of around 180 base pairs. Caspases also cleave scaffolding proteins of the nuclear envelope which results in nuclear shrinkage. Detachment of apoptotic cells is caused by cleavage of components of focal adhesion complexes. Gelsolin, an enzyme that depolymerises actin, is also activated by caspases and this causes blebbing of the plasma membrane. The localisation of phosphatidylserine at the outer leaflet of the plasma membrane is also suggested to be mediated by caspases. The exposure of phosphatidylserine at the surface of apoptotic cells is recognised by phagocytic cells and phagocytosis of apoptotic cells, unlike that of microbes, leads to stimulation of an anti-inflammatory response by the phagocytes.

Many pathogens are known to induce apoptosis of macrophages, including *Salmonella* and *Shigella*. However, macrophages undergoing apoptosis have been suggested to kill intracellular mycobacteria. The ability of *Mycobacterium tuberculosis* to prevent macrophage apoptosis has been demonstrated as a virulence factor and a number of mechanisms have been suggested (Fairbairn, 2004), including enhancement of soluble TNF receptor release preventing TNF receptor stimulation on macrophages (Fratazzi et al., 1999), upregulation of signalling pathways involved

in cell survival (Maiti, Bhattacharyya, and Basu, 2001), and stimulation of Mcl-1 production, an anti-apoptotic protein (Sly et al., 2003).

Pyroptosis ("fiery death") has been described as a pro-inflammatory programmed cell death that is initiated by activation of caspase-1 (Fink and Cookson, 2005). *Salmonella* and *Shigella* can induce pyroptosis in macrophages. Oncosis requires a signalling pathway that does not involve caspases. Signs of oncosis includes cell swelling, rapid plasma membrane breakdown and swollen nuclei without internucleosomal DNA fragmentation; this can be induced in macrophages by *Pseudomonas aeruginosa* infection.

11.1. Autophagy

Autophagy is a mechanism for degrading the cell's own proteins and organelles. The formation of double membrane structures, called autophagosomes, is characteristic of autophagy and functions to isolate the cytoplasmic constituents including mitochondria, endoplasmic reticulum and ribosomes. Fusion with lysosomes mediates degradation of the autophagosomal content. Autophagy plays a role in many processes including; homeostatic functions, production of nutrients during starvation, and killing of intracellular pathogens. Autophagy is also suggested to be a form of programmed cell death, differing from apoptosis because the signalling is caspase independent and characteristics of apoptosis, such as DNA fragmentation and loss of cell shape are not observed until very late into cell death (Levine and Yuan, 2005).

Autophagy also plays a role in the fight against pathogenic microorganisms including viruses and bacteria (Kirkegaard, Taylor, and Jackson, 2004). For instance, *M. tuberculosis* interferes with phagosome maturation creating a safe niche for replication. However, induction of autophagy resulted in myco-bacterium phagosome maturation and inhibition of mycobacterium survival (Gutierrez et al., 2004).

12. MACROPHAGE EVASION MECHANISMS

Pathogens have evolved several mechanisms to prevent killing by macrophages. Following uptake, some pathogens (eg. *Shigella* and *Listeria*) break out of the phagosome and replicate in the cytoplasm, where they use host cell actin for intracellular movement. Other pathogens (*Legionella*, *Salmonella* and *Mycobacteria*) control phagosome fusogenicity, arresting phagosome maturation at different stages. *Coxiella burnetti* and *Leishmania* species are members of a small group of pathogens that reside in mature vacuoles and depend on vacuole acidification for replication.

Fungal pathogens have also developed mechanisms to prevent killing. For instance, *Histoplasma capsulatum*, a facultative intracellular fungus, is found in soil, but can cause respiratory infections upon inhalation, especially in the immuno-compromised host. In the lung *H. capsulatum* is phagocytosed by alveolar

macrophages. The phagosomes fuse with endosomes, but not with lysosomes and the pH stays around 6. *H. capsulatum* replicates inside these phagosomes, eventually killing the macrophages and spreading infection (Newman, 1999). *H. capsulatum* interaction with the immune system is discussed in more dept in chapter 18. The mechanisms involved in the inhibition of phagosome maturation and acidification are unknown. However, it is known that pH 6 is maintained in the phagosome independent of V-ATPase (Newman, 1999). Macrophage activation with IFN-γ mediates killing of *H. capsulatum* probably by an increase in reactive nitrogen intermediates (Newman, 1999).

Another fungal pathogen, *Cryptococcus neoformans*, has been recovered from multiple environmental sources including soil and avian excreta. A major virulence factor of *C. neoformans* is its capsule. This capsule masks potential receptor ligands on the cell wall and is known to inhibit phagocytosis and killing by macrophages (Del Poeta, 2004). The capsule also reduces the respiratory burst, downregulates protective cytokine production and upregulates production of the Th2 cytokine IL-10 (Shoham and Levitz, 2005). More about this pathogen can befound in chapter 17.

Several reports have shown mechanisms used by *Candida albicans* to escape phagocytosis and killing by macrophages. *C. albicans* phospholipomannan, a surface glycolipid that is shed by *C. albicans*, was shown to mediate escape from macrophages by inducing apoptosis (Ibata-Ombetta et al., 2003a; Ibata-Ombetta et al., 2003b). It has also been shown that a soluble factor from *C. albicans* suppresses nitric oxygen production, but does not stimulate the production of immunosuppressive cytokines (IL-10 and TGF-β) (Chinen et al., 1999). Furthermore, *C. albicans* β-1,2-linked mannooligosaccharides, which are part of the *C. albicans* cell wall, have been shown to be involved in adhesion to macrophages (Fradin et al., 1996) and inhibit NO and TNFα production (Jouault et al., 2000). Once inside the macrophage, it adapts to oxidative stress and starvation and induces morphological changes; at a later stage when hyphal growth enables escape from the macrophages, it activates glycolysis and downregulates stress responses (Lorenz, Bender, and Fink, 2004). Host defence against *C. albicans* is discussed further in chapter 16.

13. MACROPHAGE INTERACTIONS WITH FUNGI

Fungi can be phagocytosed either after opsonisation with antibodies and complement or following recognition by pattern recognition receptors. Glucans and mannosylated proteins are major components of the fungal cell wall and are important PAMPs. Many macrophage receptors have been suggested to be involved in binding of fungi through recognition of these PAMPs, including scavenger receptors, CR3, mannose receptor, dectin-1 and SIGN-R1. These receptors will be discussed further in chapter 12 and 13.

Macrophages interaction with fungi is complex and, as mentioned by Vazquez-Torres et al. (Vazquez-Torres and Balish, 1997), the exact mechanism and efficiency of fungal killing are likely to depend on the following factors:

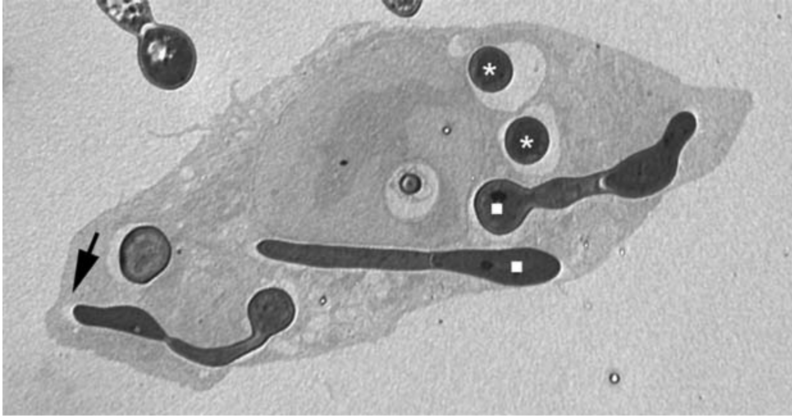

Figure 3. Electron micrograph of a thioglycollate elicited peritoneal macrophage 2 hours after *C. albicans* yeast phagocytosis. The macrophage nucleus is distinguished by the electron dense matter surrounded by a membrane in the centre of the cell. The yeasts (*) are able to grow filaments (■) inside the phagosome, and break out of the macrophage (arrow). This electron micrograph was produced at the Sir William Dunn School of Pathology with the assistance of Michael Hollinshead. Taken from Heinsbroek et al., Trends in Immunology, 2005

- Macrophage source: Human macrophages are efficient in killing *C. albicans*, while *C. albicans* is able to escape from mouse macrophages by growing hyphae that break out of the cell, releasing *C. albicans* into the extracellular environment (Kaposzta et al., 1999) (Fig. 3).
- Macrophage environment: Macrophage receptors are expressed at different levels depending on the tissue environment e.g. alveolar macrophages express high levels of Dectin-1, but not CR3. Moreover, different resident tissue macrophages such as Kupffer cells do not generate as much superoxide anion as peritoneal macrophages (Vazquez-Torres and Balish, 1997).
- State of activation: In contrast to unstimulated macrophages, IFNγ stimulated macrophages produce peroxynitrite, which has a high candidacidal activity (Vazquez-Torres and Balish, 1997). IFNγ-activated macrophages have also been shown to have enhanced anticryptococcal activity (Mody et al., 1991).
- Fungal pathogenicity and morphology: Pathogenic strains of *H. capsulatum* are able to deactivate the macrophage oxidative burst (Ikeda and Little, 1995). *C. albicans* hyphae have been shown to be more resistant to macrophage killing (Vazquez-Torres and Balish, 1997). Moreover, *C. albicans* hyphae are not recognised by Dectin-1 while dectin-1 is able to mediates phagocytosis of *C. albicans* yeast (Gantner, Simmons, and Underhill, 2005).

14. CONCLUSION AND REMAINING ISSUES

Macrophages are important cells in the host resistance to fungal pathogens. Besides their role as professional phagocytes, clearing particles from the body, these cells are also important regulators of the immune system. The interaction between

macrophages and fungi is complex, only recent research has started to unravel the molecules involved in recognition. Single receptors have been described to recognize fungi and regulate subsequent macrophage responses including phagocytosis, killing, antigen presentation, and secretion of immune modulators. Different receptors have been shown to collaborate for an optimal macrophage response, this is the start of further research to identify all the receptors involved and the exact interaction between the different signalling pathways. Moreover, fungi have many different PAMPs and are able to change the expression and exposure of these molecules thereby modulating the recognition and subsequent macrophage responses which adds to the complexity of this interaction.

In vitro experiments with macrophages are a good tool for further research. In comparison with other phagocytes, macrophages are easy to collect and assays are readily available to assess macrophage phagocytic and secretory responses. Improved methods are required to analyse cellular responses and host pathogen interactions in vivo.

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CHAPTER 2

DENDRITIC CELLS

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Abstract: In the past decade, a dramatic shift has occurred in our mechanistic understanding of immunity to fungi. It has became apparent that understanding how innate immune responses are activated will result in the construction of better vaccines and immunomodulatory strategies that are effective at eliciting protective immunity to fungi. The model has brought dendritic cells (DCs) to center stage as promising targets for immunotherapy intervention, and vaccine development and has shifted the emphasis from the "antigen" towards the "adjuvant". DCs function at three levels in the manipulation of the immune response to fungi. First, they mount an immediate or innate response to them by producing inflammatory mediators upon capture and phagocytosis; second, through these preceding innate functions, they decode the fungus-associated information and translate it in qualitatively different T helper (Th) responses, and third they have a key role in containing and dampening inflammatory responses by tolerization through the induction of regulatory T cells (Treg). This chapter will highlight how the remarkable functional plasticity of DCs in response to fungi can be exploited for the deliberate targeting of cells and pathways of cell-mediated immunity in response to fungi and candidate fungal vaccines

Abbreviations: AEDS: atopic eczema/dermatitis syndrome; CRs: complement receptors; DCs: dendritic cells; CTLA-4: cytotoxic T lymphocyte antigen-4; FcR: Fc receptors; HSCT: hematopoietic stem cell transplantation; IDO: indoleamine 2,3-dioxygenase; IL: interleukin; lDCs: lymphoid dendritic cells; MAPK: mitogen-activated protein kinases; mDCs: myeloid dendritic cells; MHC: major histocompatibility complex; MR: mannose receptors; MyD88: Drosophila myeloid differentiation primary response gene 88; NK: natural killer cells; PAMPs: pathogen-associated molecular patterns; pDCs: plasmacytoid dendritic cells; PKC: protein kinase C; PP: Payer's patches; PRRs: pattern recognition receptors; Th: T helper; Treg: regulatory T cells; TLRs: Toll-like receptors

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1. INTRODUCTION

Human beings are continuously exposed to fungi, yet they rarely get fungal diseases. Although not unique among infectious agents, fungi possess complex and unusual relationships with the vertebrate immune system, partly due to some prominent features (Romani 2001), among these, their ability to exist in different forms and to reversibly switch from one to the other in infection. Because cycling between different morphotypes is not obligatory for fungi as it is for other organisms, morphological transition is a mechanism fungi have evolved to adapt to different environments. This may explain why, although associations between morphogenesis and virulence have long been presumed for fungi that are human pathogens (Rooney and Klein 2002), no molecular data unambiguously establish a role for fungal morphogenesis as a virulence factor (Gow et al. 2002). What fungal morphogenesis implicates, through antigenic variability, phenotypic switching, and dimorphic transition, is the existence of a multitude of recognition and effector mechanisms to oppose fungal infectivity at the different body sites (Romani and Kaufmann 1998; Romani 2004).

The need for most fungi is a stable host-parasite interaction that is achieved upon the implicit agreement that the elicited immune response be strong enough to allow host survival without pathogen elimination and to establish commensalism/persistency without excessive pro-inflammatory pathology. Therefore, the balance of pro-inflammatory and anti-inflammatory signaling is a prerequisite for successful host/fungus interaction. In light of these considerations, the responsibilities for virulence is shared by the host and the fungus at the pathogen-host interface, regardless the mode of its generation and maintenance. Studies with *Candida albicans* have provided a paradigm that incorporates contributions from both the fungus and the host to explain the theme of the origin and maintenance of virulence for pathogens and commensals (Romani et al. 2002). Through a high degree of flexibility, the model accommodates the concept of virulence as an important component of fungus fitness in vivo within the plasticity of immune responses orchestrated by dendritic cells (DCs). Conceptually, this implies that the qualitative development of adaptive response to a fungus may not primarily depend on the nature of the fungal form being presented but rather on the type of cell signaling initiated by the ligand/receptor interaction in DCs. Therefore, the functional plasticity of DCs at the pathogen/host interface may offer new interpretative clues to fungal virulence.

2. IMMUNITY TO FUNGI

Protective immunity against fungal pathogens is achieved by the integration of two distinct arms of the immune system, the innate and adaptive (or antigen-specific) responses (Romani 2004). The majority of fungi are detected and destroyed within hours by innate defense mechanisms (Herring and Huffnagle 2001; Romani 2004). Most of the innate mechanisms are inducible upon infection and

their activation requires specific recognition of invariant evolutionarily conserved molecular structures shared by large groups of pathogens (also known as PAMPs, pathogen-associated molecular patterns) by a set of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (McKnight and Gordon 2000; Gordon 2002; Netea et al. 2004; Roeder et al. 2004; Levitz 2004). Antigen-independent recognition of fungi by the innate immune system leads to the immediate mobilization of immune effector and regulatory mechanisms that provide the host with three crucial survival advantages: rapid initiation of the immune response (both innate and adaptive) and creation of the inflammatory and co-stimulatory context for antigen recognition; establishment of a first line of defense, which holds the pathogen in check during the maturation of the adaptive response; and steering of the adaptive response towards the cellular or humoral elements that are most appropriate for protection against the specific pathogen. Therefore, the goal to achieve the optimal activation of the antigen-specific immunity cannot be achieved without effectively activating the pathogen-detection mechanisms of the innate immune response (Romani 2004).

In vertebrates, however, if the infectious organism can breach these early lines of defense an adaptive immune response will ensue, with generation of antigen-specific T helper (Th) effector and B cells that specifically target the pathogen and memory cells that prevent subsequent infection with the same microorganism. There is extensive plasticity in the T-cell response to fungi (Romani 2004). The flexible program of T cells leads to the production of many mediators, including cytokines. Due to their action on circulating leukocytes, the cytokines produced by fungus-specific T cells are instrumental in mobilizing and activating antifungal effectors, thus providing prompt and effective control of infectivity once the fungus has established itself in tissues or spread to internal organs. To limit the pathologic consequences of an excessive inflammatory cell-mediated immune reactions, the immune system resorts to a number of protective mechanisms, including the reciprocal cross-regulatory effects of Th1- and Th2-type effector cytokines, such as interferon (IFN)-γ and interleukin (IL)-4, and the generation of IL-10-producing regulatory T cells (Treg) capable of finely tuning antifungal inflammatory and Th reactivity (Romani 2004). Therefore, host resistance to fungi seems to depend on the induction of innate and adaptive cellular immune responses that are intimately linked and controlled by sets of molecules and receptors that act to generate the most effective form of immunity for protection against fungal pathogens Fig. 1. The dichotomous Th-cell model has proven to be a useful construct that shed light on the general principle that diverse effector functions are required for eradication of different fungal infections.

3. DENDRITIC CELLS: IMMUNOBIOLOGY

The DC system, first discovered by Ralph Steinman and Zanvil Cohn in 1973 (Steinman and Cohn, 1973), comprises a network of different subpopulations. DCs show a unique functional duality during their development, designed to ultimately

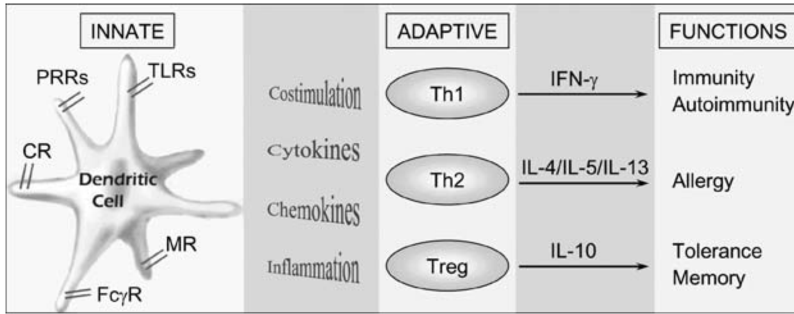

Figure 1. Th1/Th2/Treg polarization by dendritic cells in fungal infections. Essential to the successful removal of fungal pathogens is the early recognition of fungi by the innate immune system. Dendritic cells express numerous pathogen recognition receptors which enable them to sense distinct microbial stimuli, and they process this information and elicit distinct functional responses that induce different T-cell responses. DCs that produce IL-12 p70 stimulate protective Th1 responses. Those that produce IL-4 may yield allergic Th2 responses, and those that produce IL-10 may induce Treg implicated in tolerance and memory to fungi. PRRs, pattern recognition receptors; TLRs, Toll-like receptors, Th, helper T cells, Treg, regulatory T cells (See Color Section.)

provide secondary lymphoid tissues with useful information about the antigenic composition in the periphery. DCs are generated from either myeloid or lymphoid bone marrow progenitors through intermediate DC precursors that home to sites of potential antigen entry, where they differentiate locally into immature DCs. They are strategically located at epithelial barriers that often serve as major portals of pathogen entry. DCs avidly internalize non-opsonized pathogens by macropinocytosis, phagocytosis or through C-type lectins and mannose receptors (MRs) or complexes of antibody and microbial antigen via receptors for the Fc portion of immunoglobulins (FcRs). By virtue of their high phagocytic and endocytic capacities, DCs constitutively internalize samples of their antigenic microenvironment, which in the event of infection, will also include microbial antigens. After antigen capture in the presence of maturation signals associated with inflammation or infection, activated DCs undergo a complex maturation process. In infections, DCs become activated and mobilized by either direct recognition of PAMPs by TLRs or indirectly through receptors for inflammatory cytokines/chemokines and T cell products. In vivo this process is paralleled by migration of DCs to T cell-rich areas of lymphoid organs, where they present pathogen-derived information and antigen-derived peptides to antigen-specific T cells and direct their differentiation into Treg or effector cells, including $CD4^+$ Th polarization to secrete different pattern of cytokines. The ability of DCs to influence the pattern of cytokines secreted by T cells represents a critical function which can profoundly influence the final outcome of the immune response to a pathogen. Thus, cytokines secreted by Th1 cells are typically considered necessary for protection against intracellular pathogens and viruses, whereas secreted Th2 cytokines are important for protection

against multicellular nematode parasites (Reis e Sousa et al. 1999; Pulendran et al. 2001; Ardavin et al. 2004; Pulendran 2004).

Several factors appear to influence the ability of DCs to polarize T-cell cytokine responses. These include: **i**) *DC subsets*. DCs can develop along two pathways, myeloid DCs (mDCs, also called conventional DCs) and plasmacytoid DCs (pDCs). Murine lymphoid DCs (lDCs) expressing the CD11c integrin and the CD8α antigen have also been described (Shortman and Liu 2002). DC subsets differ in their phenotype, micro environmental localization, migration potential, PRR expression, responsiveness to microbes, and their capacity to induce and regulate distinct arms of the innate and adaptive immune systems (Banchereau et al. 2000; Pulendran et al. 2001; Pulendran 2004). The ability of a given DC subset to respond with flexible activating programs to the different stimuli as well as the ability of different subsets to convert into each others (Diebold et al. 2003; Zuniga et al. 2004) confers unexpected plasticity to the DC system. IL-10 production by DCs is known to favor Th2 and Treg cell development, and IL-12 production is required for Th1 cell development. Little is known about the intracellular signaling pathways within DCs that underlie differential cytokine production. Although most TLR signaling in DCs is known to prime Th1 responses, it has recently been shown that a delicate balance in signaling through different mitogen-activated kinases (MAPKs) determines IL-10 versus IL-12 production by DCs in response to microbial stimuli (Pulendran 2004; Klechevsky et al. 2005). (**ii**) *Dynamics of DC migration to the lymphoid organ*. Early after initiation of the immune response, large numbers of recently stimulated DCs actively secreting IL-12 and entering into the T-cell areas may preferentially induce Th1 cell priming. In contrast, at a later time, when DC influx decreases, and the surviving DCs in T-cell areas have down-regulated their secretion of IL-12, preferential priming for Th2 and Treg may occur (Lanzavecchia and Sallusto 2001). (**iii**) *Nature of the maturation stimuli*. TLRs function as sensors of microbial infection and play a critical role in the induction of innate and adaptive immune responses. TLR-mediated DC activation and maturation, which are dependent on nuclear factor (NK)-KB and MAPK signaling pathways, lead to up-regulation of MHC and costimulatory molecules, as well as production of cytokines and soluble factors. For instance, bacterial PAMPs induce strong production of IL-12 by DCs, which can be potently boosted by activated T cells through CD40L, other microbial stimuli induce IL-10 production and viral RNAs are potent inducers of IFN-α by pDCs (Pulendran 2004). However, DC maturation can also occur in response to signals from newly activated $CD4^+$ T cells independently of innate priming. T cell-driven DC maturation takes place both in *cis* and in *trans*, affecting all DCs in the microenvironment, irrespective of antigen-specificity. However, IL-12 production by DCs shows an absolute requirement for TLR signals (Spörri and Reis e Sousa 2003). (**iv**) *Microenvironmental factors*. Cytokine production by DCs can be influenced by mediators released in the DC microenvironment. For example, IFN-γ and IL-4 can enhance IL-12 production by activated DCs, while prostaglandins E2 and IL-10 exert an inhibitory effect (Pulendran 2004; Yao et al. 2005).

DCs and T cells can also interact through the formation of an immunological synapse in which T-cell receptors and co-stimulatory molecules congregate in a central area surrounded by a ring of adhesion molecules. Sustained signaling via these synaptic interactions is required in order for the T cell to enter the first cell division cycle (Stoll et al. 2002). It is believed that antigen presentation by DCs to T cells may involve the concerted action of multiple subsets of DCs, each of which contribute a different facet to this process through either a direct (antigen-loaded DCs that migrate to the secondary lymphoid organ could undergo apoptosis or necrosis and be phagocytosed by resident DCs) or indirect (migrating DCs could actively release antigen-bearing vescicle-exosomes-derived from the DC lysosomal compartment, which could then be captured by resident DCs) antigen presentation pathways (Pulendran 2004). The relative contribution of the direct and indirect pathways in anti-microbial immunity has not yet been resolved. In addition, mature DCs can induce Natural Killer (NK) cell activation and B cell differentiation into antibody-forming cells. In contrast, antigen capture in the absence of activation stimuli, as seen in the "steady state" migration, may lead to the induction of T cell tolerance, as a result of antigen presentation by immature DCs in the absence of costimulation (Ardavin et al. 2004). Therefore, DCs are central in the early decision-making mechanisms that result in a given type of immune response and determine the balance between immunopathology and protective immunity generated by host-microbe interactions.

4. DENDRITIC CELLS AT THE HOST/FUNGI INTERFACE

Studies in vivo suggested that DCs had the ability to internalize fungi at the sites of the infection (Bozza et al. 2002; Montagnoli et al. 2003). Soon after the infection, *Candida albicans* yeasts were found inside DCs from the gut and *Aspergillus fumigatus* conidia inside pulmonary DCs. In the case of *Candida*, the fungus appeared to translocate across the epithelial layers and to be subsequently phagocytosed by DCs (unpublished observations). For *Aspergillus*, DCs present in the alveolar spaces phagocytosed conidia, translocated to the space below, within the alveolar septal wall, and reached the draining lymph nodes where fungus-pulsed DCs instructed local development of antifungal Th reactivity (Bozza et al. 2002). It is known that DCs of the respiratory tract are specialized for uptake/processing but not for antigen presentation, the latter requiring cytokine maturation signals that are encountered after migration to regional lymph nodes (Holt et al. 1999). Studies in vitro have shown that both human and murine DCs recognize and internalize a number of fungi, including *A. fumigatus* (Bozza et al. 2002; Grazziutti et al. 2001; Bozza et al. 2003; Bozza et al. 2004; Romani et al. 2004; Serrano-Gomez 2004), *C. albicans* (Bozza et al. 2004; d'Ostiani et al. 2000; Newman and Holly 2001; Bacci et al. 2002; Cambi et al. 2003; Romani et al. 2004; Romagnoli et al. 2004; Torosantucci et al. 2004), *Cryptococcus neoformans* (Syme et al. 2002; Kelly et al. 2005), *Histoplasma capsulatum* (Gildea et al. 2001; Lin et al. 2005), *Malassezia furfur* (Buentke et al. 2000; Buentke and Scheynius 2003) and

Saccharomyces cerevisiae (Stubbs et al. 2001) and that fungi and fungal products may affect DC functioning as well (Romagnoli et al. 2004; Torosantucci et al. 2004; Macagno et al. 2001; Vecchiarelli 2003). Profiling gene expression on DCs by microarray technologies has revealed that both a shared response and a pathogen-specific gene expression program were induced upon the exposure to bacteria, viruses and fungi (Huang et al. 2001). Additional studies with *S. cerevisiae* have shown that recombinant yeast could represent an effective vaccine for the generation of broad-based cellular immune responses (Stubbs et al. 2001). It seems, therefore, that DCs are uniquely able at decoding the fungus-associated information at the host/fungus interface.

4.1. Fungal Recognition by Dendritic Cells and Receptor Cooperativity

PRRs for fungi include receptors for a variety of complement components (CRs), for mannosyl/fucosyl glycoconjugate ligands (MRs), for β-glucan (Dectin-1), C-type lectins, FcRs and TLRs (McKnight and Gordon 2000; Gordon 2002; Netea et al. 2004; Roeder et al. 2004; Levitz 2004; Poulain and Jouault 2004; Steele et al. 2003).

MRs belong to a family of lectins that mediate nonopsonic phagocytosis of fungi (Taylor et al. 2005). MR ligation by fungi can be linked to induction of effector functions, but the link is dependent on activation. C-type lectins bind carbohydrates from pathogens and also self-glycoproteins, and thus they play an important role not only in pathogen sensing but also in cell adhesion and migration. The C-type lectin DC-SIGN is widely expressed on DCs and mediates recognition of several distinct pathogens, such as viruses, bacteria and fungi (Figdor et al. 2002). The common characteristic of these pathogens is that they cause chronic infections in which the Th1/Th2/Treg balance is a critical determinant of pathogen persistency. Dectin-1 is a C-type lectin receptor that mediates attachment and ingestion of zymosan by DCs and other phagocytes (Gantner 2003). Dectin-1 cooperates with TLR2 in the recognition of zymosan, by enhancing TLR2-mediated activation of nuclear factor (NF)-KB and IL-12/TNF-α production (Gantner 2003). TLRs are type I transmembrane proteins that are grouped into the same gene family based on their sequence similarity. Eleven mammalian TLRs have been described so far, and TLR ligands include PAMPs and additional ligands, including endogenous ligands of host origin (O'Neill et al. 2003). The ability of PAMPs to induce costimulatory molecule expression on DCs suggests a permissive role of the PAMP/TLR system in the activation of T lymphocytes during antigen presentation. All TLRs activate a core set of stereotyped responses, such as inflammation. The down-stream signaling pathway utilized by most TLRs involve the recruitment of the adapter protein MyD88 (*Drosophila* myeloid differentiation primary response gene 88) culminating in activation of NF-KB and mitogen activated protein kinases (MAPKs) that activate the transcription of the inflammatory and adaptive immune responses. However, in the case of TLR3- and TLR4-dependent signaling, other proteins may also serve as adapter molecules with or in place of MyD88 (Yamamoto

et al. 2003). Evidence suggests that individual members of the TLR family or other PRRs interact with each other and cumulative effects of these interactions instruct the nature and outcome of the immune response to the provoking pathogen (Mukhopadhyay et al. 2004).

Candida and *Aspergillus* proved to be useful pathogen models to dissect events occurring at the fungus/DC interface. Murine and human mDCs and pDCs internalize *Candida* yeasts, *Aspergillus* conidia and hyphae of both Fig. 2. The uptake of the different fungal elements occurred through different receptors and forms of phagocytosis. Live unopsonized yeasts, conidia or hyphae were mainly internalized through a phagocytic process. Transmission electronic microscopy indicated that internalization of yeasts and conidia occurred predominantly by coiling phagocytosis, characterized by the presence of overlapping bilateral pseudopods which led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional zipper-type phagocytosis, characterized

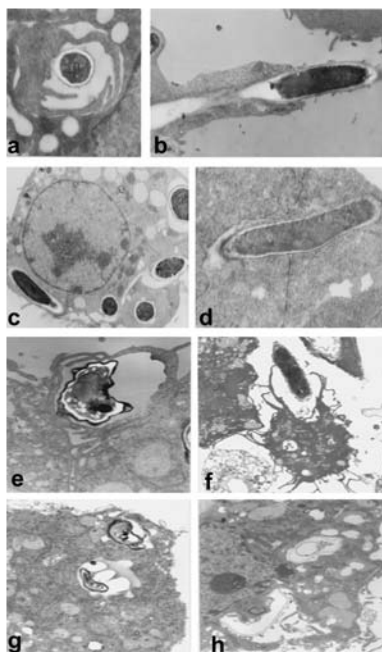

Figure 2. Murine dendritic cells phagocytose different fungal morphotypes. Transmission electron microscopy of phagocytosis of *Candida albicans* or *Aspergillus fumigatus* by dendritic cells. Murine DCs were incubated with *Candida* yeasts (a, c), *Candida* hyphae (b, d) *Aspergillus* conidia (e, g) or *Aspergillus* hyphae (f, h) for 15 min. (a) or 2 hours (b,c,d,e,f,g,h) before processing for transmission electron microscopy. Note the yeasts (a) or conidia (e) engulfment through coiling phagocytosis (magnification $\times 17,000$ in a and $\times 25,000$ in b) the hyphae uptake (b, f) through zipper-type phagocytosis (magnification, $\times 12,000$ in b and $\times 7,000$ in f); the yeasts (c) or conidia (g) inside phagolysosomes (magnification, $\times 7,000$ in c and $\times 12,000$ in g) and the hyphae lying free in the cytoplasm (d, magnification, $\times 12,000$) or being partially degraded (h, magnification, $\times 12,000$)

by the presence of symmetrical pseudopods which strictly followed the contour of the hyphae before fusion. The fate of the different forms of the fungi inside cells appeared to be quite different. Two and four hours later, the majority of *Candida* (d'Ostiani et al. 2000) and *Histoplasma* (Gildea et al. 2001) yeasts were found degraded inside phagosomes. In contrast, as early as one hour after infection, *Candida* hyphae appeared to escape the phagosome and were found lying free in the cytoplasm of cells (d'Ostiani et al. 2000). For *Aspergillus*, two hours after the exposure, numerous conidia were found inside DCs with no evidence of conidia destruction, as opposed to hyphae, that were rapidly degraded once inside cells (Bozza et al. 2002). As killing of conidia would seem to be a necessary prerequisite to obtain efficient antigen presentation, it can be postulated that either a small number of conidia are actually degraded by mature DCs thus allowing their antigen processing and presentation or, alternatively, antigens could be processed and regurgitated by other infected phagocytes and then transferred to DCs for presentation.

Recognition and internalization of unopsonized yeasts and conidia occurred through the engagement of MRs of different sugar specificity, DC-SIGN, Dectin-1 and, partly, CR3 (Serrano-Gomez et al. 2004; d'Ostiani et al. 2000; Newman and Holly 2001; Cambi et al. 2003; Romani et al. 2004). In contrast, entry of hyphae occurred by a more conventional, zipper-type phagocytosis and involved the cooperative action of FcγR II and III and CR3 (Romani et al. 2004). Phagocytosis does not require TLR2, TLR4, TLR9 and MyD88 (Bellocchio et al. 2004). For other yeasts, such as those of *Cryptococcus*, a cooperative interaction between MR and FcγR has been shown (Syme et al. 2002), whereas *Histoplasma* yeasts were found to be phagocytosed through the fibronectin receptor, very late antigen-5 (Gildea et al. 2001). Consistent with the findings that signals from protein kinase C (PKC) and/or protein tyrosine kinases are required for phagocytosis in a variety of systems (Allen and Aderem 1996), the PKC inhibitor staurosporine was required for CR- and FcγR-mediated phagocytosis, while FcγR- and, to a lesser extent, MR-mediated phagocytosis required signaling through protein tyrosine kinases (Claudia et al. 2002). The results are consistent with the view that fungi have exploited common pathways for entry into DCs, which may include a lectin-like pathway for unicellular forms and opsono-dependent pathways for filamentous forms. In terms of sugar specificity, this may vary among fungi, as DCs recognize *Candida* yeasts through a mannose-fucose receptor (Newman and Holly 2001) and *Aspergillus* conidia through a lectin receptor of galactomannan specificity (Serrano-Gomez et al. 2004; Persat et al. 2003). Actually, the sugar specificity of MRs involved in the entry of one or multiple *Aspergillus* conidia turned out to be different, as the entry of multiple conidia occurred through a pathway sensitive to galactomannan and that of one single cell through a pathway sensitive sensitive to β-glucan (Bozza et al. 2002). Therefore, fungal surface polysaccharides have a key role in the DC/fungi interactions. It also appears that unicellular fungal forms may exploit the CR3 receptor on DCs as a niche to avoid degradation through the multilectin pathway while allowing their own persistence (Ehlers 2000).

4.2. Dendritic Cell Activation

The engagement of distinct receptors by different fungal morphotypes translated into downstream signaling events, ultimately regulating cytokine production, costimulation and fungus survival. Entry of *Candida* yeasts or *Aspergillus* conidia through MRs and Dectin-1 resulted in the production of proinflammatory cytokines, including IL-12, up-regulation of costimulatory molecules and histocompatibility Class II antigens. IL-12 production by DCs required the MyD88 pathway with the implication of distinct TLRs (IL-1RI and TLR9 for *Candida* and TLR4 and TLR9 for *Aspergillus*) (Bellocchio et al. 2004; Braedel et al. 2004). These events were all suppressed upon entry through CR3. In contrast, coligation of CR3 with FcγR, as in the phagocytosis of hyphae, resulted in the production of IL-4/IL-10 and upregulation of costimulatory molecules and histocompatibility Class II antigens (Romani et al. 2002; Persat et al. 2003). The production of IL-10 was largely MyD88-independent (Romani et al. 2004; Bellocchio et al. 2004). Therefore, TLRs collaborate with other innate immune receptors in the activation of DCs against fungi through MyD88-dependent and -independent pathways Fig. 3. It is of interest that TLR gene expression on DCs could be affected upon fungal exposure in a morphotype-dependent manner (Bozza et al. 2004) and that the TLR9 agonist CpG-ODN could convert an *Aspergillus* allergen to a potential protective antigen

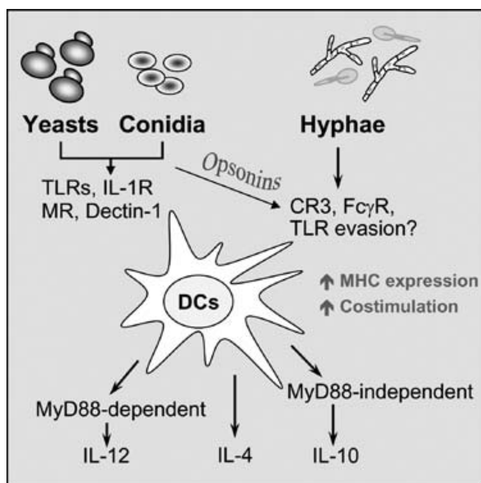

Figure 3. The exploitation of distinct recognition receptors in dendritic cells by the different fungal morphotypes. Dendritic cells sense fungi in a morphotype-dependent manner. The engagement of distinct receptors on dendritic cells translated into downstream signaling events that differentially affect cytokine production. The exploitation of a specific receptor invariably leads to the occurrence of a specific type of T helper cell reactivity. Fungal opsonins may subvert the receptor exploitation by fungal morphotypes. TLRs, Toll-like receptors; IL-1R, IL-1 receptor; MR, mannose receptors; CR3, complement receptor 3; FcγR, receptor for the Fc portion of immunoglobulins; MyD88, Drosophila myeloid differentiation primary response gene 88 (See Color Section.)

(Bozza et al. 2002). These observations points to the potential for TLR agonists to act upon the degree of flexibility of the immune recognition pathways to fungal antigens and allergens.

It is known that MAPKs participate in signal transduction events associated with a number of stimuli, such as mitogens, growth factors, and pathogen-derived products (Chang and Karin 2001). p38 and JNK 1/2 MAPKs are known to regulate IL-12/IL-10 expression on DCs (Pulendran et al. 2001). Our own data suggest that the production of IL-12/IL-10 in response to yeasts and hyphae of *C. albicans* is directly associated with the activation/inhibition of certain MAPKs. The production of IL-12 in response to yeasts was associated with the selective inhibition of extra-cellular signal-regulated kinases (ERK 44/42) while the down-regulated production of IL-12 and up-regulated production of IL-10 in response to hyphae was associated with the activation of ERK 44/42 and the selective inhibition of p38MAPKs (Bonifazi P., personal communication). These data are consistent with the notion that ERK suppresses the induction of IL-12 and enhances IL-10 production in DCs (Pulendran et al. 2001) and parallel similar data obtained with human monocytes exposed to *C. albicans* (Tang et al. 2004).

In terms of survival, fungi were rapidly degraded upon entry through MR or FcγR, a finding in line with the notion that ligation of these receptors is usually sufficient to trigger a vigorous oxidative burst and generation of proinflammatory signals on innate phagocytes (Romani 2004). For CR3, it is of interest that *Candida* exploited this receptor to survive inside DCs (Romani et al. 2002), while *H. capsulatum* used this receptor to survive in macrophages (Long et al. 2003) but not in DCs (Gildea et al. 2001). This is consistent with the observation that CR3 engagement is one most efficient uptake of opsonized fungi but it has the remarkable characteristic of a broad capacity for recognition diverse fungal ligands. In this regard, it is worth mentioning that *C. albicans* possesses fungal molecules mediating interaction with CR3 but avoiding production of nitric oxide (Romani 2004). The multiplicity of binding sites and the existence of different activation states enables CR3 of disparate (both positive and negative) effector activities against fungi (McKnight and Gordon 2000).

A remarkable and important feature of Payer's patches (PP)-DCs is the production of IL-10 in response to *Candida*, an event occurring by signaling through CR3 in the presence of opsonizing antibodies. These IL-10-producing PP-DCs activate $CD4^{+}CD25^{+}$ Treg that negatively affect antifungal Th1 reactivity (Hori et al. 2002; Montagnoli et al. 2002). It is conceivable that tissue-dependent factors and opsonins (see below) may modulate receptor usage by DCs at different body sites, thus contributing to the functional plasticity of DCs at the host/pathogen interface.

It has recently been shown that fungal RNA acts as potent DC activator (Gilboa and Vieweg 2004). Although extracellular mRNA induced DC activation by signaling through a nucleotide receptor (Ni et al. 2002), fungal RNA also activated TLR expression on DCs (Bozza et al. 2004). Upon exposure to fungal RNA, DCs underwent functional maturation, as indicated by the

upregulated expression of costimulatory molecules and MHC class II antigens and cytokine production (Bozza et al. 2003; Bacci et al. 2002).

4.3. Dendritic Cell Conditioning

4.3.1. Opsonins

Fungal opsonins are known to affect the uptake of fungi by phagocytic cells (Romani 2004). Opsonization with mannose binding lectin (MBL), C3 and/or antibodies subverted the receptor exploitation on DCs by the different fungal morphotypes and, ultimately, affected DC activation (Romani et al. 2004). MBL, a member of the collectin family of proteins, bind through multiple sites to various carbohydrate structures on fungal surfaces and promote complement activation through the lectin pathway (Turner 1996). Opsonization with MBL or C3 and/or or IgG greatly modified the receptor exploitation by fungi (Romani et al. 2004). Opsonization with MBL and C3, by favoring entry through CR3, reduced the expression of costimulatory molecules and IL-12 production, while C3 and IgG opsonization, by favoring the entry through CR3 and FcγR, significantly reduced production of IL-12, increased that of IL-4 and induced that of IL-10. Thus, collectins appear to favor the phagocytosis of the fungus without implicating the production of cytokine messengers to the immune system, an activity compatible with a primitive mechanism of host defense and in line with their ability to down-regulate the inflammatory response to fungi (Turner 1996). Antifungal antibodies also modified the receptor usage and DC activation in response to fungi (Montagnoli et al. 2003). Opsonization with protective anticandidal antibodies, specifically reacting to the phosphomannan protein complex of *C. albicans* (Montagnoli et al. 2003), while not affecting the phagocytosis, greatly affected the fungal internalization through CR3 and increased IL-10 production by PP-DCs, a finding consistent with the notion that the protective potential of opsonizing IgM antibodies may rely on their ability to fix complement C3 on the fungal surface. All together, opsonins, by subverting the morphotype-specific program of activation of DCs, may qualitatively affect DC functioning in response to fungi.

4.3.2. Tryptophan metabolic pathway

Recent evidence suggest that the inflammatory/anti-inflammatory state of DCs in response to fungi is strictly controlled by the metabolic pathway involved in tryptophan catabolism and mediated by the enzyme indoleamine 2,3-dioxygenase (IDO) (Bozza et al. 2004). IDO has a complex role in immunoregulation in infection, pregnancy, autoimmunity, transplantation, and neoplasia (Grohmann et al. 2003). IDO expressing DCs are regarded as regulatory DCs specialized to cause antigen-specific deletional tolerance or otherwise negatively regulating responding T cells (Mellor and Munn 2004). IFN-γ is required for functional IDO enzymatic activity in DCs (Fallarino et al. 2003; Grohmann et al. 2002).

In candidiasis, IDO activity was induced at sites of infection as well as in DCs via IFN-γ- and cytotoxic T lymphocyte-associate antigen (CTLA) 4-dependent mechanisms. IDO inhibition greatly exacerbated the infection and associated inflammatory pathology, as a result of deregulated innate and adaptive immune responses. In vitro, IDO blockade reduced IL-10 production in response to hyphae and increased IL-6/IL-12 production in response to yeasts by PP-DCs. Consistent with the finding that PP-DCs producing IL-10 are absolutely required for the activation of $CD4^+CD25^+$ Treg capable of negatively regulating the inflammatory response and antifungal Th1 immunity upon adoptive transfer in vivo (Montagnoli et al. 2002), the number of IL-10-producing $CD4^+CD25^+$ Treg was significantly decreased in the number of $CD4^+$ T cells producing IFN-γ of $CD4^+$ T cells producing IFN-γ increased, while that of cells producing IL-4 would decrease. It appears that the activation of IL-10-producing $CD4^+CD25^+$ T IFN-γ/IDO-dependent pathway may control the local IFN-γ/IDO-dependent pathway may control the local inflammatory pathology and Th1 reactivity to the fungus. These results provide novel mechanistic insights into complex events of host adaptation to the fungus. The production of IFN-γ may be squarely placed at this interface, where IDO activation likely exerts a fine control over inflammatory and noninflammatory activation likely exerts a fine control over inflammatory and noninflammatory antifungal responses Fig. 4. Therefore, the selective expression of IDO in the gut may represent the missing tissue-dependent factor that conditions the ability of DCs to produce IL-10 upon exposure to *Candida* hyphae, ultimately dictating the local pattern of both cytokine production and Th reactivity to the fungus. In addition, as IFN-γ is an important mediator of protective immunity to the fungus (Romani 2004), the IFN-γ/IDO axys may accommodate fungal persistence in a host environment rich in IFN-γ.

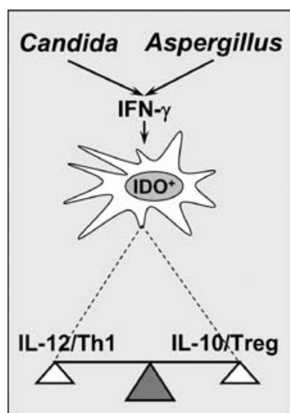

Figure 4. The crucial role for the IFN-γ/IDO-dependent metabolic pathway in *Candida albicans* and *Aspergillus fumigatus* infections. The production of IFN-γ is squarely placed at the host/pathogen interface where IDO activation exerts a fine control over dendritic cell activation and the resulting adaptive immunity to the fungi. IDO, Indoleamine 2,3-dioxygenase

In its ability to down-regulate antifungal Th1 host environment rich in IFN-γ. In its ability to down-regulate antifungal Th1 response in the gastrointstinal tract, IDO behaves in a fashion similar to that described in mice with colitis where IDO expression correlates with the occurrence of local tolerogenic responses (Gurtner et al. 2003). In the basal state, the gut is the site of the highest levels of IDO expression which is believed to be required to allow for microbial colonization and ingestion of dietary antigens without tissue-damaging inflammatory responses. The expression of IDO is indeed up-regulated in active inflammatory bowel disease (Barcelo-Batlori et al. 2002).

Recent evidence suggest a crucial role for IDO at the interface between *A. fumigatus* and the host Fig. 4. The inherent resistance to diseases caused by the fungus suggests the occurrence of regulatory mechanisms that provide the host with adequate defense without necessarily eliminating the fungus or causing unacceptable levels of host damage. A division of labor occurred between functionally distinct Treg that were coordinately activated by a CD28/B.7-dependent costimulatory pathway after exposure of mice to *Aspergillus* dormant conidia. Early in infection, inflammation was controlled by the expansion, activation and local recruitment of suppressive $CD4^{+}CD25^{+}$ Treg acting on IDO through the combined actions of IL-10 and CTLA-4. The levels of IFN-γ produced in this early phase set the subsequent adaptive stage by conditioning the IDO-dependent tolerogenic program of DCs and the subsequent activation and expansion of tolerogenic Treg, which produced IL-10 and TGF-β, inhibited Th2 cells and prevented and TGF-β, inhibited Th2 cells and prevented allergy to the Thus, regulation is an essential component of host responses in infection and allergy to the fungus, and its manipulation allows the pathogen to overcome host resistance and promote infection and allergy.

4.3.3. T cell ligands

Bidirectional signaling through the B7-CTLA-4 co-receptor pair has been shown to down-regulate immune responses and favor tolerance to alloantigens as well as tumor and self peptides, providing a mechanistic clue to the action of CTLA-4-expressing Treg (Orabona 2004). Recent data showed that both CD28 forward signaling and B7 reverse signaling positively affect the induction of immune responses after B7 engagement by CD28, providing an unexpected symmetry to the actions of CTLA-4 and CD28 in respective negative and positive costimulation of T cells. In particular, using soluble CD28-Ig and CTLA-4-Ig evidence has been provided that the two fusion proteins exert opposite effects on DC activating programmes in response to *C. albicans*. This translated in a different vaccinating ability of conditioned DCs when used as a vaccine preparation. Protective vaccination with yeast-pulsed DCs was rendered nonprotective by CTLA-4-Ig, which required IFN-γ and resulted in a Th2-dominated overwhelming infection. In contrast, nonprotective DCs pulsed with hyphae were made protective by exposure to CD28-Ig, through mechanisms contingent on autocrine IL-6. Compared with that of mice receiving DCs pulsed with hyphae in the absence

of CD28-Ig, the frequency of IFN-γ-producing T cells was increased and that of IFN-γ-producing T cells was increased and that of anticandidal protection (Orabona 2004). Therefore, different ligands of B7, through different cytokine responses in target DCs, may induce qualitatively different Th cell responses to fungi.

4.3.4. Other cells

A reciprocal activating interaction between Natural Killer (NK) cells and DCs has been suggested to play a role in the functional regulation of these cells in immunity to infections (Moretta 2002). A study in patients with atopic eczema/dermatitis syndrome (AEDS) has shown a close contact between NK cells ($CD56^+/CD3^-$) and $CD1a^+$DCs in vivo, in biopsies from *Malassezia* atopy patch test-positive skin (Buentke et al. 2002). DCs prestimulated with the yeast were less susceptible to NK cell-induced cell death and soluble yeast-derived factors decreased the cytotoxic potential of NK cells. Therefore, these findings indicate that an interaction may occur between NK and DCs in the skin of AEDS patients, upon which the fungus may exert a fine control.

5. DENDRITIC CELLS TRANSLATE FUNGUS-ASSOCIATED INFORMATION TO TH1, TH2 AND TREG CELLS

Fungus-pulsed DCs activated different types of naive $CD4^+$ Th cells in vitro and in vivo (Romani et al. 2002; Bozza et al. 2004; Bauman et al. 2000; Ferreira et al. 2003). In vitro, $CD4^+$ T murine splenocytes co-cultured with yeast- or conidia-pulsed DCs produced high levels of IFN-γ, but not IL-4 or IL-10. In contrast, DCs exposed to hyphae induced low levels of IFN-γ, but high levels of IL-4 and IL-10 in $CD4^+$ T cells. Monocyte-derived human DCs also activated different types of cytokine-producing cells upon pulsing with the different fungal morphotypes (Bozza et al. 2003; Romani et al. 2004; Perruccio et al. 2004). Interestingly, upon pulsing with yeasts or conidia, mDCs mainly activated IFN-γ-producing $CD4^+$ Th1 cells, whereas pDCs activated IFN-γ- and IL-10-producing $CD4^+$ cells (Perruccio et al. 2004). In vivo, the balance among the different DC subsets determined whether protective or nonprotective antifungal cell-mediated immune responses developed (Baumann et al. 2000). Langerhans cells, mDCs, and lDCs were present in the draining lymph node of mice immunized with protective or nonprotective cryptococcal antigen (Baumann et al. 2000). Draining lymph node lDC:mDC ratios induced by the protective immunogen were significantly lower than the ratios induced by either immunization in which the nonprotective immunogen was present. In contrast, mice given the nonprotective immunogen had lDC:mDC ratios similar to those of naive mice. Therefore, Langerhans cells and mDCs were needed for induction of the protective response, whereas lDC acted as negative regulators of cell mediated- immune responses.

Fungus-pulsed DCs activated different $CD4^+$ Th cells upon adoptive transfer into immunocompetent mice (Bozza et al. 2003; Bozza et al. 2004; Bacci et al.

2002. Adoptive transfer of purified ex-vivo DCs pulsed with yeasts/conidia or hyphae, resulted in priming of $CD4^+$ T cells for Th1 or Th2 cytokine production, respectively. The analysis of antigen specific proliferation and cytokine production by $CD4^+$ T cells from draining lymph nodes and spleens revealed that levels of IFN-γ were higher, and those of IL-4 lower, in mice immunized with yeast- or conidia-pulsed DCs as compared to mice receiving unpulsed or hypha-pulsed DCs. The ability of fungus-pulsed DCs to prime for Th1 and Th2 cell activation upon adoptive transfer in vivo correlated with the occurrence of resistance and susceptibility to the infections (Bozza et al. 2003; Bozza et al. 2004; Bacci et al. 2002). Antifungal protective immunity in vivo was also observed upon adoptive transfer of ex-vivo DCs transfected with fungal RNA. The efficacy was restricted to DCs transfected with RNA from yeasts or conidia but not with hyphal RNA. The effect was fungus-specific, as no cross-protection was observed upon adoptive transfer of DCs pulsed with either fungal species. The frequency of IFN-γ-producing occasion, also induced protection in a murine model of pulmonary cryptococcosis (Bozza et al. 2004). The frequency of IFN-γ-producing Th1 cells was increased and that of IL-4-producing cells decreased in protected mice, a finding suggesting the occurrence of a Th1-dependent antifungal resistance. It is of interest that yeast or conidial RNA, more efficiently than live fungi, concurrently activated IL-10-producing Treg. These findings expand upon the vaccinating potential of DCs in fungal infections.

6. EXPLOITING DENDRITIC CELLS AS FUNGAL VACCINES

The infusion of RNA-transfected DCs accelerated the recovery of functional antifungal Th1 responses in mice with allogeneic hematopoietic stem cell transplantation (HSCT), an experimental model in which autologous reconstitution of host stem cells is greatly reduced to the benefit of a long-term, donor type chimerism in more than 95% of the mice and low incidence of graft versus host disease (Mencacci et al. 2001). Patients receiving T cell-depleted HSCT are unable to develop antigen-specific T cell responses soon after transplant (Velardi et al. 1988) and showed a defective DC functioning (Reddy et al. 2004). However, functional recovery of the T cell system after T cell-depleted allogeneic HSCT has been demonstrated (Verfuerth et al. 2000) and both donor and recipient DCs may participate to the reconstitution of the T cell repertoire in transplantation through distinct pathways of antigen presentation (Lechler 2001). We have demonstrated that an imbalanced production of Th1 and Th2 cytokines was responsible for the susceptibility to fungal infections in the murine HSCT model (Mencacci et al. 2001). However, readdressing the balance between Th1 and Th2 subsets, as by treatment with Th2 cytokine antagonists, accelerated the recovery of Th1-mediated antifungal resistance (Mencacci et al. 2001). The recovery of functional Th1 cells producing IFN-γ was also accelerated by the infusion of fungus-pulsed or RNA-transfected DCs, a finding suggesting that DCs may pivotally determine the Th/Treg balance in HSCT (Bozza et al. 2003; Bacci et al. 2002). We have also found that the ability of

either mDCs or pDCs to phagocytose and respond to *Candida* or *Aspergillus* was defective soon after allogeneic HSCT (unpublished data). In contrast, both murine and human donor mDCs and pDCs phagocytosed fungi and underwent functional maturation in response to them. However, their activation program for cytokine production was different, being IL-12 produced mainly by mDCs and IL-12,IL-10 and IFN-α produced by pDCs. This resulted in a distinct ability for T cell priming in vitro, being Th1, Th2 and Treg differently activated by the different DC subsets (Perruccio et al. 2004). More recent data have shown that the infusion of fungus-pulsed purified DCs of either subset accelerated the recovery of peripheral antifungal Th1 immunity and increased resistance to fungal infections in mice with HSCT. However, only the co-infusion of DCs of both subsets resulted in: i) induction of Treg capable of a fine control over the inflammatory pathology; ii) tolerization toward alloantigens and iii) diversion from alloantigen-specific to antigen-specific T cell responses in the presence of donor T lymphocytes (unpublished observations). Thus, the adoptive transfer of DCs may restore antifungal immunocompetence in HSCT by contributing to the educational program of T cells through the combined action of activating and tolerizing DCs. These results, along with the finding that fungus-pulsed DCs could reverse T cells anergy of patients with fungal diseases (Grazziutti et al. 2001; Richards et al. 2002), may suggest the utility of DCs for fungal vaccines and vaccination.

7. CONCLUSIONS AND PERSPECTIVES

In the past decades, the frequency of opportunistic fungal infections has increased (Singh 2001). The increasing number of susceptible hosts, the introduction of newer modalities for HSCT, the evolution of organ transplantation practices, the use of novel immunosuppressive agents, and current antimicrobial prophylactic strategies have likely contributed to the changing epidemiology of invasive mycoses. The therapeutic efficacy of antifungals is limited without the help of host immune reactivity. Various cytokines, including chemokines and growth factors, have proved to be beneficial in experimental and human refractory fungal infections (Roilides et al. 2002; Kullberg et al. 2004). The Th1-Th2 balance itself can be the target of immunotherapy (Puccetti et al. 1995; Koguchi and Kawakami 2002). The inhibition of Th2 cytokines, or the addition of Th1 cytokines, can increase the efficacy of antifungals, such as polyens and azoles, in experimental mycoses (Romani 2001).

The appreciation that activation of the innate immune system initiates, amplifies and drives antigen-specific immune responses together with the identification of discrete cell types, specific receptors and the signaling pathways involved in the activation of innate immunity has provided a multitudo of new targets for exploitation by the developments of adjuvants for vaccines (Deepe 2004). Developments in DC biology are providing opportunities for improved strategies for the prevention and management of fungal diseases in immunocompromised patients. The ultimate challenge will be to design fungal vaccines capable of inducing

optimal immune responses by targeting specific receptors on DCs. This will require, however, further studies aimed at elucidating the convergence and divergence of pathways of immune protection elicited in infections or upon vaccination.

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CHAPTER 3

THE NEUTROPHIL

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Abstract: The neutrophil provides a crucial defence against fungal infections. This numerous phagocyte is recruited rapidly to sites of infection, where it detects pathogens through a range of pathogen-recognition receptors. Phagocytosis and the generation of a range of microbicidal molecules neutralises the pathogen, following which neutrophil death by apoptosis triggers an injury-limiting resolution process facilitating the restoration of normal tissue architecture

1. GENERAL INTRODUCTION

Neutrophils are our most numerous professional phagocyte, whose role is host defence against infections, principally those caused by bacteria and fungi. The importance of the neutrophil in fungal infection is demonstrated in patients rendered acutely neutropaenic through chemotherapy or following bone marrow transplantation, when susceptibility to, and mortality from, fungal infections dramatically increases.

1.1. The History of the Neutrophil

In the 18th century, light microscopes were able to identify some of the basic constituents of blood, namely the red cells and the white cells, or white corpuscles, as they were first described. Further progress was made in the 19th century when Paul Ehrlich used staining techniques to identify different types of corpuscles that he termed the acidophile, basophile and neutrophile because of their acidic, alkaline and negative staining respectively. It was at this time that Erhlich first described the neutrophil as a polynuclear cell. Later, evidence pointed to a single nucleus, but with many lobes, and Metchnikoff described the cell as a polymorphonuclear leukocyte. This term became popular with scientists and

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book authors at the time, despite the fact Metchnikoff himself preferred the term microphage to distinguish it from its similar, yet larger, phagocytosing relative, the macrophage.

As time progressed, investigators described these polymorphonuclear leukocytes leaving the circulation through a process of diapedesis. Observing infected sites led Waller to speculate that pus cells assisted bacteria in their transport, growth and survival. This theory was further strengthened by the fact that bacteria were seen in white corpuscles, presumably being transported to new sites of infection. Metchnikoff disagreed with Waller's theory, believing the white corpuscles were in fact the bacteria's enemy.

Many pathologists wondered how the bacteria gained access to these cells. Metchnikoff observed starfish larvae contained specialised cells that appeared to attempt to ingest and destroy foreign tissue (in his case, a rose thorn inserted into a starfish gastrula). He also noted that the *Amoeba*, a unicellular organism, engulfed its prey and digested it intracellularly, its appearance closely resembling a leukocyte ingesting bacteria. As a result, Metchnikoff proposed that neutrophils patrolled the circulation and mobilised to infected tissues and engaged in a process of phagocytosis of bacteria. From these seminal early studies, our understanding of the neutrophil has moved forward to develop a detailed understanding of its crucial role in host defence, its potential to cause disease, and the exquisite regulation of its life and function. The importance of the neutrophil in microbial defence is shown by those congenital syndromes in which neutrophil function is impaired or neutrophil numbers are reduced, and where microbial infection is a major cause of morbidity and mortality. In the Chediak-Higashi syndrome, an autosomal recessive condition, granules are unable to fuse with the phagosome and thus cannot exert their functional role. This condition is associated with recurrent microbial infections, albinism, hepatoslenomegaly, and lymphoproliferative malignancy. Giant cytoplasmic inclusions of granules in leukocytes are observed under light microscopy. Disorders with marked abnormalities of neutrophil function tend to be rare, presumably because of a substantial negative pressure for their persistence across the generations.

1.2. The Structure of the Neutrophil

Neutrophils mature in the bone marrow over a 7–14 day period. They evolve through six morphological stages: myeloblast, promyeloblast, myelocyte, metamyelocyte, non-segmented neutrophil and segmented neutrophil. In the myeloblast stage, the nucleus is very large and round, taking up much of the intracellular space and allowing only a small amount of cytoplasm, which does not contain any granules. Several nucleoli are present. The promyeloblast stage is characterised by a larger cell with a round or oval nucleus, in which the nucleoli are less obvious, and during this stage the azurophilic granules begin to appear within the cytoplasm. The secondary granules appear in the myelocyte stage. These contain large amounts of

glycoprotein which give rise to the pink colouration upon staining with classical H&E based stains. The nucleus becomes almost horseshoe shaped in the metamyelocyte stage. The chromatin becomes denser. The final stages of development give rise to the segmented neutrophil. Strand bridges join two or more nuclear lobes. The cytoplasm stains pink due to the presence of primary (azurophilic), secondary (specific) and tertiary granules. Primary (Azurophil) granules contain cationic proteins, myeloperoxidase (MPO), matrix metalloproteinases (MMPs) and hydrolases. Secondary (specific) granules contain collagenase, lactoferrin and histaminase. Immature neutrophils have vast reserves dedicated to the synthesis of granules with a large and active Golgi apparatus and endoplasmic reticulum that disappear as the neutrophils age rendering the mature neutrophil unable to synthesis new granules.

1.3. Regulation of Neutrophil Numbers

The neutrophil is our most numerous professional phagocyte, with $2.5 - 7.5 \times 10^9$ cells/litre in the circulation. Once the neutrophil is released from the cytokine rich environment of the bone marrow, their inactivated state allows them a lifespan of between six to ten hours in the circulation. The bone marrow manufactures 1×10^{11} neutrophils per day in healthy adults (Figure 1). The capacity to dramatically increase these numbers exists within the functional system of the marrow. At any given time, approximately 60% of the marrow activity is dedicated to neutrophil production with approximately 20% of activity reserved for erythrocyte production. The bone marrow has a large store of neutrophils that can be mobilised rapidly to target infections as soon as they are detected. Retention and release are regulated by adhesion molecule expression, with evidence for roles for both CD11b/CD18 and VLA-4 in the control of these processes (Burdon et al., 2005). Additional roles in these processes are evident for chemoattractant cytokines (chemokines), with evidence that chemokines acting on CXCR2, the major receptor regulating neutrophil recruitment from the microcirculation into tissues, also cause mobilisation of neutrophils from the marrow into the circulation (Burdon et al., 2005). Interestingly, chemokines acting on the receptor CXCR4 also appear to be important in regulating neutrophil release from the marrow, but in contrast to CXCR2 ligands,

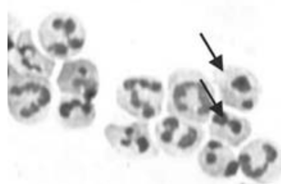

Figure 1. Neutrophils in culture. Healthy viable neutrophils exhibit the classical polymorphonuclear phenotype. As they age, they undergo constitutive apoptosis, with shrinking of the nucleus (pyknosis). Two apoptotic neutrophils, showing nuclear condensation, are marked by arrows (See Color Section.)

activation of CXCR4 serves to firstly retain immature cells in the marrow, and secondly to direct the return of senescent neutrophils from the circulation to the marrow for destruction (Martin et al., 2003). Once released by the bone marrow, neutrophils appear to be retained in the circulation unless recruited to inflammatory sites, though a substantial proportion of cells may be held, at least transiently, in a marginating pool in capillary beds such as those of the lung. Without an inflammatory stimulus, circulating senescent neutrophils upregulate expression of CXCR4, which provides a directional cue moving cells back to the bone marrow for removal, with analogous events likely to occur in the phagocytic systems in the spleen and liver.

1.4. Regulation of Neutrophil Recruitment

Neutrophil recruitment to sites of infection is rapid and efficient. Migration from the microvasculature occurs along gradients of chemotactic factors that are generated by the host and potentially the pathogen. Host-generated chemotactic mediators include chemotactic cytokines (chemokines) and complement fragments, whose generation and efficacy are regulated by multiple mechanisms, presumably with the aim of delivering neutrophils to the site of inflammation whilst minimising bystander tissue damage en route. Interestingly, a hierarchy regulates neutrophil recruitment and elimination in ways permitting careful control of neutrophil function and numbers. Factors that directly cause neutrophil recruitment, such as chemokines, tend to be poor inducers of neutrophil survival; conversely, cytokines that delay neutrophil apoptosis tend to show little efficacy in assays of chemotaxis (though may prime responses to chemotactic factors). Overarching proinflammatory mediators and pathways orchestrate the production of recruitment and survival factors, and on cessation of the inflammatory insult these may be withdrawn to favour resolution.

1.4.1. Chemokines

Chemokines have major roles in driving the selective recruitment of leukocytes. The first chemokines to be identified and characterised in the late 1980s and early 1990s included monocyte-chemoattractant protein-1 (MCP-1) (Yoshimura et al., 1989), RANTES (Schall et al., 1988) (named in honour of its near-mystical powers of cell recruitment (Cohen, 1996)), and interleukin-8 (IL-8) (Matsushima et al., 1988; Yoshimura et al., 1987, Yoshimura et al., 1987); these chemokines recruiting principally monocytes, lymphocytes, and neutrophils respectively. Now renamed according to structural considerations as part of a rational nomenclature as CCL2, CCL5 and CXCL8 respectively (Sabroe et al., 2002), these chemokines provided hope that the mechanisms regulating selective cell recruitment could be identified and therapeutically targeted. Inevitably, passing time has revealed a complex network of chemokines showing redundancy and overlapping spectra of activity, which has been complicated by important differences between experimental species (e.g. mice) and men. In man, neutrophils principally express two chemokine receptor types, CXCR1 and CXCR2 (Chuntharapai and Kim, 1995). The best-known

neutrophil-recruiting chemokine, CXCL8, acts upon both of these receptors, but there appears to be some division of responsibilities between CXCR1 and CXCR2. The latter receptor is relatively promiscuous, binding a range of CXC chemokines containing an ELR amino acid motif, including CXCL8, CXCL1 (MGSA), CXCL7 (NAP-2), CXCL5 (ENA-78), and CXCL6 (GCP-2). Activation of CXCR2 appears to be crucial for neutrophil recruitment (Chuntharapai and Kim, 1995; Pease and Sabroe, 2002; Del Rio et al., 2001), and small molecule inhibitors of CXCR2 are showing substantial promise as effective therapies to inhibit neutrophil recruitment where neutrophilic inflammation is destructive (e.g., potentially, in asthma, chronic obstructive pulmonary disease, and the acute respiratory distress syndrome) (Pease and Sabroe, 2002; White et al., 1998; Jones et al., 1997; Podolin et al., 2002). Although CXCR1 and CXCR2 are both high-affinity receptors for CXCL8, it appears that activation of CXCR1 is linked more with neutrophil activation and respiratory burst generation rather than recruitment (Chuntharapai and Kim, 1995; Pease and Sabroe, 2002). CXCR1 is relatively fastidious, binding CXCL8 and CXCL6 only. CXCR1 and CXCR2 have high homology, yet their expression is independently regulated by proinflammatory stimuli, with CXCR2 showing rapid downregulation on activation of neutrophils by a variety of chemokines, cytokines, and pathogen-derived molecules, whilst CXCR1 expression is relatively preserved (Doroshenko et al., 2002; Sabroe et al., 2005, Sabroe et al., 1997; Ali et al., 1999; Richardson et al., 2003; Tomhave et al., 1994). Such divisions of roles between these two receptors are unlikely to be absolute, and it is conceivable that the properties of these receptors are arranged such that CXCR2 function is responsible for initial cell recruitment, and after its downregulation, signalling via CXCR1 takes over to regulate activation and, potentially, further tissue positioning (Ludwig et al., 1997). There is limited evidence that activated neutrophils may express other chemokine receptors, such as CCR1 (Bonecchi et al., 1999), which may contribute to regulation of their recruitment, tissue positioning, or activation, but in general it is thought CCR1 has little role in human neutrophil function (Hall et al., 2001). This is in contrast to results obtained from studies in mice, where it is clear that CC chemokines, acting on receptors including CCR1, may have important roles in neutrophil recruitment (Gao et al., 1997). As a further complication, a mouse homologue of CXCR1 has only just been identified (Fu et al., 2005), making dissection of the individual roles of these receptors difficult. In general, it is probably true that CC chemokines have a very minor or no role in the direct induction of neutrophil recruitment in humans, and thus caution is required when extrapolating from data generated in the mouse. Indirect roles for CC chemokines, through the recruitment of monocytes and T cells and their subsequent induction of neutrophil recruitment, are, however, likely.

Neutrophil-recruiting chemokines are themselves generated from an extremely wide range of tissues. There are few cell types that cannot make CXCL8, and at sites of inflammation, epithelial cells, endothelial cells, fibroblasts, and infiltrating leukocytes will all represent potentially important sources of chemokines. Heat-killed opsonised *Candida albicans* and *Saccharomyces cerevisiae* as well as zymosan are

able to induce significant production of CXCL8 from human neutrophil cultures *in vitro* (Hachicha et al., 1998). Production of the CC chemokine, CCL3, following challenge in the same experiments, was however only minimally increased after exposure to *C. albicans* and was not increased compared to control following exposure to *S. cerevisiae* and zymosan. These results contrasted with findings for most of the bacterial pathogens tested, which induced potent induction of both chemokines. Theses findings held true even after priming of neutrophils with TNFα after which *S. cerevisiae* and zymosan still failed to stimulate CCL3, and in fact induced lower levels of the chemokine after neutrophil priming than did control cells. The importance of CXCL8 to neutrophil recruitment in yeast infections has been confirmed in genetically modified BALB/c mice which lack the CXCR2 orthologue (Balish et al., 1999). These CXCR2$^{-/-}$ mice demonstrated increased susceptibility to invasive and gastric candidiasis. Furthermore, neutrophil recruitment was retarded compared to wild-type mice both in tissues and in peritoneal exudate following challenge with heat-killed *Candida albicans*.

The role of CC chemokines in neutrophil recruitment, as noted above, remains challenging to dissect. Most studies are based in mice, which have a different repertoire of neutrophil chemokine receptors compared to man, moreover, CC chemokine-mediated monocyte recruitment may indirectly result in downstream amplification of inflammation and subsequent tissue neutrophilia. The lack of CC chemokine production in response to infectious challenge has been confirmed for many other yeasts. Whether this is a host-specific tuning of the immune response, or an evolved response of the pathogen to try to minimise phagocyte recruitment, is not known. *Cryptococcal neoformans* infection of murine lungs, for example, fails to induce significant mRNA or protein for a variety of CC chemokines including CCL2, CCL3, and CCL5 (Kawakami et al., 1999). Although for many yeasts production of CCL3 is minimal and seems unnecessary for neutrophil recruitment, CCL3 production has been shown to be an important determinant of neutrophil recruitment into the peritoneal cavity of mice challenged with the yeast form of the dimorphic fungus *Histoplasma capsulatum* (Medeiros et al., 2004). In this case, CCL3 production was induced by live yeast but not β-glucan from the cell wall, and production was regulated by leukotrienes (Medeiros et al., 2004), and in part mediated neutrophil recruitment indirectly via recruitment of mononuclear cells that released second chemotactic signals.

Some yeasts are capable of inhibiting chemokine-induced migration of neutrophils. A constituent of the capsule of *Cryptococcus neoformans*, mannoprotein (MP)-4, inhibits migration of neutrophils towards CXCL8 (as well as to other chemotactic factors such as fMLP and platelet activating factor) possibly through a mechanism involving chemoattractant cross-desensitisation and premature neutrophil activation (Coenjaerts et al., 2001). Thus, although cryptococcal capsule polysaccharides, such as glucuronoxylomannan, may be potent inducers of CXCL8, cryptococcal meningitis is associated with a paucity of recruited neutrophils to the cerebrospinal fluid (Lipovsky et al., 1998). Therefore *Cryptococcus neoformans* is well equipped to inhibit neutrophil recruitment, both by preventing responses

to CXCL8, and by inducing only low levels of many CC chemokines that might amplify inflammatory responses.

CXC chemokines are also important in responses to filamentous fungi. Invasive aspergillosis is associated with neutropenia or neutrophil dysfunction. Following exposure to *Aspergillus fumigatus*, the murine CXCR2 agonists KC and MIP-2 are critical to prevention of invasive aspergillosis in the lung (Kawakami et al., 1999). In this model, blocking antibodies against CXCR2 induced invasive aspergillosis in immunocompetent mice. Similarly, transient overexpression of KC improved fungal clearance in a model of murine invasive aspergillosis (Mehrad et al., 2002). Roles for CC chemokines in neutrophil recruitment and host defence against *Aspergillus fumigatus* (Gao et al., 1997) have also been observed. Mice lacking CCR1, which binds CCL3 and CCL5, also demonstrate increased mortality suggesting that for this infection (Gao et al., 1997), though again CCL3 effects may in part be mediated through actions on monocyte recruitment, cells that in themselves also have potent phagocytic, antimicrobial properties (Mehrad et al., 2000).

1.4.2. Other chemotactic factors

A variety of other molecules of various classes also serve to regulate neutrophil recruitment, including lipid mediators such as platelet activating factor (PAF) and leukotriene B_4 (LTB_4). The activated complement fragment, C5a, is also a potent stimulus of neutrophil recruitment, potentially acting sequentially with chemokines (Hopken et al., 1996; Ivey et al., 1995). In contrast to chemokines, C5a is an extremely potent inducer of respiratory burst. Chemoattractants such as chemokines, C5a, and bacterial peptides acting on the fMLPR, exhibit a hierarchy of function, whereby, for example, C5a signalling will desensitise responses to chemokines, but chemokines cannot desensitise responses to C5a (Sabroe et al., 1997). The upshot of this complicated interaction between chemoattractants is probably that multiple chemoattractant molecules are responsible for tissue positioning of neutrophils, acting in a sequential manner, and these mechanisms may also allow neutrophils to continue to migrate along chemotactic gradients of molecules that may vary over many orders of magnitude of concentration (Foxman et al., 1997). Signalling via the C5a receptor is important for clearance of some bacterial infections (Hopken et al., 1996), and likewise has a role in fungal infections since during infection with *Cryptococcus neoformans* C5a enhances killing of yeast (Lovchik and Lipscomb, 1993). This effect is influenced by the site of inoculation, with a role for C5a demonstrated in a murine model of intravenous infection in which C5 deficient mice had decreased recruitment of neutrophils into pulmonary vessels and decreased clearance of yeast as compared to wild-type mice. In keeping with lower concentrations of complement in the alveolar space, however, C5 deficient mice demonstrated no defect in early recruitment or killing in response to intratracheal fungal challenge (Lovchik and Lipscomb, 1993). *Cryptoccocus neoformans* has developed adaptations to prevent this host response: the capsule polysaccharide glucuronoxylomannan (GXM) downregulates neutrophil expression of the C5a

receptor (C5aR/CD88) (Monari et al., 2002). In contrast, unencapsulated strains induce upregulation of C5aR expression, emphasising the importance of the cryptococcal capsule as a virulence determinant that inhibits neutrophil recruitment. Filamentous fungi also appear capable of inducing complement-mediated neutrophil chemotaxis, since, following germination, *Aspergillus fumigatus* conidia and *Rhizopus oryzae* spores activate chemotactic complement components (Waldorf and Diamond, 1985). In addition, extracts of the dimorphic fungus *Coccidioides immitis* demonstrate chemotactic activity for neutrophils in the presence of serum but not heat-inactivated serum, suggesting a complement-mediated effect (Galgiani et al., 1978).

Bacterial peptides are initiated by a formlyated methionine residue, and small peptides with this motif, such as fMLP, are potent neutrophil recruiting and activating factors. *Candida albicans* also produces a chemotactic factor that is capable of inducing neutrophil recruitment via the formyl peptide receptor (FPR) (Edens et al., 1999). A variety of *Candida* spp., including C. *tropicalis, C. parapsilosis* and *C. glabrata*, produce these factors but *Saccharomyces cerevisiae* does not (Geiger et al., 2004). The chemotactic factor is approximately 1kDa in size and further analysis suggests it is a low molecular mass polypeptide, like fMLP. Since this factor is produced by the white phase but not the opaque phase (mating competent) phenotype it has been suggested that *C. albicans* downregulates production of this factor during mating to prevent neutrophil recruitment during this critical stage of the life-cycle.

2. OVERARCHING REGULATORY MECHANISMS

It is evident that cytokines such as TNFα and IL-1β have major roles in the orchestration of neutrophil recruitment, through the production of cytokines such as CXCL8 from other leukocytes and tissue cells. We have shown that activation of tissue cells by monocytes exposed to TLR agonists results in marked CXCL8 generation, dependent upon TLR-induced IL-1β secretion from the monocyte that drives CXCL8 production from tissue cells (Morris et al., 2005). TNFα/lymphotoxin-α double knock-out mice have increased susceptibility to *Candida albicans* infection in a model of intra-peritoneal challenge (Netea et al., 1999). While these factors are well recognised to contribute to activation of neutrophils, these mice also demonstrated significant impairment in neutrophil recruitment, and phagocytosis of yeast, although killing of ingested yeast was not altered (Netea et al., 1999). Production of TNFα in response to fungal infection is regulated at multiple levels, as illustrated by studies of mice deficient in Fas, which produce more TNFα and show increased neutrophil recruitment in fungal infection, resulting in greater fungal clearance and host survival (Netea et al., 1999).

IL-17A is increasingly recognised as playing an important role in neutrophil granulopoiesis and recruitment. It is produced by T-lymphocytes and therefore interconnects lymphoid and myeloid lineages in host defence. T-lymphocytes

are important in host defence against a variety of fungi, in particular yeast and dimorphic fungi. Accordingly, mice lacking the IL-17 receptor demonstrated increased fungal loads and decreased neutrophil recruitment to target organs during systemic challenge with *Candida albicans* (Huang et al., 2004).

2.1. Neutrophil Adherence

Neutrophil recruitment from the microvasculature proceeds according to the classical three-stage model, whereby selectin-mediated interactions between the neutrophil and endothelium allow the neutrophil to roll along the vessel wall. Upon encountering chemoattractants such as chemokines, probably displayed bound to glycosaminoglycans (GAGs) on the vessel wall, upregulation of integrin-mediated adhesion results in tight bonding of the leukocyte to the vessel wall, followed by transmigration (diapedesis) between endothelial cells and into tissue (Luster, 1998). Effective migration relies upon L-selectin-mediated rolling of neutrophils, but subsequently L-selectin is shed from the neutrophil, a process that is probably important in allowing effective transmigration of the vessel wall. Engagement of selectins may also activate specific signalling pathways contributing to the regulation of leukocyte recruitment (Simon et al., 1999). Activation of pattern recognition receptors and chemoattractant receptors results in L-selectin shedding, and inappropriate L-selectin shedding could impede effective leukocyte recruitment. Many components of fungi activate pattern recognition receptors such as TLRs, which will result in L-selectin shedding (Sabroe et al., 2002, 2003), and this has been proposed as a mechanism that might explain reduced leukocyte recruitment in disseminated cryptococcal infection. *Cryptococcus neoformans* capsule polysaccharides glucuronoxylomannan (GXM) and galactoxylomannan as well as mannoprotein induce shedding of L-selectin from neutrophils (Dong and Murphy, 1996). GXM also induces shedding of the TNF receptor, TNFR p75–80 (Dong and Murphy, 1996), and interferes with E-selectin-mediated binding of neutrophils to endothelial cells (Ellerbroek et al., 2004), in a CD14/TLR4-dependent fashion. In keeping with these *in vitro* results, individuals with systemic cryptococcal infection and detectable serum levels of cryptococcal polysaccharide have decreased levels of L-selectin in peripheral blood neutrophils and increased levels of soluble L-selectin in serum (Jackson et al., 2005). GXM also binds to a principal neutrophil integrin, CD18, and may inhibit the interaction of β2 integrins with their ligand ICAM-1 (Dong and Murphy, 1997). Leukocyte adhesion deficiency results from deficiencies in CD11b/CD18, giving rise to recurrent infections by bacteria and fungi, and in severe forms are associated with a very abbreviated lifespan.

2.2. Neutrophil Activation

Recruited neutrophils that encounter pathogens respond by induction of antimicrobial systems and the production of further chemokines, in particular CXCL8, to amplify the innate immune response. The processes of transmigration of the

vessel wall and local tissues under the influence of chemotactic factors, and the exposure to cytokines such as GM-CSF and TNFα and lipid mediators such as PAF, results in enhanced (primed) responses to microbial factors and other neutrophil activators, seen for example in marked increases in ROS production in primed cells (Cadwallader et al., 2002; Condliffe et al., 1996; Fuhler et al., 2004; Kitchen et al., 1996). Extensive evidence links cytokines known to prime neutrophil function with enhanced killing of a variety of fungal species. In particular, G-CSF and IFNγ cause enhanced killing of fungal components both in vitro and in vivo (Gil-Lamaignere et al., 2005; Liles et al., 1997; Roilides et al., 1995). Interestingly, although increased killing can reflect increased respiratory burst, this is not always a direct relationship, and a marked discordance between effects of TNFα and IFNγ on pathogen killing and respiratory burst has been reported (Diamond et al., 1991). The detection of pathogens is potentially mediated by molecules including CD11b/CD18, CD14, the fMLPR, and TLRs, in cooperation with Fc receptors that interact with opsonised particles. Signalling via these pathways triggers phagocytosis and delivery to the phagosome of toxic antimicrobial molecules, including proteases and reactive ions such as reactive oxygen species. Neutrophil activation, as characterised by alterations in adhesion molecule expression, regulation of chemotaxis, production of ROS and the potential to degranulate, can be triggered by a variety of factors that signal inflammation as well as direct encounter with pathogens. Molecules such as PAF and C5a serve roles both as endogenous chemoattractants and neutrophil activators, and generation of such factors at sites of inflammation is likely to be an important mechanism amplifying antimicrobial responses.

2.2.1. Toll-like receptors (TLRs)

The biology and roles of TLRs is covered in chapter 11. In brief, TLRs enable responses to a broad range of pathogen-associated molecules, ranging from lipoproteins (TLR2) to LPS (TLR4) to foreign DNA (TLR9) and viral RNA (TLRs 3, 7, and 8) (Sabroe et al., 2003; Akira and Takeda, 2004). Signalling via TLRs activates multiple pathways of host defence, regulating macrophage phagocytosis, cytokine production, cell survival, and ROS production. In the neutrophil, TLR activation has been shown to influence many aspects of neutrophil function, including the ability to (a) prolong cell survival both directly and indirectly via monocyte activation (Sabroe et al., 2003); (b) regulate neutrophil recruitment directly through effects on chemokine receptor expression and indirectly through effects on CXCL8 generation by neutrophils, monocytes, and tissue cells (Morris et al., 2005; Sabroe et al., 2002, 2003); (c) modulate neutrophil expression of adhesion molecules (Sabroe et al., 2002, 2003); and (d) prime neutrophils to induce ROS production (Sabroe et al., 2002, 2003). Neutrophils express the majority of TLRs (except TLR3), and their function can be primed by GM-CSF (Sabroe et al., 2002; Hayashi et al., 2003; Kurt-Jones et al., 2002). There is particularly strong evidence for important roles for TLRs 2 & 4 in neutrophil function. The relative contribution of other TLRs to neutrophil antimicrobial responses will no doubt become clearer over time. TLRs contribute to antifungal host defence (Romani,

2004; Roeder et al., 2004). TLR2 recognises a cell-wall glycolipid in *Candida albicans* phospholipomannan (Jouault et al., 2003). β-glucans, found in yeast cell wall preparations such as zymosan, and which exist as carbohydrate polymers in the cell walls of fungi such as *Saccharomyces cerevisiae, Candida albicans* and *Paracoccidioides brasiliensis* (Brown et al., 2003), cause activation of leukocytes that is dependent, at least in monocytic cells, on collaborative signalling of TLR2 and dectin-1. In the neutrophil, zymosan signalling also involves the CD11b/CD18 integrin (which can also act as a signalling molecule and a partner to other signalling molecules such as TLR4), and autocrine generation of platelet-activating factor (PAF), a potent chemoattractant lipid and leukocyte activator (Au et al., 1994). CD14, a non-signalling protein that is required for efficient TLR4-mediated responses to LPS, also appears to be involved in the TLR-mediated recognition of fungal components (Mambula et al., 2002; Wang et al., 2001). In contrast the *Cryptococcus neoformans* capsular polysaccharide GXM appears to activate TLR4/CD14 but not TLR2 (Shoham et al., 2001). *Aspergillus* species activate macrophages via both TLR2 and TLR4 (Meier et al, 2003) although the complexity of hyphal and conidial cell walls has meant that the polysaccharide and protein PAMPs responsible are still being evaluated (Roeder et al., 2004). TLR9, which recognises CpG DNA, is also activated by fungi, including *Candida albicans* and *Aspergillus fumigatus* (Bellocchio et al., 2004, Bellocchio et al., 2004). Thus, neutrophils respond to zymosan in a complex fashion that is likely to involve TLR signalling, and it is highly probable that neutrophil activation by fungal components, acting via a range of TLRs including TLR2, 4, and 9, will contribute to the induction of a neutrophil anti-fungal response. The pattern of TLR activated determines the specific responses of neutrophils to fungal challenge. For example, in responding to *Aspergillus fumigatus*, TLR2 may govern fungal killing via release of extracellular gelatinases such as MMP-9, and production of proinflammatory cytokines, while TLR4 may govern both the fungicidal effects mediated by reactive oxygen species generated in myeloperoxidase-positive granules, and also production of anti-inflammatory cytokines such as IL-10 (Bellocchio et al., 2004).

TLRs are highly expressed by other immune cells such as monocytes, which appear likely to play an important role in the orchestration of neutrophilic inflammation by the indirect regulation of neutrophil recruitment, activation, and survival (Morris et al., 2005; Sabroe et al., 2004). In a model of disseminated candidiasis in C3H/HeJ mice, which lack functional TLR4, decreased production of KC and MIP-2 (Netea et al., 2002) was observed compared to wild type mice, that was associated with decreased neutrophil recruitment and decreased fungal clearance. In the same study it was shown that TNFα and IL-1β production by macrophages was TLR2-dependent, demonstrating roles for both TLRs in the production of mediators influencing neutrophilic inflammation. In genetically modified mice optimal neutrophil recruitment in response to *Aspergillus fumigatus* required both TLR2 and TLR4 activation (Meier et al, 2003). Regardless of the specific TLRs involved, fungicidal pathways to both yeast and filamentous fungi

appear to be dependent on signalling via MyD88, the signalling adapter that is a major component of TLR signalling, and an essential component of IL-1R signalling (Bellocchio et al., 2004).

2.2.2. Phagocytosis

One of the important consequences of neutrophil activation is to ensure effector functions such as phagocytosis of fungi (Figure 2). In general, yeasts are phagocytosed well, with similar rates of internalization reported for a variety of *Candida* spp. (Lyman and Walsh, 1994). *Candida krusei*, an important medical pathogen appears, however, to be significantly less efficiently phagocytosed than is *Candida albicans* (Richardson and Donaldson, 1994). *Cryptococcus neoformans* is less well phagocytosed than other yeasts and, as is also the case for many bacteria, the presence of a polysaccharide capsule is likely to be a significant adaptation to prevent phagocytosis (Lyman and Walsh, 1994). Of interest, *Trichosporon beigelii*, which is phylogenetically related to *Cryptococcus neoformans*, is also less well phagocytosed even though it lacks a capsule, so other surface antigens may also modify internalization (Lyman and Walsh, 1994). Another fungal adaptation to inhibit phagocytosis by neutrophils is the glycoprotein extracellular fibrillar matrix on the spherules of *Coccioides immitis* (Frey and Drutz, 1986).

Opsonization enhances fungal internalization. Although various monoclonal antibodies against *Candida* spp. enhance phagocytosis of yeast *in vitro* (Wellington et al., 2003) their effect *in vivo* is less marked (Casadevall, 1995), but nonetheless potentially important (Valerius et al., 1997; van Spriel et al., 1999, 2001). Opsonization is particularly important for *Cryptococcus neoformans* in view of its capsule, but also mediates non-significant alterations in phagocytosis of unencapsulated *Cryptococcus neoformans* or *Candida albicans* in some studies (Monari et al., 1999). Opsonized *Cryptococcus neoformans* is taken up predominantly via the FcγRI (CD16) and FcγRIII (CD64) receptors, at least in HIV-infected individuals

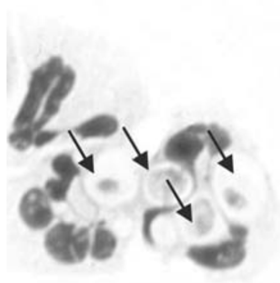

Figure 2. Neutrophils engulfing fungal particles. This photomicrograph shows neutrophils that have taken up particles of zymosan (derived from yeast cell walls) into phagosomes (arrowed) for destruction. The ability of the neutrophil to engulf multiple foreign particles in an attempt to neutralise infection is shown in the cell on the right (See Color Section.)

in whom a monoclonal antibody against this organism enhances phagocytosis and killing of yeast (Monari et al., 1999).

Other factors implicated in the opsonization of fungi include complement components and surfactant proteins. Complement components generated by the alternative pathway enhance phagocytosis of *Aspergillus fumigatus* conidia (Sturtevant and Latge, 1992). Receptors involved in recognising opsonized fungi can cooperate in order to induce specific effector functions. For example, CR3 (Mac-1, CD11b/CD18), which recognises *Candida albicans* opsonized with C3bi, does not appear to further enhance levels of FcR-mediated yeast internalization when yeast are optimally opsonized (van Spriel et al., 2001). However activation of both FcRs and CR3 during phagocytosis can modify some effector functions, such as antibody-dependent cellular cytotoxicity, though respiratory burst and degranulation are not effected by simultaneous engagement of CR3 and FcRs (van Spriel et al., 2001). Surfactant protein D can opsonize fungi, in which context it enhances phagocytosis of *Aspergillus fumigatus* (as does surfactant protein A), but not phagocytosis of *Candida albicans* (Tacken et al., 2004; Madan et al., 1997).

A complete overview of all the downstream signalling pathways activated within the neutrophil by encounter with fungi is beyond the scope of this work. Evidence to date suggests that signalling pathways activated by TLRs are similar in neutrophils and monocytes, this latter cell type being better characterised with respect to TLR signalling. Certainly engagement of TLRs, activation of integrins, production of autocrine activators such as PAF, and engagement of a phagocytosis programme will activate a broad range of signalling pathways within the neutrophil, including PI-3 kinases, MAP kinases, generation of free NF-κB, etc., as reviewed in many excellent articles including those cited here (Akira and Takeda, 2004; Kawai and Akira, 2005). Small G proteins such as Rac and Cdc42 are involved in MAPK activation following *Candida albicans* phagocytosis, and contribute to effective internalisation and microbicidal responses (Zhong et al., 2003).

2.2.3. Killing

Reactive oxygen and nitrogen species and the proteases activated in association with their generation represent major mechanisms of microbial killing by neutrophils (Christin et al., 1997; Fierro et al.). Antimicrobial proteins such as defensins also contribute to the ability of the neutrophil to kill fungi (Schneider et al., 2005), and neutrophils can release complement proteins that contribute to the formation of a membrane attack complex on the surface of yeasts (Lukasser-Vogl et al., 2000). Proteins present in azurophil granules, including defensins and also cathepsin G and BPI, can work together to inhibit the grown of yeasts such as *Histoplasma capsulatum* (Newman et al.), 2000). Fungistasis is a property of other proteins that can be derived from neutrophils, such as lactoferrin (Palma et al., 1992). Both the myeloperoxidase (MPO) and NADPH-oxidase systems generate reactive ions that contribute to defence against fungal infections (Aratani et al., 2002). Relative resistance to neutrophil hydrogen peroxide is a significant virulence factor with respect to pathogenic dimorphic fungi (Schaffner et al., 1986).

Deficiencies in the NADPH oxidase system are a feature of chronic granulomatous disease (CGD), and result in a markedly enhanced susceptibility to *Aspergillus* spp. This X-linked condition usually manifests itself in early childhood. Individuals are susceptible to most pathogens, including less virulent species such as *Staphylococcus epidermidis*. The defect occurs in the respiratory burst pathway, such that sufferers are unable to produce hydrogen peroxide. Clinically, the individual develops chronic severe forms of pneumonia, abscesses, osteomyeltis and lymphadenitis. Diagnosis may involve a combination of enzyme assays and phagocytosis function tests including quantitative nitroblue tetrazolium (NBT), chemiluminence and quantitative intracellular killing curve. Mouse models of CGD, such as the gp91 phox knock-out mice, demonstrate that deficiency of the NADPH oxidase system results in enhanced susceptibility to pulmonary disease after intratracheal challenge and greater degrees of lung inflammation (Morgenstern et al., 1997). Glucose – 6 – phosphate dehydrogenase deficiency is an X- linked inherited disorder resulting in the absence of this enzyme's activity. This results in inadequate production of NADPH, which is needed for the respiratory burst. The clinical picture is very similar to CGD. Genetic myeloperoxidase deficiencies, with varying consequences for susceptibility to microbial infection (including *Candida spp.*), have also been described.

Unsurprisingly, there is also evidence fungi may have attempted to evolve strategies that limit their killing by neutrophils (Du et al., 2005; Levitz and Diamond, 1985; Murayama et al., 1996; Smail et al., 1992). The basis of the respiratory burst generated in response to fungi involves a response to common cell wall constituents such as mannans and zymosan and can be inhibited by mannose (Danley and Hilger, 1981). However the dimorphic fungi may have evolved mechanisms to downregulate this host response. The spherule form of *Coccidioides immitis* is relatively resistant to hydrogen peroxide (Galgiani, 1986). In addition, mature *Coccidioides immitis* spherules appear to be able to inhibit hydrogen peroxide and hypochlorous acid generation, despite being capable of inducing superoxide generation (Galgiani, 1995). *Histoplasma capsulatum* binding to neutrophils is enhanced by complement-mediated opsonization and occurs via CD18, but despite effective phagocytosis under these conditions superoxide generation is minimal (Schnur and Newman, 1990). This is despite multiple lines of experimental evidence suggesting a respiratory burst occurs in neutrophils and phagolysosomal fusion occurs under these conditions, suggesting that this dimorphic fungus may have developed mechanisms to trap intracellular superoxide to subvert oxidative host defence in neutrophils (Schnur and Newman, 1990; Kurita et al., 1991).

2.2.4. Resolution of inflammation and regulation of neutrophil lifespan

Neutrophils are the most short-lived of all cell types, with a half-life in the circulation of a few hours. In culture, neutrophils die rapidly, with a half-life of around 12 hours (there being some variations in death rates between laboratories) (Sabroe et al., 2004), and thereafter die rapidly by apoptosis (Figure 3). Apoptosis can result from activation of either an 'intrinsic' or an extrinsic pathway, the former

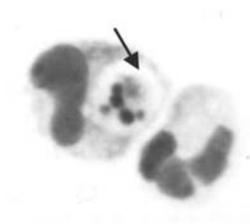

Figure 3. Removal of aptoptotic neutrophils. In this smear, leukocytes were isolated from the joint of a patient with rheumatoid arthritis. The two cells shown here are monocytes, one of which has engulfed an apoptotic neutrophil (arrowed), for removal in an injury-limiting fashion that is associated with downregulation of proinflammatory macrophage function (See Color Section.)

mediated via ligation of death receptors such as Fas and TRAIL and the latter via stress-induced changes in mitochondrial permeability and activation of both caspase-dependent and independent pathways. This intrinsic pathway is regulated by both pro- and anti-apoptotic members of the Bcl-2 family. These complex processes are reviewed in a number of excellent articles to which the reader's attention is directed (Simon, 2003; Adams and Cory, 1998; Ashkenazi and Dixit, 1998; Thornberry and Lazebnik, 1998; Green and Reed, 1998).

The key role of apoptosis in resolution of neutrophilic inflammation was first appreciated by Savill and Haslett (Savill et al., 1989), allowing safe clearance of these potentially dangerous cells from tissues. Ideally, in the context of infection, neutrophils will remain viable until invading pathogens are killed, but thereafter apoptosis would proceed promptly to abrogate inflammation and avoid tissue damage (Haslett, 1997). With resolution of inflammation, apoptotic neutrophils are cleared by phagocytes, particularly macrophages (Savill and Fadok, 2000). In a further layer of anti-inflammatory regulation, macrophage ingestion of apoptotic neutrophils results in induction of an anti-inflammatory cytokine profile in these cells. In contrast, neutrophils that die by necrosis rather than apoptosis are proinflammatory, both through release of cell contents into the local milieu, and through induction of proinflammatory cytokines from monocytes and macrophages (Fadok et al., 2001). In the majority of circumstances, as illustrated by the extraordinary manner in which a bacterial or fungal pneumonia resolves to leave normal or near-normal lung architecture, the process of removal of neutrophils by apoptotic cell death is an exceptionally efficient resolution mechanism (Haslett, 1997).

Activated neutrophils show a marked relative prolongation of their lifespan (Lee et al., 1993; Colotta et al., 1992). The ability to directly prolong lifespan is largely restricted to a group of proinflammatory cytokines such as TNFα and GM-CSF: it is interesting that chemokines such as CXCL8 are relatively poor stimulators of enhanced survival, implying compartmentalised regulation of neutrophil recruitment, activation, and survival. Growth factor-mediated delay of apoptosis thus acts in concert with effects on bone marrow to increase the numbers of functionally competent neutrophils present at a site of infection. Pathogens themselves can cause enhanced survival by direct engagement of TLRs, but this

survival response appears to be relatively small compared to that induced by pathogen activation of TLRs on bystander monocytes, which results in the release of potent survival factors from these cells (Sabroe et al., 2002). It is plausible that the direct survival response to TLR agonists is relatively weak in order to prevent a single contact with a pathogen resulting in the generation of an activated neutrophil with a very long lifespan, whose production of antimicrobial factors could outlive the pathogen. The regulation of neutrophil lifespan through TLR signalling of other cell types such as monocytes provides an attractive external control mechanism, allowing withdrawal of survival factors and resolution of inflammation once the infective insult has been cleared.

Some pathogens have evolved strategies to manipulate neutrophil apoptosis to their advantage. For example, the Gram-negative bacterium, *Pseudomonas aeruginosa*, secretes a toxin that accelerates neutrophil apoptosis *in vitro* (Usher et al., 2002) and may favour bacterial persistence at inflammatory sites (Allen et al., 2005). There is also some evidence that the interaction between fungi and neutrophils may change rates of apoptosis to the advantage of the pathogen (Medeiros et al., 2004).

3. EXCESSIVE ACTIVATION AND DISEASE

Although defective neutrophil function can result in disseminated infection, inappropriate or overwhelming neutrophil activation can also be a major cause of disease. Destructive neutrophilic inflammation is thought to contribute to a range of pathologies, from asthma to rheumatoid arthritis to vasculitis. In the context of fungal disease, the role of the neutrophil is beneficial to the host, but progession of sepsis to syndromes such as the acute respiratory distress syndrome (ARDS) is associated with an extremely high mortality. ARDS is a neutrophil-driven acute inflammatory disease, in which neutrophil-mediated capillary damage results in a dramatic non-cardiogenic pulmonary oedema and severe respiratory failure. In this context, it is relevant that anecdotally, engraftment of bone marrow and reappearance of neutrophils in the peripheral circulation can, in the context of pulmonary infection, be associated with a transient worsening of oxygenation and a deterioration in respiratory status. Clearly, neutralisation of neutrophil function in this setting would not be desirable, but such scenarios demonstrate the requirement for careful regulation of neutrophil function by the host in health and disease.

4. CONCLUSION

The neutrophil is central to our defence against fungal infection and disease. Its function is regulated at all levels from production to recruitment to activation and survival, providing a robust defence mechanism that is relatively rarely implicated in unwanted disease. Therapeutic manipulation of neutrophilic inflammation, for example by the administration of G-CSF to encourage bone marrow production and prime neutrophil function, is already an established strategy in the treatment of fungal disease.

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CHAPTER 4

LYMPHOCYTES

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Abstract: Lymphocytes play a critical role in the defense against viral, bacterial and fungal infections. In the last few years our understanding of lymphocyte involvement in response to fungi has dramatically increased. Recent studies examining the immunopathogenesis of fungal infections have provided major insight into the crucial role of different T lymphocyte subsets in protection. The controlled activation of the Thl subset is the common denominator for ensuring protection against most fungal infections. However, nature of fungal organism (commensal opportunistic or frank pathogen), site of infection (local or systemic) and host susceptibility dictate kinetics, magnitude, and character of T lymphocyte responses in a highly complex, intricate regulatory pattern. This chapter describes progress in some of the major experimental models of lymphocyte-mediated immunity to fungal infections

Abbreviations: Ab: antibody; Ag: antigen; MHC: major histocompatibility complex; MHC–I: major histocompatibility complex class I; MHC–II: major histocompatibility complex class II; pMHC: peptide-major histocompatibility complex; Th: T helper cells; Th1: type I T helper cells; Th2: type II T helper cells; T_{reg}: regulatory T cells; TCR: T cell receptor; Hsp60: heat shock protein 60; ABPA: allergic bronchopulmonary aspergillosis; HVEM: herpes virus Entry Mediator; ICOS: inducible co-stimulator; SLAM: signaling lymphocyte activation molecule; CTLA-4: cytotoxic T lymphocyte antigen-4; PD-1: programmed death-1; BTLA: B and T lymphocyte attenuator; APC: antigen presenting cells; DC: dendritic cells; IL: interleukin; PBMC: peripheral blood mononuclear cells; IFN: interferon; TNF: tumor necrosis factor; Tr1: type I regulatory T cells; Th3: type III T helper cells; GITR: glucocorticoid inducible tumor necrosis factor receptor; PCP: *P. carinii* pneumonia; BCR: B cell receptor; CD40 L: CD 40 ligand

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1. CHARACTERISTICS AND MORPHOLOGY OF LYMPHOCYTES

Lymphocytes are small white blood cells that bear the major responsibility for carrying out immune system activities. They are derived from bone marrow pluripotent stem cells. Lymphocytes are produced at a high rate (10^9 per day) and migrate via circulation to secondary lymphoid tissues. Lymphoid cells represent 20% of total white blood cells present in adult blood circulation.

The two major classes of lymphocytes are: T cells, which are processed in the thymus where they acquire CD4 or CD8 molecules, and B cells, which grow to maturity independent of the thymus.

The term CD stands for "cluster differentiation" and was introduced to classify monoclonal antibodies (Abs) that recognize identical subgroups from a panel of a leukocyte cell line. The CD nomenclature is now used almost exclusively in referring to leukocyte antigens (Ags). CD4 and CD8, the most intensively studied of the CD Ags, are glycoproteins expressed on thymus derived lymphocytes.

B cells and T cells are able to recognize specific Ag targets. Both have a large nucleus and agranular cytoplasm and are indistinguishable on the basis of their morphological characteristics, although they have different functions and surface markers. B cells are featured by the cell surface presence of Ig, class II major histocompatibility complex (MHC-II) molecules, and receptors for products of complement cascade (C3b and C3d). These cells work chiefly by secreting Abs into the body fluids (humoral immunity). Conversely, T cells show the ability to interact directly with their targets (cellular immunity).

Mature T cells carry a CD3 marker, most T helper (Th) cells also carry a CD4 marker, while a CD8 marker is found on many suppressor/cytotoxic T cells. All T cells express an Ag-binding T cell receptor (TCR) on their cellular surface. The TCR is very similar in structure to immunoglobulin. It has two paired polypeptide chains (alpha/beta (α/β) or gamma/delta (γ/δ)) that have a constant (c) or variable (v) portion. Most lymphocytes (CD4 and CD8) express (α/β)-TCR and a secondary population of T cells expresses the alternative (γ/δ)-TCR. γ/δ T cells are a minor population in the peripheral blood but constitute a major population among intestinal intraepithelial lymphocytes.

2. RECOGNITION OF ANTIGENS BY T CELLS

T lymphocytes recognize fragments of antigenic proteins when these are bound to products of MHC genes (Kourilsky and Claverie, 1989) on antigen presenting cells (APC). The recognition by T lymphocytes of peptide fragments (Ags) derived from foreign pathogens is mediated by the TCR, which transduces extracellular signals by initiating a wide array of intracellular signaling pathways. Two main types of MHC proteins have been described, referred to as class I (MHC–I) and class II (MHC–II). CD4 T cells respond to Ag in association with MHC-II molecules and CD8 T cells respond to Ag in association with MHC-I molecules. The peptide-major histocompatibility complex (pMHC) interacts with T cell surface CD4 and

CD8 which bind to MHC-I and MHC-II respectively. However, the binding sites for CD4 and CD8 are separate from the TCR recognized pMHC and allow a single MHC molecule to be bound simultaneously by both TCR and either CD4 or CD8 (Gao et al., 1997; Wang et al., 2001b).

2.1. T Cell Receptor Usage to a Protective Antigen from Fungi

Very little information is available regarding TCR usage to a protective Ag from fungi. An elegant paper from Deepe's group examined a TCR repertoire of cells that react with F3, an immunodominant heat shock protein 60 (Hsp60) Ag from *H. capsulatum*, that confers protection against a lethal *H. capsulatum* challenge. The researchers reported that Vβ6 T cells are instrumental both to generation of Ag-reactive cells, and to protective efficacy against *H. capsulatum* (Deepe and Gibbons, 2001a). Furthermore, this group demonstrated that immunization with recombinant Hsp60 produced a very different profile in the T cell repertoire, compared to immunization with F3. The response to recombinant Hsp60 was dominated by generation of Vβ 8.1/8.2 T cells, while F3 response was characterized by preponderant Vβ6 T cells. These two types of Vβ cells were both important in the generation of protective responses induced by specific Ag (Scheckelhoff and Deepe, 2002).

A study of TCR bias in patients with allergic bronchopulmonary aspergillosis (ABPA) was carried out by Chauhan B. et al (Chauhan et al., 2002). The majority of ABPA patients expressed allergen-specific T cells with Vβ13 genes, whereas Vβ1 gene T cell repertoires were predominantly expressed in non ABPA controls. These data suggest that increased usage of Vβ13 in ABPA and Vβ1 in non ABPA could be important in susceptibility and resistance, respectively.

A study regarding *P. carinii*-specific CD4 T cell repertoire in HIV infected patients has been performed by Li Pira G. et al. (Li Pira et al., 2002). The Authors observed that clonal heterogeneity of *P. carinii*-specific T cell repertoires was preserved in seropositive asymptomatic individuals.

2.2. T Cell Costimulation

T cells require more than the signal generated by the TCR complex in order to decide whether and how to respond to an encountered Ag. The contribution of the many molecules involved in this decision, either on primary or secondary encounter of Ag, is well established. Recent data have reported that the activation (or down-regulation) of T cells is a process of multisignal integration, the TCR being mainly responsible for the specificity of the response.

The T cell costimulatory molecules can be defined as cell surface molecules that cannot functionally activate T cells on their own, but rather amplify or counteract signals provided by the TCR complex. Molecules that facilitate adhesion, but do not regulate TCR signal, are not considered as co-stimulatory.

The large majority of T cell co-stimulators belong to either the Ig (CD28-like) or the tumor necrosis factor (TNF) receptor superfamilies of molecules (Croft, 2003). They can be considered as constitutively expressed (CD28, CD27, HVEM) on T cells, or induced after Ag recognition by the TCR. Constitutively expressed molecules are positive regulators, whereas inducible co-stimulators are positive (ICOS, CD30, OX40, 4-1BB, SLAM, CD40L) as well as negative (CTLA-4, PD-1, BTLA) regulators (Kroczek et al., 2004). The respective ligands of these molecules are expressed on all professional APC, including dendritic cells (DC), B cells and macrophages (Coyle and Gutierrez-Ramos, 2001).

A number of data evidence a critical role for constitutive and inducible costimulatory molecules in regulating susceptibility or resistance to fungal infections.

2.2.1. *T cell costimulation in* Candida albicans

The role of T cell costimulation via CTLA-4 has been studied in an experimental model of murine candidiasis. It has been observed that production of interleukin (IL)-4 by CD4 T cells is critically dependent upon CTLA-4/B7 interaction (Spaccapelo et al., 1997). Interference with this costimulatory pathway during infection could result in regulation of CD4 type I T helper cell (Th1) cell response (Spaccapelo et al., 1997). Further studies demonstrated that blockage of this costimulatory pathway results in decreased IL-4 production by infected IL-12-deficient mice (Mencacci et al., 1998b). The role of costimulation in T cell response was also studied by evaluating peripheral blood mononuclear cells (PBMC) proliferative response by blocking various costimulatory molecules. PBMC were obtained from patients with atopic dermatitis and were stimulated with *C. albicans*. The results showed that costimulatory molecules play a role in regulating proliferative response of PBMC (Kawamura et al., 1998). In addition, it has been demonstrated that neutrophils expressing CD80 may adversely affect Th1-dependent resistance in fungal infections, through inhibition of interferon (IFN)-γ-producing CD4 T cells via CD80/CD28-dependent mechanisms (Mencacci et al., 2002).

2.2.2. *T cell costimulation in* Cryptococcus neoformans

In an in vitro experimental system, an association between presence of capsule and rapid induction of costimulatory CTLA-4 molecules on T cells, has been observed by using PBMC from healthy donors stimulated with *C. neoformans*. These results suggest a new mechanism that enables *C. neoformans* to elude host immune response (Pietrella et al., 2001a). Moreover, a positive role for costimulation via CD40L in promoting protective response against *C. neoformans* has been suggested in another study (Vecchiarelli, 2000a).

In a more recent paper, these results were expanded and experiments were conducted by using mice genetically lacking CD40L. This study showed that IL-12 and IFN-γ were decreased in these mice with respect to wild-type counterpart. This was correlated with scarce inflammatory response and enhancement of fungal growth (Pietrella et al., 2004).

2.2.3. *T cell costimulation in* Pneumocystis carinii

P. carinii (P. jirovecii) infections, referred to overall as pneumocystosis, are observed in four clinical forms: asymptomatic infections, infantile (interstitial plasma cell) pneumonia, pneumonia in immunocompromised host, and extrapulmonary infections (Stringer et al., 2002). The requirement for T cell costimulation in *P carinii* infection was underlined by demonstrating that costimulation-dependent T cell-mediated inflammation plays an important role in both susceptibility to, and pathogenesis of *P. carinii* pneumonia (PCP) (Baumgartner et al., 2002).

In addition, it has been demonstrated that after intratracheal injection of *P. carinii*, mice deficient in both costimulatory molecules CD2 and CD28, spontaneously developed PCP, despite normal T cell numbers. However, double deficient mice retained sufficient immune function to clear the infection. Thus, costimulatory molecule function is critical in determining initial susceptibility to infection with *P. carinii* (Beck et al., 2003).

3. CD4 LYMPHOCYTES

The two major subsets of CD4 Th, type I (Th1) and type II (Th2), are characterized by different cytokine production patterns and have different roles in immune response. Each subset induces functions that are effective in handling certain types of pathogens, but can be ineffective or pathological when responding to other pathogens (Mosmann, 1996). It is well known that Th1 secrete IFN-γ, IL-2, TNF-α and TNF-β. TNF is also produced by Th2, but it is considered a Th1 associated cytokine. Th1 cytokines promote activation of natural effector cells and delayed hypersensitivity, they induce resistance to intracellular pathogens, including fungi. (Mosmann and Sad, 1996; Bot et al., 2004; Fidel, 2002; Romani, 1999). Th2 secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Th2 cytokines encourage Ab production, important in fighting extracellular pathogens (Mosmann and Sad, 1996).

Most studies have focused on regulatory T cells (T_{reg}) that account for 5–10% of circulating T cells. This population of CD4 T cells expresses IL-2α receptor (CD25), and is able to maintain tolerance to self Ags. However CD25 expression does not fully characterize T_{reg} because CD25 is expressed by T cells in cultures including Th1 and Th2. As a consequence, it is impossible to clone Ag-specific CD4CD25 cells with T suppressor activity or with effector function. Regulatory T cells have been shown to be capable of suppressing proliferation and cytokine production by other populations of T cells, limiting tissue damage caused by a strong anti infectious immune response (Belkaid and Rouse, 2005; Mittrucker and Kaufmann, 2004).

There are distinct T cell subtypes of T_{reg} of which some are induced in response to infectious agents, while others are natural regulators. Inducible T_{reg} such as type I regulatory T cells (Tr1) and type III T helper cells (Th3) can develop from conventional CD4 T cells that are exposed to specific stimulatory conditions such as deactivating cytokines and blockage of costimulatory signals. Natural T_{reg} are generated from the normal process of maturation in the thymus and constitutively

express CD25, CTLA-4 and the glucocorticoid inducible tumor necrosis factor receptor (GITR). These cells have been described in appropriate reviews (Bluestone and Abbas, 2003; O'Garra et al., 2004; Mills and McGuirk, 2004).

3.1. T Helper Immune Response to Fungi

The establishment of the Th1/Th2 paradigm has been one of the major advances in the field of microbial immunology, providing a framework for studying the distinct immune responses against different pathogens. There is a great deal of clinical evidence and experimental data indicating that susceptibility and resistance to different infections may be associated with occurrence of distinct cross-regulated Th1/Th2 responses: Th1 intervene in cell-mediated responses, such as delayed type hypersensitivity and macrophage activation, and play a central role in the host defense mechanism against intracellular pathogens; Th2 mediate humoral and allergic responses and are involved in the host defense against helminthic infection and in the pathogenesis of allergic diseases by inducing IL-4 and IL-5 production (Mosmann and Sad, 1996; Mosmann and Coffman, 1989). Moreover, Th2 inhibit the development and biological activities of Th1 cells. Thus, according to this paradigm, a successful immune response to an infectious agent can depend on the activation of the appropriate, more than a sufficient, immune effector function (Powrie and Coffman, 1993) and, as a corollary concept in microbial pathogenesis, microorganisms may evade host-protective immunity throughout activation of an inappropriate Th response (Powrie and Coffman, 1993).

As regards fungal infections, it is now clearly demonstrated that efficient host defense results from an optimal interplay between innate and adaptive immune system, intimately linked and controlled by sets of molecules and receptors. Neutrophils and monocytes are involved in the non-specific clearance of yeasts (e.g. *C. albicans* and *C. neoformans*), while Th1 responses mediate specific protection via release of IFN-γ, which in turn enhances antifungal effector functions of phagocytes. By contrast, Th2 responses and IL-4 and IL-10 release correlate with disease exacerbation and pathology (Altamura et al., 2001). In the last few years, many studies have provided insight into the important regulatory role of Th and cytokines to different fungal pathogens, and into their immunoprophylactic or therapeutical potential.

3.1.1. T helper immune response in Candida albicans

Both innate resistance and acquired immunity play a role in maintaining *C. albicans* in the commensal state and protecting from systemic spreading.

Current opinion is that Th1 responses characterize the carriage of saprophytic yeast and the resistance to disease seen in healthy humans, whereas Th2 responses are associated predominantly with pathology observed in immunocompromised hosts (Puccetti et al., 1995; Fidel and Sobel, 1994). Although Th1 cytokines have been shown to direct the overall outcome of infection, the precise role of the Th1/Th2

response in systemic candidiasis, appears to apply mainly to the development of resistance to re-infection (Mencacci et al., 1998b; Mencacci et al., 1998a).

The involvement of T cell response against *C. albicans* has been studied in local and systemic infections.

Local infection. The role of T cells in local candidiasis is often controversial and not well defined.

Regarding vaginal infections, studies by Fidel's group illustrated a lack of a protective role of T cells against *C. albicans* vaginitis. This assertion was due to the evidence that most T cell-immunodeficient or knockout mice had a vaginal fungal burden similar to that of wild-type strains (Wormley et al., 2003). In support of the missing T cell involvement at vaginal level, they demonstrated that following intravaginal challenge with *Candida* Ag in adult women, there was no evidence of local immune stimulation, including changes in Th (Fidel et al., 2003).

A role for oral and vaginal epithelial cells in retarding or arresting the growth of *C. albicans*, has been described (Nomanbhoy et al., 2002). It was also observed that antifungal activity is dependent on contact of the yeast with intact, but not necessarily live, epithelial cells (Yano et al., 2005). On the other hand, Fidel's group reported the presence of CD8 T cells in oropharyngeal candidiasis lesions, of HIV-positive patients suggesting evidence for a local response, despite reduced levels of CD4 T cells (Lilly et al., 2004).

Data on local immunity were also provided by Cassone's group, demonstrating the presence of protective Abs and Th1 cytokines in vaginal fluids, and suggesting local humoral and cellular responses involved in anticandidal protection at vaginal level (de Bernardis et al., 2000). As a matter of fact, a role for CD4 T cells in accelerating the clearance of the fungus from the vagina, has been reported (Santoni et al., 2002). In addition, the presence of *C. albicans*-specific Th1 in vaginal mucosa of women with recurrent vaginal candidiasis was documented (Piccinni et al., 2002). Furthermore, a clear critical role for humoral immunity against vaginal candidiasis has been stressed (De Bernardis et al., 2002; Torosantucci et al., 2005).

Systemic infection: Previous reports indicate that cell-mediated (Th1) immunity, rather than humoral immunity is the key to host protection against murine disseminated candidiasis (Kuruganti et al., 1988; Cenci et al., 1997; Romani et al., 1992; Spellberg et al., 2003; Spellberg, 2001). As a matter of fact, some important virulence traits of *C. albicans* such as mycelial transition, could influence the development of T cell response. In particular, different forms of *C. albicans* oppositely trigger Th response. Pulsing murine DC with yeast or hyphae resulted in induction of Th1 or Th2, respectively (d'Ostiani et al., 2000). However, recent studies highlight the induction of substantially similar functional patterns in human DC encountering the different forms of growth of *C. albicans*, both seemingly activating Th1 immunity (Romagnoli et al., 2004).

Even though the presence of Th2 response and T_{reg} could be regarded as deleterious for the development of protective response (Mills and McGuirk, 2004; Claudia et al., 2002), this type of response could be considered beneficial under particular conditions. For instance, Th2 cytokines, such as IL-4 and IL-10, are required for

the maintenance of Th1-mediated immune resistance to the fungus (Mencacci et al., 2000; Mencacci et al., 1998b; Mencacci et al., 1998a) and T_{reg} that are generated in mice with candidiasis are essential components of host-protective antifungal Th1 immunity (Montagnoli et al., 2002).

In summary, T cell mediated immunity is the key to host defense against *C. albicans* infection. The induction and maintenance of Th1 immune response is a critical event for protective response at systemic level.

3.1.2. T helper immune response in Aspergillus fumigatus

A number of experimental data point to the protective role of Th1 reactivity in aspergillosis and to the deleterious role of Th2 development. In particular, the roles of Th1 and Th2 have been elucidated in a murine model of invasive pulmonary aspergillosis in which resistance to infection is associated with IFN-γ-producing interstitial lung lymphocytes and high-level production of TNF-α and IL-12 from lung phagocytes (Cenci et al., 1999; Cenci et al., 1998; Mehrad et al., 1999), whereas dominant release of Th2 cytokines of interstitial lung lymphocytes is associated with progressive disease (Cenci et al., 1998). Th1-mediated resistance to invasive aspergillosis is further confirmed by using IL-4 and IL-10 knockout animals for the associated cytokines such as IL-4 and IL-10 (Clemons et al., 2000; Cenci et al., 1999). Protective immunity is documented in animals sensitized with a sublethal challenge of *A. fumigatus* conidia, developing a Th1 response on subsequent exposure to lethal infection (Cenci et al., 1999; Cenci et al., 1998).

A crucial role in driving the type and the strength of T cell response has been ascribed to DC. As a matter of fact, Th polarization is dependent on the interaction of immune cells with conidia and hyphae of *A. fumigatus*. In particular, murine and human DC phagocytose conidia and hyphae of *A. fumigatus* through distinct recognition receptors (Bozza et al., 2002). DC transport conidia and hyphae of *A. fumigatus* from the airways to the draining lymph nodes, and initiate disparate Th responses to the fungus. The entry mode of fungi into DC is responsible for Th polarization and patterns of susceptibility or resistance to infection (Bellocchio et al., 2005). It is noteworthy that the role of DC in induction of T cell response is further emphasized, given that the infusion of fungus-pulsed or RNA-transfected DC also accelerates recovery of functional antifungal Th1 responses in mice with allogeneic hematopoietic stem cell transplantation (Bozza et al., 2003) and that DC pulsed with *A. fumigatus* f16 allergen are able to induce specific T cells that may be capable of conferring immunity to invasive aspergillosis (Ramadan et al., 2005).

However, *A. fumigatus* can suppress the human cellular immune response via gliotoxin-mediated apoptosis of APC (Stanzani et al., 2005). Interestingly, the doses at which gliotoxin targeted and killed peripheral APC in vitro were significantly below those demonstrated in serum of patients with documented invasive aspergillosis (Stanzani et al., 2005).

In conclusion, there is evidence for Th1-dependent resistance against *A. fumigatus* and for a pivotal role for DC in sustaining, driving and transferring the T cell

response. It is noteworthy that fungal components such as gliotoxin affect the viability of APC, and this observation could be important to revisit the *A. fumigatus*/APC interaction and the subsequent induction of T cell-mediated response.

3.1.3. T helper immune response in Cryptococcus neoformans

There is evidence for the essential role of T cell mediated immunity in host defense against cryptococcal infection (Collins and Bancroft, 1991; Lim and Murphy, 1980). In particular, direct anticryptococcal activity has been observed for T lymphocytes (Levitz et al., 1995; Muth and Murphy, 1995b; Muth and Murphy, 1995a; Levitz and Dupont, 1993; Murphy et al., 1993) and the development of Th1 response is considered critical for positive outcome of cryptococcosis. In the development of T cell response, a critical step is represented by APC interaction with this fungus; as a matter of fact, the impact of *C. neoformans* with APC could be considered decisive for driving protective or non protective T cell response (Vecchiarelli, 2000b; Vecchiarelli, 2000a). It is noteworthy that the capsule represents the principal virulence factor of *C. neoformans*. Its major constituent is glucuronoxylomannan (GXM) together with other minor components, namely mannoproteins (MP) and galactoxylomannan (GalXM). These compounds have different and even somewhat opposite effects on immune response to the fungus. Studies performed using purified GXM, GalXM and MP and an acapsular *C. neoformans* strain evidenced that GXM is able to inhibit T cell response and development of Th1, by inhibiting APC functions (Vecchiarelli et al., 2003; Retini et al., 2001; Retini et al., 1998; Vecchiarelli et al., 1996; Vecchiarelli et al., 1994), including IL-12 production (Vecchiarelli, 2000b; Retini et al., 1999). Conversely, MP, a minor constituent of capsular material, promotes the efficiency of APC favoring Th1 response (Pietrella et al., 2005; Mansour et al., 2004; Mansour et al., 2002; Levitz et al., 2001; Pietrella et al., 2001b; Pitzurra et al., 2000). Differently from GXM and MP, GalXM exerts a direct inhibitory activity on T cells bypassing APC (Pericolini et al., 2005).

C. neoformans infection usually occurs via inhalation and particular attention has been devoted to immune defenses at lung level. It has been established that pulmonary clearance of *C. neoformans* requires the presence of CD4 T cells (Hill and Aguirre, 1994) and development of Th1 immunity (Hernandez et al., 2004; Huffnagle, 1996). CD8 T cells are also involved in favoring the clearance of *C. neoformans* from the lung (Huffnagle et al., 1994; Mody et al., 1993), and CD8 T cell function could be independent of CD4 T cells (Lindell et al., 2005).

In conclusion, like for other fungi, the development of Th1 response is considered critical in fighting the *C. neoformans* infection at systemic, as well as at pulmonary, level. T lymphocytes seem to have direct anticryptococcal activity, and convincing evidence exists for the role of CD8 T cells in circumventing the infection at lung level.

3.1.4. *T helper immune response in* Histoplasma capsulatum

The efficacy of the protective immune response to *H. capsulatum* requires an orchestration of numerous cellular and soluble effectors. CD4 and CD8 T cells contribute to the clearance of the organism in both primary and secondary infection (Allendorfer et al., 1999; Deepe, 1994; Gomez et al., 1988; Wuthrich et al., 2003). In addition, the role of Th1 immune responses in mediating protection against many intracellular infections including *H. capsulatum* has been documented (Zhou and Seder, 1998; Zhou et al., 1995). Noteworthy are interesting studies performed recently, which report that *H. capsulatum* induces apoptosis in the lungs of mice, T cells being the dominant apoptotic population. Apoptosis of T cells blunts clearance of *H. capsulatum* (Allen and Deepe, 2005).

3.1.5. *T helper immune response in* Pneumocystis carinii

Clinical and experimental studies have documented that host defense against *P. carinii* involves a concerted effort between innate, cell-mediated and humoral responses (Steele et al., 2005), but the exact role of Th immunity to this fungus is not yet completely clear.

The fact that patients receiving immunosuppressive therapy with corticosteroids and cytotoxic agents may develop PCP indicates that a severe T cell defect is a major predisposing factor for this infection (Steele et al., 2005). The risk of acquiring PCP seems to increase when CD4 lymphocyte counts drop below 200 per microliter, regardless of the underlying disease (Gluck et al., 2000).

In a murine model of PCP it was found that an early T lymphocyte response is present in draining lymph nodes, followed by later recruitment of Th1 and Th2 lymphocytes into lung tissue, suggesting that both Th1 and Th2 lymphocyte responses are involved in clearance of *P. carinii* (Shellito et al., 2000). Moreover, mice deficient in T cell costimulation are susceptible to acute infection with PCP (Beck et al., 2003).

3.2. Regulatory T Cells

There is some evidence for a CD4CD25 T_{reg} cell role in fungal infections. These cells are locally generated during vaginal candidiasis (de Bernardis et al., 2000), and have been proved to be an essential component for inducing memory-protective immunity to *C. albicans* (Montagnoli et al., 2002). Further studies in B cell deficient mice supported previous observations by showing that mice did not survive re-infection with *C. albicans*; this circumstance was concurrent with the failure to generate IL-10-producing DC and CD4CD25 T_{reg} (Bozza et al., 2003).

More recent results could imply that the effects of CD4CD25 T_{reg} are deleterious when the pathogen penetrates the mucosa and disseminates throughout the bloodstream (Netea et al., 2004). In addition, a deleterious effect of CD4CD25 cells in human coccidioidomycosis has been suggested in a recent study. By using immunohistochemical staining for lymphocyte subsets, discrete perigranulomatous lymphocytic clusters were observed and CD4CD25 T lymphocytes were identified,

suggesting that down-regulation of the cellular immune response occurs within coccidioidal granulomata (Li et al., 2005).

In conclusion, the role of T_{reg} has been principally studied in *C. albicans* infections and it seems as though these cells may have different, even opposite roles, depending on whether infection is local or systemic and on the development of primary or memory immune responses.

4. CD8 LYMPHOCYTES

T lymphocyte immunity, considered the main defense against fungal infections, is predominantly carried forth by CD4 T cells, therefore any defects in their many functions increase susceptibility. However, although the critical role of CD4 T cells is indubitable, there is evidence showing that CD8 T cells also mediate a protective response against fungi. In particular it has been demonstrated that CD8, similarly to CD4 cells, show plasticity in their capacity to regulate antifungal immunity.

4.1. CD8 Response in *Candida albicans*

The role of CD8 T cells has been studied particularly in mucosal *C. albicans* infections. In vaginal candidiasis, the importance of CD8 T cells in participating to local immune response is not clear. Indeed it has been suggested that CD8 cells could play a marginal role in anti-*Candida* immunity at the vaginal level (Santoni et al., 2002). Moreover, a considerable and persistent increase of CD8 T cells in vaginal candidiasis has been observed, but their role has not yet been defined (Ghaleb et al., 2003). A controversial role for CD8 T cells has been reported in oropharyngeal candidiasis, in a mouse experimental model and in HIV positive subjects. In the mouse experimental model, depletion of CD4, but not CD8, prolonged infection in the compromised host (Farah et al., 2001; Farah et al., 2002). Human studies in HIV positive and negative subjects provide convincing evidence on the important role of CD8 T cells in mucosal host defense against oropharyngeal candidiasis, especially when CD4 cell numbers are reduced (McNulty et al., 2005; de Repentigny et al., 2004; Lilly et al., 2004; Myers et al., 2003).

In summary, although CD8 cells are an important population evidenced at local level, their role in local candidiasis is not clearly defined.

4.2. CD8 Response in *Aspergillus fumigatus*

CD8 T cells have been attributed a role in induction of cytotoxic response against epitopes from *A. fumigatus* allergen, suggesting that these cells may be capable of conferring immunity to invasive aspergillosis (Ramadan et al., 2005). Recent studies in a model of intranasal *A. fumigatus* infection showed that the pulmonary surfactant protein affected CD4, but not CD8 T cells, acting as a natural immunosuppressant in the lung, with direct inhibitory actions on allergen-stimulated CD4 Th2 lymphocytes (Scanlon et al., 2005). In addition, in an experimental model of dogs with nasal

aspergillosis, the immunohistochemical findings from the inflammatory infiltrate showed the presence of CD4 and CD8 T cells (Peeters et al., 2005).

In conclusion, the role of CD8 T cells against aspergillosis is not defined, although some data suggest the presence of CD8 in the inflammatory infiltrate and a role for CD8 T cells in conferring immunity to invasive aspergillosis.

4.3. CD8 Response in *Cryptococcus neoformans*

Compelling evidence underlines that a T cell response, involving both CD4 and CD8 T cell recruitment, is required in order to prevent *C. neoformans* dissemination, to restrict pathogen to the lung and to resolve infection (Hill and Harmsen, 1991; Huffnagle et al., 1991). Very recently, a role for CD8 against *C. neoformans* infections has been suggested (Edwards et al., 2005; Miyagi et al., 2005). An excellent recent paper by Huffnagle's group supports the hypothesis that effector CD8 T cell function is independent of CD4 T cells and that IFN-γ production from CD8 T cells plays a role in controlling *C. neoformans* by limiting survival of *C. neoformans* within macrophages (Lindell et al., 2005). The role of CD8 in mediating resistance to *C. neoformans* was also stressed by using an experimental model of mice lacking of CD4 (Aguirre et al., 2004).

4.4. CD8 Response in *Histoplasma capsulatum*

Activation of CD8 has been shown after infection with *H. capsulatum*. Magnitude of CD8 T cell response was lower than that of CD4 T cell response, but expansion and contraction of both cell types followed the same kinetics (Lin and Wu-Hsieh, 2004). The role of CD8 in limiting *H. capsulatum* replication has been recently suggested. In particular, CD8 T cells from *H. capsulatum*-infected mice exhibited cytotoxic activity against macrophage targets containing *H. capsulatum*. Furthermore, CD8 T cells can be stimulated by DC that present exogenous *H. capsulatum* Ags, either through direct ingestion of yeasts or through uptake of apoptotic macrophage-associated fungal Ags (Lin et al., 2005).

In summary, CD8 participate to immunity to *H. capsulatum* through their cytotoxic activity and by mediating a state of vaccine resistance against this fungus (Deepe and Gibbons, 2002; Deepe and Gibbons, 2001b).

4.5. CD8 Response in *Pneumocystis carinii*

CD8 T cells can participate in defense against *P. carinii* when CD4 T cells are unavailable (Beck and Harmsen, 1998). A dual role in response to *P. carinii* was proposed for CD8 T cells recruited in the lung: a positive one ascribed to cytotoxic CD8, which facilitates the clearance of *P. carinii*, and a negative one ascribed to non cytotoxic CD8, which contributes to lung injury (McAllister et al., 2004).

Further investigation indicated that CD8 T cell recruitment and lung damage is type I IFN (IFN-α/β) dependent (Meissner et al., 2005).

5. γ/δ LYMPHOCYTES

The thymus generates two major lineages of mature α/β T cells, defined by CD4 and CD8 expression, and differing in MHC-restricted Ag recognition and peripheral effector functions. While most T cells use a CD3-associated α/β TCR as the Ag recognition structure, a secondary population of T cells expresses the alternative γ/δ TCR. T lymphocytes bearing γ/δ TCR are a minor fraction of T cells in peripheral blood, and are rarely found in lymph nodes and spleen; conversely, they are abundant in intestine, skin, tongue, esophagus, trachea, lungs and genital epithelia (Hayday, 2000; Triebel and Hercend, 1989).

Most γ/δ T cells recognize ligands, which are different from the short peptides recognized by α/β T cells in the context of MHC-I or MHC-II molecules. The biological activity of γ/δ T cells includes the production of a variety of cytokines and potent cytotoxic activity, also against many tumor cells.

There are controversial data regarding the role of γ/δ T cells in *C. albicans* infections. In particular, it has been demonstrated that in mucosal candidiasis, γ/δ T cells have positive influence on macrophage function by favoring nitric oxide production and anticandidal activity (Jones-Carson et al., 1995). A more recent paper reported a negative role for γ/δ T cells in an experimental model of vaginal candidiasis, by demonstrating that TCR γ-chain-knockout mice had significantly less vaginal fungal burden, when compared to wild-type mice (Wormley et al., 2001).

Results recently reported by Fidel's group showed that considerable numbers of γ/δ TCR cells at mucosal level have been observed during oropharyngeal candidiasis. Similarly, high levels of γ/δ TCR cells were also observed in the vagina (McNulty et al., 2005).

A beneficial role for γ/δ T cells has been suggested against *A. fumigatus*. In particular *A. fumigatus* Ags have been described to stimulate γ/δ T cells, suggesting that these cells may contribute to the protective immune response against invasive aspergillosis (Hebart et al., 2002).

A participation in negative response against *C. neoformans* pulmonary infections has been suggested for γ/δ cells. In fact, it has been demonstrated that these cells accumulate in the lungs after cryptococcal infection, and play a down-regulatory role in the development of Th1 response and host resistance against this fungal pathogen (Kawakami, 2004; Uezu et al., 2004).

γ/δ cells were found in high proportion in bronchoalveolar lavage and peripheral blood of HIV-infected patients with PCP (Kagi et al., 1993). Some years afterward it was demonstrated that mice deficient in both α/β and γ/δ TCR naturally acquired PCP with lethal consequences. Therefore, the role of α/β and γ/δ cells in generation of Abs was considered important for resistance to *P. carinii* infection (Hanano and Kaufmann, 1999). A negative role for γ/δ T cells was reported in a subsequent

paper, which demonstrated an increased resistance against PCP in γ/δ T cell deficient mice (Steele et al., 2002).

6. B LYMPHOCYTES

B cells are an important component of adaptive immunity. They produce and secrete millions of different Ab molecules, each of which recognizes a different foreign Ag. B cells also cooperate with other cells of the immune system, including macrophages, DC and T cells, to eliminate foreign Ags. B cells express a B cell receptor (BCR) that is an integral membrane protein complex composed of two Ig heavy chains, two Ig light chains, and two heterodimers of Igα and Igβ. The various BCR forms and their downstream signaling molecules act as critical checkpoint guards by allowing to continue differentiation or preventing developmental progression (Spanopoulou et al., 1994; Young et al., 1994).

B cells are able to capture and present Ags to T lymphocytes, they are important APC in the propagation of B and T cell response. The critical role of BCR in ensuring that Ags are efficiently captured, processed and loaded into MHC-II, has recently been reported (Clark et al., 2004). The Ag-loaded MHC-II complexes can participate in T cell activation. The process involved in BCR capture and presentation of Ag to T cells is quite clear, but the underlying mechanisms are still obscure. Recent evidence indicates that in addition to innate stimuli and T cell-derived signals, B lymphocytes exert a profound regulatory effect on the Ag-presenting function of DC. B cells produce cytokines and chemokines, critically involved in the process of maturation, migration, and function of DC (Lund et al., 2005). Furthermore, B cells could modulate the availability of effector T cells, a role that would be consistent with the concept that Abs, and/or B cells, can regulate the outcome of cell-mediated immune responses.

6.1. B Lymphocytes in *Candida albicans*

The detection of B lymphocytes and protective Abs at vaginal level indicate their involvement in local anti *Candida* responses (de Bernardis et al., 2000). Furthermore, women with recurrent vaginal candidiasis have normal peripheral blood B and T lymphocyte subset levels (White et al., 1997).

More recently, the main role of CD4 T cells in helping vaginal B cells produce Abs, has been envisaged in an experimental model of vaginal candidiasis (Santoni et al., 2002). It has been suggested that B cells also play a role in chronic mucocutaneous candidiasis. In particular, an impaired B cell response to pokeweed mitogen has been reported in an immunosuppressed patient with chronic mucocutaneous candidiasis (von Bernuth et al., 2002).

The role of B cells in systemic candidiasis has been demonstrated in an experimental model of B cell-deficient mice. These animals are unable to resist re-infection with certain fungi, such as *C. albicans*, despite their resistance to primary infection and their ability to generate activated Th1 cells (Montagnoli et al., 2003).

6.2. B Lymphocytes in *Aspergillus fumigatus*

An immunodominant B-cell epitope of a major *A. fumigatus* allergen, (Asp f 2), has been identified (Banerjee et al., 1999). In addition, it has been reported that B cells from PBMC of ABPA patients, were found to be significantly more sensitive to IL-4 stimulation, compared to those from atopic and non-atopic patients, with upregulation of CD23 (low affinity IgE receptor) (Khan et al., 2000). The elevated number of CD23 molecules in these patients was also confirmed in a more recent paper (Knutsen et al., 2004). Furthermore, it has been suggested that B cells are involved in the innate host response to *A. fumigatus* (Wang et al., 2001a). This is consistent with the recent discovery that members of the toll-like receptor family are expressed on mammalian and B cells (Ogata et al., 2000).

6.3. B Lymphocytes in *C. neoformans*

It has been difficult to unequivocally establish the role of B cells in defense against fungal pathogens. Studies have shown no differences between the outcome of fungal infections in B cell-deficient and in normal mice (Allendorfer et al., 1999; Wagner et al., 1996; Monga et al., 1979). However Aguirre and Johnson were able to document an effect that suggested a role for B cells in resistance to *C. neoformans* infection, under conditions in which both T and B cell functions were impaired, and B cells were reconstituted from normal mice (Aguirre and Johnson, 1997). A role for B cells was also evidenced in a recent paper from Casadevall's group, through examination of susceptibility to pulmonary *C. neoformans* infection in B cell-deficient mice. The authors report an enhanced susceptibility, with respect to that of wild-type mice. Furthermore, passive Ab administration against *C. neoformans* in B cell-deficient mice was ineffective in prolonging survival, suggesting that B cells contribute to host defense against *C. neoformans* and that Ab efficacy is dependent on the presence of B cells (Rivera et al., 2005).

6.4. B Lymphocytes in *H. capsulatum*

B cells do not seem to play a critical role in pulmonary histoplasmosis, as suggested by a study conducted by Deepe's group. In an experimental model, B cell-deficient mice did not exhibit any differences in survival or infectious burden compared with controls in either primary or secondary histoplasmosis. Thus, neither the production of Abs, nor the Ag-presenting capacity of B cells contributed to the protective immune response (Allendorfer et al., 1999).

6.5. B Lymphocytes in *P. carinii*

B lymphocytes participate in host defense against *P. carinii* in different ways (Harmsen and Stankiewicz, 1991), including production of Abs against this important opportunistic pathogen. Although serum Abs directed against *P. carinii*

are ubiquitous in humans, including human immunodeficiency virus-infected individuals, recent experimental evidence shows that B cells and Abs can contribute significantly to host defense against *P. carinii* (Beck and Harmsen, 1998). The importance of B cells in helping the clearance of *P. carinii* is also underlined in various papers (Hanano and Kaufmann, 1999; Gigliotti et al., 1998). Moreover, a recent report suggests a role for B cells in efficiently presenting Ags to T cells, as well as in regulating activation or expansion of primed Ag-specific CD4 T cells, suggesting that B cells also mediate host defense against *P. carinii* by facilitating CD4 T cell activation or expansion (Lund et al., 2003).

In conclusion, there is evidence for the involvement of CD4 T cells, CD8 T cells and B cells in protection against *P carinii*. However, the precise role of CD4 and CD8 remains obscure; conversely the role of B cells appears to be critical for the resolution of *P. carinii* infection.

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CHAPTER 5

OTHER CELLS: THE ROLE OF NON-NEUTROPHILIC GRANULOCYTES, NK AND NKT CELLS IN FUNGAL IMMUNOLOGY

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Abstract: The immune response to fungal infection is diverse and necessitates the participation of both innate and adaptive immunity. Furthermore, fungal infections are closely related to a variety of allergic fungal diseases, primarily in the respiratory tract. This chapter evaluates the role of important innate leukocytes in response to fungal infection and in the immunopathogenesis of allergic fungal disease. We first evaluate the participation of mast cells, basophils and eosinophils (non-neutrophilic granulocytes) in allergic fungal disease of the upper and lower respiratory tracts, in addition the contribution of these cells to host defense against fungal infection is discussed. In the second part of the chapter we evaluate the role of innate lymphocytes, specifically natural killer and natural killer T-lymphocytes and summarize what is understood about the important immunoregulatory and effector functions contributed by these cells during host immune responses fungal infection

1. INTRODUCTION

The host immune response to fungal challenge is diverse, involving both adaptive and innate branches of the immune system. This chapter evaluates the role of some of the important innate cell types in the immune responses against fungi.

We will evaluate the role of mast cells, basophils and eosinophils, which represent the non-neutrophilic granulocytes. These cells are central to the allergic manifestations of fungal disease, and to a lesser extent can contribute to host defense against fungal pathogens. In addition, natural killer T cell (NKT cells) and natural killer cells (NK cells) are innate cytotoxic lymphocytes, and make important contributions to the regulation of host immune responses to a variety of fungi. In conjunction with a regulatory effector function, NK and NKT cells play a direct role in killing of fungal organisms through direct cytotoxicity. Consideration of the role these important

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innate leukocytes make to the host immune response to fungal challenge will greatly enhance the overall understanding of immunologic response to fungal infections.

2. MAST CELLS AND BASOPHILS

Mast cells and basophils are responsible for the initiation of IgE-mediated hypersensitivity responses. Many fungal organisms produce allergens that result in allergic fungal diseases including rhinitis, fungal sinusitis, asthma, and allergic bronchopulmonary mycosis (ABPM). These diseases are associated with elevated serum and fungal specific IgE, and by implication, type-1 hypersensitivity (Horner et al. 1995; Bush et al. 2004; Khun and Swain 2003 Kurup 2000). Although it is presumed that IgE-mediated mast cell and basophil reactions are central to fungal allergy, the strict requirement for IgE-mediated reactions is still debated. There is even more limited information on the contribution of mast cells or basophils to host defense against fungal pathogens, though there is the potential to regulate immune responses. Overall, in the host response to fungi, mast cells and basophils make versatile contributions to allergy, inflammation and host defense (Figure 1).

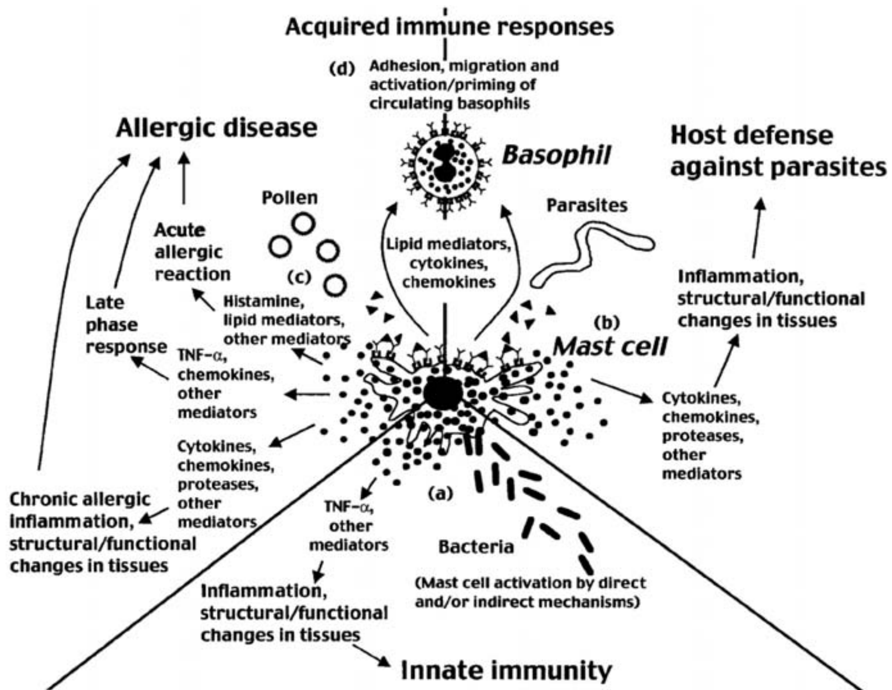

Figure 1. The diverse role of mast cells and basophils in the regulation of host defense, allergy and innate responses. From Gali et al. 2001. "Analyzing the Roles of Mast cells and Basophils in Host Defense and Other Biological Responses". This figure is reprinted by courtesy of International Journal of Hematology."

3. MAST CELLS AND BASOPHILS IN ALLERGIC FUNGAL DISEASE

Mast cell and basophil activation by fungal allergens and organisms is evidenced by the release of histamine in response to fungi. This activity, often attributable to immediate hypersensitivity, is associated with a number of fungal diseases, specifically, rhinitis, asthma, cutaneous hypersensitivity and ABPM.

Fungal rhinitis is characterized by immediate hypersensitivity to fungal allergens. As such, the cross-linking of allergen-specific IgE on sensitized mast cells or basophils, is a proximal event in this typically mild pathology (Bush, 2004). This may be caused by allergens derived from many different fungal species, and up to 6% of the population may exhibit this form of hypersensitivity (Horner et al. 1995). (For an excellent review of fungal allergens see: Kurup, 2002).

ABPM is another allergic fungal disease that involves the release of mast cell and basophil mediators. Diagnostic markers, and our current understanding of the immunopathogenic mechanisms of ABPM, strongly suggest an important role for T-helper type 2 (Th2)-driven IgE production and immediate hypersensitivity (Knutsen and Slavin, 1992). However, it should be noted that the detailed mechanism by which *A. fumigatus* allergen or antigen induces “allergic” disease is more complicated.

The observation that the clinical severity of ABPM correlates with fungal antigen-specific IgE supports the idea that IgE-sensitized mast cells or basophils contribute to allergic inflammation (Kumar, 2003). Additional evidence lies in the finding that surfactant proteins have been demonstrated to bind to *A. fumigatus* antigen and block both binding of the allergen to IgE and the release of histamine (Madan et al. 1997.) In this way, surfactant proteins are able to attenuate pathology in ABPM, highlighting the importance of immediate hypersensitivity in ABPM (Kishor et al. 2002; Madan et al. 2001[A], 2001[B], 2005; Strong et al. 2002). Furthermore, synthetic peptides derived from *A. fumigatus* antigen-1 react with sera and induces histamine release from mast cells of patients with ABPM, but do not induce responses in other atopic patients or healthy individuals (Madan et al. 2004). This evidence strongly indicates a crucial role for sensitized mast cells in ABPM. There is; however, evidence that basophils and mast cells may be important independently of IgE.

An early study attempting to highlight differences between mold-sensitive asthma and ABPM reported that histamine release in response to anti-IgE or a mixture of *Aspergillus fumigatus* antigens was elevated in patients with ABPM (Ricketti et al. 1983). Surprisingly, there was no correlation between histamine release in response to *A. fumigatus* antigen mixture and total serum levels of IgE, antigen-specific IgG and IgE, or the ratio of specific Ig to serum levels of histamine (Ricketti et al. 1983). Nonetheless, hyperreactivity, as evidenced by histamine release from the basophils of ABPM patients when stimulated with *A. fumigatus* antigen was significantly elevated compared to asthmatics sensitive to mold, but without ABPM (Ricketti et al. 1983). This suggests that pulmonary mast cells may be accountable for the hyperreactivity, but offer no suggestion that this is IgE-dependent and therefore

traditional type-1 hypersensitivity to the fungus. Some of the potential mechanisms for mast cell and basophil activation in the absence of IgE are discussed below in the context of other fungal organisms, and the role of IgE in the pathogenesis of ABPM is elaborated in the section on eosinophils.

Mast cell degranulation and histamine release occurs *in vitro* in response to *C. albicans* antigen occurring both directly, or indirectly through the production of mast cell sensitizing antibodies (Segal et al. 1975; Varinon et al. 1975). Upon challenge with *C. albicans* antigen, basophil histamine release has been correlated to serum levels of IgE, and circulating levels of basophils and LTC4 were shown to peak prior to the late phase response (Tanizaki et al. 1985; Takahashi et al. 1992).

Immediate hypersensitivity to *C. albicans* is also associated with cutaneous infection. In experimental models of cutaneous candidiasis, basophils accumulated in the skin and released histamine. The mechanism by which they are attracted was not determined; though, it was independent of antibody, complement or direct chemotactic effects of the fungi (Sohnle et al. 1977; Greenburg et al. 1980). Since basophils are known to express the chemokine receptor for eotaxin, this chemokine may be involved (Uguccioni et al. 1997).

As emphasized above, the mechanism by which fungi activate mast cells and basophils can depend upon the antigen and occur in an IgE-dependent or independent fashion. For example, the response to *Alternaria* spores has been reported to induce histamine release from basophils, but mycelia were more potent, demonstrating antigenicity (Fadel et al. 1992). Fungal spores of *Trichoderma viridae* were found to interact directly with human mast cells and basophils to induce the release of histamine in an IgE-independent fashion when highly concentrated, and in an IgE-dependent fashion in lower concentration, demonstrating both fungal antigenicity and IgE-independent mast cell activation (Larsen et al. 1996). In IgE-independent mast cell or basophil activation, fungal proteases are likely to be responsible through stimulation of protease activated receptors and the release of mediators from endothelial cells at the site of exposure (Reed and Kita, 2004). Although serum levels of IgE and fungal specific IgE are often used as indicators for allergic fungal disease, the ability of mast cells and basophils to degranulate independently of IgE suggests that immediate hypersensitivity is not a strict requirement for the contribution of mast cells and basophils to allergic fungal disease.

4. MAST CELLS AND BASOPHILS IN HOST DEFENSE

The role of the mast cell and basophils in host defense against fungi has been largely unexplored. This is unfortunate considering the potential of mast cells and basophils to directly recognize and respond to fungi through the specific production of regulatory mediators and clearance of fungal organisms.

Only one report demonstrates that mast cells phagocytose *C. neoformans* in the presence of specific IgE (Feldmesser et al. 1997). Nonetheless, the potential ability of mast cells to recognize fungi through pattern recognition receptors is suggested

by the expression of toll-like receptors (TLR) (Marshall, 2004). The fungal product glucuronoxyulomannan from the capsule of *C. neoformans* is recognized by TLR-4, which is expressed by mast cells (Shoham et al. 2001). Furthermore, mast cells have the ability to recognize yeast-derived zymosan through TLR-2. Zymosan stimulation of mast cells results in the production of GM-CSF, IL-1β and cysteinyl leukotrienes (McCurdy et al. 2003). The importance in regulating inflammation is exemplified by mast cells that are crucial in zymosan-induced peritioneal inflammation (Kolaczkowska et al. 2001). In addition to potential recognition of fungi, mast cells produce antimicrobial peptides. These include members of the cathelicidin and defensin families, and defensins exhibit antifungal activity against *C. neoformans*, indicating the potential to contribute to fungal clearance (Di Nardo et al. 2003; Marshall and Jawdat, 2004). Surprisingly, to date, there is no indication of the importance of TLR or other pattern recognition by mast cells in fungal infection, nor have any studies attempted to thoroughly evaluate the requirement of mast cells in the clearance of fungal pathogens.

5. SUMMARY

Mast cells and basophils are important cells in the proximal events associated with immediate hypersensitivity. Many fungal diseases are associated with this type of hypersensitivity; however, literature establishing a firm role for immediate hypersensitivity in fungal disease, with the exception of mast cell and basophil degranulation in rhinitis and ABPM is lacking. Future work will be required to define a role in candidiasis. Information on the contribution of mast cells and basophils to host defense against fungi is also lacking, though future investigation of mast cells in the innate response to fungi and host defense is likely to be very fruitful.

6. EOSINOPHILS

The eosinophil is a highly toxic granulocyte with phagocytic capability that is typically present during the late phase of allergic inflammation or during parasitic infection. The eosinophil may contribute to host immune responses against infection through degranulation and phagocytic activity. In contrast to mast cells and basophils, there exists a substantial body of information on eosinophil responses to fungi.

7. EOSINOPHIL MEDIATORS AND GRANULE PROTEINS

Eosinophils posses a number of mediators stored within specific and primary granules that contribute to the pathophysiology of fungal disease. Upon activation, eosinophils can synthesize and release cytokines, chemokines, and lipid mediators of the arachidonic acid pathway, causing allergic inflammation in response to

fungi. It is likely these mediators participate in fungal disease; however, the exact contribution of eosinophil derived-mediators remains unclear.

Important intracellular constituents of the eosinophil include the membrane-disrupting major basic protein (MBP). MBP is a marker for eosinophilic degranulation, and is toxic to both microorganisms and mammalian cells. Degranulation is evidenced by extracellular deposition in biopsy specimens from patients infected with *Paracoccidoides brasiliensis* (Wagner et al. 1998). In patients with allergic fungal sinusitis, chronic rhinosinusitis and ABPM, it may contribute to the pathogenesis of fungal disease (Khan et al. 2000; Manning and Holman, 1998; Ponikau et al. 2005; Slavin et al. 1988).

Another important mediator is eosinophil peroxidase (EPO), commonly evaluated to determine eosinophilic activity. As such, it is often measured in allergic fungal disease. Interestingly, it has been reported to enhance the fungicidal activity of macrophages though it reduced their secretion of the pro-inflammatory cytokines TNF-α and IL-6 (Kurup et al. 1997[C]; Murali et al. 1998; Lefkowitz et al. 1997; Lincoln et al. 1999).

Eosinophil cationic protein, a toxin secreted by activated eosinophils, is significantly increased in the mucin of patients with allergic fungal sinusitis and at even higher levels in patients with nasal polyposis of fungal etiology, making it a potentially important contributor to these clinical conditions (Feger et al. 1997; Di Lorenzo et al. 2001). Additionally, treatment of ABPM with itraconazole resulted in decreased sputum eosinophil cationic protein levels suggesting a role in the pathogenesis of the disease (Wark et al. 2003).

A common feature of eosinophilic disease is the presence of Charcot-Leyden crystals, composed of an eosinophilic acylhydrolase and indicative of eosinophilic degranulation. These crystals are seen in the sputum and mucin of patients with allergic fungal disease of the upper airway and ABPM. Intracellular Charcot-Leyden-like crystals have been reported in macrophages in a murine model of pulmonary cryptococcosis (Bosken et al. 1988; Chen, 1993; Granville et al., 2004; Pantanowitz and Balough, 2004; Rane et al. 2003; Huffnagle et al. 1998[A]).

Other mediators stored in the granules of eosinophils include eosinophil-derived ribonucleases. Although increased levels of eosinophil derived neurotoxin have been described during the late-phase allergic response to fungal allergen, and in the nasal lavage fluids of patients with allergic fungal sinusitis, the majority of the literature regarding eosinophil derived ribonucleases is confined to viral disease (Bascom et al. 1989; Manning and Holman, 1998; Rosenberg and Domachowske, 2001).

8. EOSINOPHILS AND THE UPPER RESPIRATORY TRACT

In fungal disease of the upper respiratory tract the presence of eosinophils is a critical diagnostic feature. Diseases of fungal etiology in the upper respiratory tract primarily involve fungal sinusitis, and both superficial “allergic” diseases and invasive fungal sinusitis are associated with eosinophilia (Currens et al. 2002; Ferguson 2004; Granville et al. 2004. As reported by the Ponikau et al. (Ponikau

et al. 1999 and subsequently by other investigators, allergic fungal sinusitis (AFS) is manifest as nasal polyposis and a strong eosinophilic response. AFS is considered to be among the most prevelant forms of chronic rhinosinusitis (Braun et al. 2003[A], 2003[B]; Granville et al. 2004; Ponikau et al. 1999). The condition results from a superficial infection of the sino-nasal mucosal tissue by a variety of different fungal organisms, dematiaceous fungi being the most common (Clark et al. 1996; Katzenstien et al. 1983[A], 1983[B]; Gourley et al. 1990; MacMillan et al. 1987; Torres et al. 1996).

Eosinophilia in the mucous and activation of eosinophils, as evidenced by deposition of Charcot-Leyden crystals (allergic mucin), was an original diagnostic feature of AFS and has remained important (Katzenstien et al. 1983[A], 1983[B]; Kuhn and Swain, 2003; Waxman et al. 1987). It is not understood; however, what factors are strictly required for the induction of eosinophilia and recruitment of eosinophils to the sino-nasal tissues during fungal infection of the upper respiratory tract.

Elevated serum IgE and fungal specific IgE are often included in the major diagnostic criteria of AFS. Fungal proteases from *Aspergillus* have been implicated in the amplification of IgE response to fungal antigens in ABPM; however, the role of IgE-mediated hypersensitivity in the development of an eosinophilic response is not clear (Corey, 1992; Deutsch and Hevron, 2004; Khun and Swain, 2003; Reed and Kita, 2004). In some patients with AFS, systemic allergy to fungal organisms may not be present (Collins et al. 2004; Sasama et al. 2005). Local production of fungal specific IgE in the absence of elevated total serum levels has been suggested as a possible mechanism for the development of an eosinophilic response in individuals without evidence of systemic allergy (Collins et al. 2004). Because serum IgE levels, and fungal specific IgE have been traditionally considered diagnostic for AFS, it is possible to misdiagnose chronic eosinophilic rhinosinusitis in patients lacking these clinical features. Additionally, there is an emerging consensus that fungi may be responsible for non-IgE mediated chronic rhinosinusitis, where eosinophilic inflammation is linked to the presence of certain fungi in the sino-nasal passage through an IgE independent mechanism (Sasama et al. 2005). A study of fungal sinusitis using IgE-deficient mice demonstrated no difference in the recruitment of eosinophils to the nasal mucosa compared to wild type mice (van de Rijn et al. 1998). This concept is further supported by a recent study characterizing the humoral response in AFS, where fungal specific IgG3 was elevated in the study group, but IgE levels remained unchanged, suggesting other immunoglobulin isotypes can mediate eosinophilic inflammation (Pant et al. 2005). Thus, eosinophilic sinus disease with fungal etiology should not always be considered an IgE-mediated allergic condition, but rather hypersensitivity characterized by eosinophilic recruitment in a Th2 dominated response (Ferguson, 2004).

Keeping this in consideration, the cytokine and chemokine response is crucial in mediating eosinophilic inflammation in fungal sinusitis. Data regarding the mechanisms of cytokine and chemokine mediated recruitment of eosinophils in fungal sinusitis are lacking. One study examined eosinophilic recruitment in response to a protein extract from *A. fumigatus*, and reported that eotaxin was able to recruit

eosinophils to the nasal mucosal tissues in an IL-5 dependent manner, but little else has been reported (van de Rijn, 1998).

The presence of eosinophils in the blood of patients with fungal sinus disease is associated with worse disease (Zadeh et al. 2002). This may be a result of eosinophil activation and degranulation at the site of infection. The activation of eosinophils in the sino-nasal mucosa is presumed by the presence of Charcot-Leyden crystals in allergic mucin, but there are other markers for eosinophil activity in AFS (Klatzenstien et al. 1983; Kuhn and Swain 2003; Waxman et al. 1987). Levels of MBP have been correlated with disease activity suggesting a contribution of eosinophils to the pathogenesis (Khan et al. 2000). ECP has also been detected at elevated levels in patients with AFS and this also correlated to disease severity (Feger et al. 1997; Collins et al. 2004).

Conversely, the mechanisms of eosinophil activity, and the potential contribution of eosinophils to host defense in infectious sinusitis are poorly understood. During allergic disease potentially pathogenic organisms must be prevented from invading the host. In an examination of mucin from patients with AFS, eosinophils were found to be associated with fungal hyphae, and appeared to phagocytose the cuticular substance of the fungal hyphae into a sheet-like invaginated space into which, granules were released (Watanabe et al. 2004). In nasal polyposis, recruited eosinophils were reported to phagocytose and kill fungal conidia and damage fungal hyphae, processes that were amplified by TLR-2 and TLR-4 stimulation (Pitzurra et al. 2004). There is no solid evidence; however, that the anti-fungal activity of eosinophils in upper respiratory tract mycoses is protective.

Although there is an appreciation that eosinophils participate in fungal sinusitis, the detailed mechanisms of recruitment and activation in sinusitis have not been explored. To this end, the eosinophilic response to fungal infections of the lung and other sites provides some additional information.

9. EOSINOPHILS AND ALLERGIC FUNGAL DISEASES OF THE LUNG

Asthma and ABPM are associated with significant morbidity and rarely mortality. One of the hallmarks of ABPM is the presence of eosinophils, illustrating the centrality of these cells in the immune response to allergic fungal disease of the lung.

The mechanisms of eosinophil induction and recruitment have been intensely investigated because these cells are such a prominent feature of allergic fungal disease of the lung. Both in patients with ABPM, and in murine models of the disease, eosinophilia in the bone marrow, peripheral blood and lung have been described. Investigations have included the mechanisms of eosinophilopoesis and recruitment in an attempt to understand this phenomenon and to elucidate the role of these cells in the pathophysiology of lung disease. There are a number of different cytokines, chemokines, and growth factors that have been identified in the recruitment and activation of eosinophils in ABPM (Table 1).

Table 1. Factors Regulating eosinophilia Cytokines and chemokines and other factors influencing the development of eosinophilia in response to fungal challenge. (+) indicates positive regulation, (-) negative regulation, and (+i) indirect positive regulation. [1]Blood eosinophilia, [2]tissue eosinophil recruitment, [3]Bone marrow eosinophilopoeisis

	Mediator	+/−	Fungal Species	Site of Induction	Ref's
Cytokines	EO-CSF	+	*A. fumigatus*	Blood[1]	42
	GM-CSF	++	*A. fumigatus*	Lung[2]	29
	IL-5	+++	*A. fumigatus, C. neoformans*	Bone Marrow[3], Blood, Lung	19, 29, 49, 74, 202, 104, 108, 109, 146, 204
	IL-4	+/−	*A. fumigatus*	Bone Marrow, Blood, lung	106–110, 204
	IFN-γ	−	*A. fumigatus, C. neoformans*	Lung, blood	28, 76, 104
	IL-1β/TNF-α	+i	*A. fumigatus*	Lung	204
	IL-9	+i	*A. fumigatus*	Lung	137
	IL-10	−	*A. fumigatus*	Lung	60
	IL-25	+i	*A. fumigatus*	Lung	77
	IL-13	+	*A. fumigatus*	Lung	20, 77
	IL-18	−	*A. fumigatus*	Lung	18
	TGF-β	−	*C. neoformans*	Lung	212
Chemokines	Eotaxin/CCL11	++	*A. fumigatus*	Lung	184, 191
	MCP-1/CCL2	++	*A. fumigatus*	Blood, Lung	19, 20, 184
	MCP-4	+	*A. fumigatus*	Lung	191
	MIP-1α	+	*A. fumigatus, C. neoformans*	Blood, Lung	21, 54, 154
	RANTES/CCL5	+i	*A. fumigatus*	Blood, Lung	20, 54, 182, 184
	CXCR2	+i	*A. fumigatus*	Lung	182
Other Factors	IgE	+/−		Blood, Lung	106–111, 141, 204
	Complement	++	*A. fumigatus*	Lung	7, 41
	Surfactant proteins	−	*A. fumigatus*	Blood, Lung	62, 94, 127, 128, 130, 192

At least one report has examined the role of eosinophil colony stimulating factor (EO-CSF) in the induction of eosinophils. T-cells from patients with ABPM were demonstrated to produce EO-CSF but not neutrophil colony stimulating factor (N-CSF) in response to challenge with *A. fumigatus* antigen, demonstrating a specific T-cell driven induction of eosinophils in the bone marrow (Enokihara et al. 1985). The role of cytokines and chemokines; however, has been more comprehensively documented.

IL-5 is the most important cytokine involved in the induction and recruitment of eosinophils in response to fungal challenge. *A. fumigatus* antigen stimulates a blood and bone marrow eosinophilia in a murine model. *In vitro*, bone marrow cells from treated mice responded to IL-5 with increased production of eosinophils, indicating the potential for *A. fumigatus* antigen to mediate IL-5-dependent commitment to the eosinophil lineage (Murali et al. 1992). Later studies based on this model confirmed IL-5-mediated eosinophilopoesis, demonstrating increased numbers of bone marrow eosinophils in correlation with increased IL-5 transcription in the bone marrow. Further, intra-nasal challenge induced recruitment to the lung, supporting the effect of a distant cytokine (Murali et al. 1997). In murine models of ABPM, T-cell derived IL-5 accompanied a robust eosinophilic influx in the lung, and particulate antigen stimulated higher levels of IL-5, which correlated with increased numbers of eosinophils in the blood (Chu et al. 1996; Kurup et al. 1994[B]). Furthermore, neutralization of IL-5 through single or multiple doses of mAb significantly reduced eosinophil numbers or activity as determined by EPO levels in the bone marrow, blood and lung (Foster et al. 1996; Kurup et al. 1994[A]; Kurup et al. 1997[C]) Finally, in patients with ABPM, IL-5 levels strongly correlate with eosinophilia, which substantiates the observation in human fungal disease (Walker et al. 1994).

The role of IL-4, and by implication IgE, is not as clear, but appears not to be crucial in mediating eosinophilic inflammation in ABPM. In atopic patients, IL-4 levels were associated with eosinophilia (Walker et al. 1994). In mice; however, neutralizing IL-4 only moderately reduced the number of eosinophils in response to challenge with *A. fumigatus* antigen (Kurup et al. 1994[A], 1997[C]). More convincingly, studies using IL-4 knockout mice demonstrated similar levels of blood and lung eosinophilia, suggesting a mechanism of eosinophilic inflammation independent of IgE-mediated hypersensitivity (Kurup et al. 1997[B]). Thus, it is clear that although elevated serum IgE and fungal specific IgE are diagnostic features of ABPM, in the context of these experimental models, IgE mediated hypersensitivity is not required for eosinophilic inflammation, but that various other cytokines contribute to recruitment of eosinophils (Mehlhop et al. 1997).

Neutralizing IFN-γ enhanced the eosinophilic response to *A. fumigatus* antigen in the murine model (Kurup et al. 1994[A]). By contrast, T-cell derived GM-CSF in the lung is associated with maintenance and activation of eosinophils (Chu et al. 1996). Increased expression of IL-1α and TNF-α is associated with increased numbers of eosinophils in murine ABPM (Chu et al. 1996), and the transgenic overexpression of IL-9 during *A. fumigatus* antigen challenge promoted the eosinophilic response (Mclane et al. 1998). IL-10 knockout mice display increased eosinophilia in response

to *A. fumigatus* antigen challenge, and IL-25 production in response to *A. fumigatus* infection induced the recruitment of eosinophils in an IL-5 and IL-13-dependent fashion (Grunig et al. 1997; Hurst et al. 2002). IL-18 depletion causes increased eosinophilia and down regulated TLR-2 in a murine model of ABPM, indicating that pattern recognition receptor-stimulation of cytokine responses may be important in regulating the eosinophilic response (Blease et al. 2001[A]). In mice pre-treated with CpG oligodeoxynucleotide, down regulation of peripheral blood eosinophilia, likely through the production of IFN-γ by CD4+ T-cells, would support a negative regulatory role for IFN-γ (Banergee et al. 2004). In addition to cytokine responses, chemokines are pivotal in regulating the eosinophilic response to fungi.

Eotaxin is an important chemokine in the recruitment of eosinophils, so, it is not surprising that in eotaxin knockout mice challenged with *A. fumigatus* antigen, airway responsiveness and eosinophil recruitment are significantly reduced 24 hours after inoculation (Schuh et al. 2002[B]). The development of chronic disease was unchanged, suggesting eotaxin may be important in early recruitment of eosinophils, but not in the later phases of the response (Schuh et al. 2002[B]). Eotaxin levels may be indirectly effected by MCP-1/CCL2, another important chemokine in the regulation of eosinophils in ABPM (Blease et al. 2001[B]). In sensitized mice challenged with live conidia, overexpression of MCP-1 resulted in decreased eosinophilia and fungal burden, indicating a protective role for MCP-1 in non-allergic hosts (Blease et al. 2001[B]). Although soluble antigen sensitization in MCP-1 knockout mice did not alleviate eosinophilia, MCP-1 knockout mice were more susceptible to live *A. fumigatus* conidia, and were found to produce increased levels of Th2 cytokines: IL-5, IL-13 and chemokines, eotaxin and RANTES in addition to increased pulmonary eosinophilic infiltration (Blease et al. 2000[B]; Koth et al. 2004). MCP-4 and MIP-1α are important chemokines in the recruitment of eosinophils, and neutralization of RANTES/CCL5 sensitive cells has been demonstrated to alleviate pulmonary eosinophilia upon challenge with *A. fumigatus* conidia (Gonzalo et al. 1996; Schuh et al. 2003; Stellato et al. 1997). It follows that chemokine receptors are also important in regulation of eosinophils in ABPM and fungal asthma. CXCR2 positively regulated Th2 type cytokines and pulmonary eosinophilia in response to *A. fumigatus*, as evidenced by challenging knockout mice (Schuh et al. 2002[A]). The receptors for RANTES and eotaxin, expressed on eosinophils also positively regulate their recruitment; CCR1 knockout mice displayed decreased levels of Th2 cytokines and peripheral blood eosinophilia, and neutralization of CCR3 inhibited the eosinophil recruitment to the lung (Blease et al. 2000[B]; Forssmann et al. 2004).

Other soluble factors including complement and surfactant are important in regulating the eosinophilic response in the lung following challenge with fungal organisms or antigen. Mice deficient in the receptor for C3a displayed reduced airway eosinophilia following stimulation with *A. fumigatus* antigen (Drouin et al. 2002). This was an effect likely resulting from dampened production of Th2 cytokines including IL-4, IL-5 and IL-13, which were also decreased (Drouin et al. 2002). The pharmacological inhibition of C3a or C5a through blockade of their specific receptors

also reduced the number of eosinophils in the airway, indicating the contribution of these anaphylatoxins to the recruitment of eosinophil (Baelder et al. 2005).

Surfactant proteins also appear to be important in regulating eosinophilic response in the lung. Airway hyperresponsiveness and eosinophilia in a model of fungal asthma induced by *A. fumigatus* antigen was preceded by a reduction in surfactant protein B (SP-B) levels, suggesting that modulation of surfactant biosynthesis can alter eosinophilic responses (Haczku et al. 2002). Furthermore, treatment with surfactant protein A (SP-A) or surfactant protein D (SP-D) in several models has been demonstrated to reduce peripheral blood and lung eosinophilia, as well as Th2 cytokines IL-4, IL-5 and IL-13 (Kishor et al. 2002; Madan et al. 2001[A], 2001[B]; Strong et al. 2002). Interestingly, in the knockout models of SP-A and SP-D, both mouse strains displayed increased intrinsic eosinophilia and Th2 secretion, but only the SP-D knockout mouse displayed more pulmonary hypersensitivity in response to *A. fumigatus* antigen compared to wild type. The SP-A knockout mouse was resistant, and upon treatment with recombinant SP-A displayed increased eosinophilia and lung damage (Madan et al. 2005). A role for SP-A in humans is suggested by the association of specific polymorphisms in SP-A2 with increased levels of IgE and eosinophilia in patients with ABPM (Saxena et al. 2003).

The regulation of eosinophils in allergic disease is mediated by a complex network of chemokines, cytokines and other soluble factors induced by exposure to fungal antigens or fungal organisms in the lung. The complete mechanism is complicated and beyond the scope of this discussion; however, activation of lung endothelial cells and other inflammatory cells may be largely attributable to the activity of fungal proteases in the lung (Reed and Kita, 2004). Furthermore, the histopathology includes the presence of Charcot-Leyden crystals in the bronchial mucus plugs of patients with ABPM indicating activity of these cells, and in one report eosinophil degranulation via CD32 (FcγRII) has been demonstrated *in vitro* with *Candida* specific IgG (Bosken et al. 1988; Chen, 1993; Ikeda et al. 1999).

Eosinophils are present in the lung, blood, and bone marrow in patients with fungal asthma or ABPM and in experimental models of the disease, but what is their contribution to the pathophysiology and disease outcome? In the numerous studies analyzing the mechanisms of eosinophil recruitment to the lung, decreased numbers of eosinophils are nearly always associated with reduced pathology. Eosinophil numbers and inflammatory pathology were unchanged in IL-4 knockout mice, excluding a role for other responses downstream of IL-4, and implicating eosinophils as the major contributor to the disease (Kurup et al. 1997[B], 1999[A], 1999[B]). It is has been suggested that airway hyperreactivity characteristic of fungal asthma, can occur in the absence of IL-5 or eosinophils, and is more closely linked to IgE (Kurup et al. 1999[B], Corry et al. 1996). However, the majority of reports indicate that IL-5 and eosinophils are required for the bulk of the adverse effects in fungal asthma and ABPM. In a mouse model of fungal asthma, IL-5 deficiency abolished airway hyperreactivity and lung damage. Although allergen specific IgE was required for the development of airway hyperresponsiveness in some models of fungal asthma, this is not always the case, rather, IL-5 and eosinophils appear to be the common denominator responsible for the

global spectrum of symptoms and pathology associated with fungal asthma and ABPM, including airway hyperresponsiveness, bronchial hyperreactivity and the associated tissue damage and remodeling (Foster et al. 1996, Hamelmann et al. 1997; Hogan et al. 1997, 1999; Hogaboam et al. 2000)

Atopic individuals are prone to mounting allergic Th2 responses promoting the recruitment of eosinophils, and this Th2 skewed response may in turn promote the ability of *A. fumigatus* to colonize and persist in the airway, resulting in the development of a more chronic disease such as ABPM. Mechanistically, the experimental models indicate that IgE is dispensable in the development of ABPM or at least eosinophilic inflammation, and implicate the eosinophil as a key mediator of pathology. Thus, it may be more appropriate to describe IgE-mediated hypersensitivity as a risk factor for the development of chronic eosinophilic fungal disease in the lung.

10. EOSINOPHILS AND INFECTIONS OF THE LUNG

The eosinophil participates in the response to pulmonary cryptococcal infection. While the protective response to *Cryptococcus* occurs via cell-mediated immunity, cryptococcal infection can induce hypereosinophilia, and eosinophilic pneumonia, which is not protective (Feldmesser et al. 2001; Kobayashi et al. 2001; Marwaha et al. 1995; Starr et al. 1995). The balance of Th1/Th2 immunity governs the pathophysiology of cryptococcal infections, and disease progression often correlates to the presence of eosinophils, and by implication a Th2 response. By contrast, some reports in rats suggest a protective role for eosinophils. Brown Norway rats with Th2 skewed immune responses display eosinophilic infiltration, and reduced lung CFU compared to Lewis rats with a Th1-polarized innate response. This suggested that eosinophils together with the humoral response contribute to resistance in some circumstances (Kobayashi et al. 2001). These findings are in keeping with reports that eosinophils associate with *C. neoformans in vivo*, and that eosinophils phagocytosed *C. neoformans* in the presence of fungal specific IgG1 or IgE *in vitro* (Feldmesser et al. 1997). In mice; however, there is more convincing evidence that eosinophils are associated with adverse disease outcome. BALB/c mice, which clear *C. neoformans* slower that congenic C.B-17 mice, were found to have similar leukocyte recruitment profiles except for the increased recruitment of eosinophils in BALB/c mice (Lovchik et al. 1999). Further, in highly resistant CBA/J, moderately resistant BALB/c, and susceptible C57BL/6 mice, resistance correlated inversely with the duration of eosinophilic infiltrate (Huffnagle et al. 1998[B]). The presence of eosinophils, and disease outcome are highly dependent upon the nature of the cytokine response in the lung. IFN-γ receptor knockout mice recruited higher numbers of eosinophils than wild-type mice and displayed an inability to resolve infection, whereas, increased IFN-γ production following administration of an OX40L (CD134):Ig fusion protein, reduced pulmonary eosinophilia, a finding that correlated to reduced fungal burden in pulmonary cryptococcal infection (Chen et al. 2005; Humphreyes et al. 2003). Other cytokines and chemokines have also been implicated in the recruitment of eosinophils during pulmonary

cryptococcal infection. Macrophage inflammatory protein-1α (MIP-1α) knockout mice display significantly increased pulmonary eosinophilia and profound lung damage associated with deposition of Charcot-Leyden crystals (Olszewski et al. 2000). Finally, pharmacologic inhibition of IL-5, or anti-IL-5 mAb reduced both recruitment and crystal deposition during pulmonary cryptococcal infection, but did not alter the clearance of the organism, probably because the depletion of IL-5 reduced the recruitment of other cells important in clearance of *C. neoformans* including macrophages, neutrophils and $CD8^+$ T-lymphocytes (Huffnagle et al. 1998[A]; Van Wauwe et al. 2000). A similar finding was noted with the administration of the cytokine TGF-β, which suppressed the eosinophilic response to *C. neoformans* and impaired pathogen clearance (Williams et al. 2005).

Though the mechanism linking the presence of eosinophils with poor disease outcome in cryptococcal infection of the lung has not been explicitly described, the formation of crystalline structures in the lung tissue has been postulated to contribute to the pathologic features of the disease (Feldmesser et al. 2001). It is likely that the contents of eosinophil granules contribute to tissue damage, and together with eosinophil derived cytokines promotes susceptible Th2 type responses to fungi.

11. EOSINOPHILS OUTSIDE THE LUNG

Peritoneal invasion with a variety of fungal organisms can occur in patients receiving continuous ambulatory peritoneal dialysis (CAPD) and in some cases is associated with an eosinophilic infiltrate (Ampel et al. 1988; Lee et al. 1997; Nankivell et al. 1991). The cell wall fraction from *H. capsulatum* induced peritoneal eosinophilia through an IL-5 dependent mechanism. In a murine model of infection with *H. capsulatum* or inoculation with *H. capsulatum* derived beta-glucan, leukotrienes were demonstrated to play and important role in the recruitment of eosinophils and other inflammatory cells (Medeiros et al. 2004, 2004; Sa-Nunes et al. 2004).

Fungal infection of the central nervous system (CNS) has been reported to cause eosinophilic meningitis and blood eosinophilia. Patients infected with *Coccidiodes immitis* that has disseminated to the CNS may demonstrate an eosinophilic pleocytosis, or eosinophilic meningitis (Ismail and Arsura 1993; Ragland et al. 1993). In cryptococcal invasion of the CNS, blood and CSF eosinophilia have been reported, and histological examination of cerebral granuloma revealed the presence of eosinophils (Anderson et al. 1985; Kamezawa et al. 2000; Gross et al. 2003). Other reports of CSF eosinophilia include a case of chronic eosinophilic meningitis associated with positive culture for *Candida guillermondii*, and cases of eosinophilic meningitis associated with *Aspergillus* sinusitis or disseminated histoplasmosis (Chan et al. 2004; Livramento et al. 1993; Paz-Sendin et al. 1999). The purpose of eosinophils in the CNS is not well understood, nor has it been extensively examined, but is felt to be associated with adverse clinical outcome.

12. SUMMARY

Fungal allergy and infection are often associated with increased numbers of eosinophils in the serum and at the sites of disease. In most cases, eosinophils and secreted granule constituents, contribute adversely to the pathophysiology of fungal diseases; however, the eosinophil may also contribute to host defense, as it has been reported to phagocytose fungi at the site of infection and produce products toxic to fungi. It remains to be determined what distinguishes protective versus adverse eosinophilic responses to fungal infection.

13. INNATE CYTOTOXIC LYMPHOCYTES

Innate cytotoxic lymphocytes include the semi-invariable TCR-bearing natural killer T-lymphocytes (NKT) and natural killer (NK) cells. Both these cell types are important regulators and effectors in the immune response to fungal infection. They are able to participate in the absence of adaptive responses through their cytotoxic ability or the secretion of cytokines to help create a resistant environment and clear fungal pathogens.

14. NKT CELLS IN FUNGAL INFECTION

NKT cells are innate cytotoxic lymphocytes with a semi-invariable T-cell receptor (TCR). The TCR consists of a conserved Vα chain and semi-conserved β chains that recognize lipid antigen in the context of the CD1 family of surface antigen on APC, and more recently have been reported to recognize microbial antigen exogenously (Kinjo et al. 2005; Mattner et al. 2005). Literature regarding the role of these cells in fungal infection is incomplete; though there is evidence they exhibit anti-microbial activity, and participate in host response to pulmonary infection with *C. neoformans* (Gansert et al. 2003; Kawakami, 2002).

NKT cells contribute to the host response against cryptococcal infection primarily through the secretion of cytokines and subsequent regulation of Th1/Th2 response to fungal infection. Originally, cytokine production by NKT cells was analyzed in response to stimulation with alpha-galactosylcerimide (α-gal-cer) in murine models of cryptococcosis. α-gal-cer is a marine-sponge-derived lipid that is recognized specifically by NKT cells when presented by CD1, and stimulates the production of both Th2 and Th1 type cytokines (Kawano et al., 1997). In a murine model of cryptococcal infection, serum IFN-γ production was stimulated using α-gal-cer, and levels of IFN-γ were attributable to NKT cells, as NKT-knockout mice did not display the observed increases (Kawakami et al. 2001[B]). Upon restimulation with live *C. neoformans*, spleen cells of α-gal-cer-treated mice produced a large amount of IFN-γ not seen in NKT-knockout mice. Furthermore, the fungal burden was significantly reduced in the lung and spleen of α-gal-cer-treated mice compared to treated or untreated NKT-KO mice, establishing the contribution of IFN-γ-producing NKT cells in the host response against *C. neoformans* (Kawakami et al. 2001[B]).

The IFN-γ response of α-gal-cer stimulated mice was further characterized in IL-18 knockout mice. IFN-γ production and cryptococcal clearance were augmented in an IL-12 and IL-4-dependent fashion (Kawakami et al. 2001[C]). The role of NKT cells in cryptococcal infection under naïve conditions was subsequently analyzed (Kawakami et al. 2001[A]). It was determined that Vα14+ NKT cells accumulated in the lung of mice infected with *C. neoformans* in an MCP-1-dependent fashion, and contributed to the development of fungal-specific acquired cellular responses (Kawakami et al. 2001[A]). It has since been reported that in the C57BL/6 mouse, conflicting data regarding the ability of this mouse to clear *C. neoformans* is at least partially attributable to the development of NKT cells (Blackstock and Murphy, 2004). Elegant experiments involving the adoptive transfer of thymocytes from mice at early and later stages of development have demonstrated the importance of mature NKT cells (Blackstone and Murphy, 2004). It is becoming increasingly clear that the recruitment of mature NKT cells capable of producing large quantities of IFN-γ is an important feature of host defense against pulmonary infection with *C. neoformans* (Blackstone and Murphy, 2004). In addition to a defined role for cytokine production, NKT cell cytotoxic activity against *C. neoformans* may also contribute to host defense.

Despite an understanding of the contribution of NKT cell-derived cytokine regulation of the host immune response to fungal pathogens, there is little known regarding other potentially important effector functions of these cells. NKT cells are known to express granulysin, to be cytolytic, and important in the defense against intracellular pathogens such as *Mycobacteria* (Krensky, 2000; Sugawara et al. 2002). Furthermore, NKT cells have been demonstrated to exhibit direct anti-microbial activity against mycobacterium and express the cytolytic molecule ganulysin; known to mediate the direct anti-cryptococcal activity of $CD8^+$ T-lymphocytes (Gansert et al. 2003; Ma et al. 2002). Together, this information suggests that NKT cells may participate in direct anti-fungal activity.

Thus, NKT cells appear to regulate the response to infection with *C. neoformans* through the production of the Th1 inflammatory cytokine IFN-γ, an important feature of protective host responses against fungal pathogens, and exhibit, at least the potential, to mediate direct anti-fungal activity.

15. NK CELLS IN FUNGAL INFECTION

Natural killer (NK) cells are innate cytotoxic lymphocytes widely recognized for their role in immune responses to tumor cells, viral or intracellular bacterial infection. Although less appreciated, NK cells are also central to the host response against a wide variety of fungal pathogens.

There is evidence from the earliest era of NK cell biology that these cells contributed to the fate of a host challenged by fungal pathogens. In response to *Aspergillus* infection, increased NK cell proliferation was dependent upon the production of IL-2 and associated with decreased fungal burden (Romano et al. 1986; Benedetto et al. 1988). In addition, invasive infection with *Aspergillus* in

the sinuses or lung is associated with decreased numbers and cytotoxic function of NK cells (Krishnaraj and Svanborg, 1993; Liodolt et al. 1989; Morrison et al. 2003). In *H. capsulatum* infection, NK cells have an important role (Tewari et al. 2000). Though at least one report has indicated otherwise, the beige mouse (*bg/bg*) deficient in NK cells displays heightened susceptibility to *H. capsulatum*, as do perforin deficient mice (Patino et al. 1987; Suchyta et al. 1988; Zhou et al. 2001). In paracoccidioiodomycoses, the decreased function of NK cells, despite normal numbers, is associated with disease, and NK cells have been demonstrated to exhibit anti-fungal activity against *C. immitis* (Jimenez and Murphy, 1984; Peracoli et al. 1991). Infection with *P. carinii* is associated with depressed NK cell function (Bonagura et al., 1989; Staugus et al., 1988). Finally, there is extensive literature on the role of NK cells in cryptococcal infection, and the immunomodulatory effects of *Candida* on NK cell populations.

Though it is well understood that NK cells participate in the response to fungal infection, there is a surprisingly little known about the mechanisms of recruitment. Only one report has explicitly examined NK cell recruitment in the context of fungal infection. In response to invasive aspergillosis, NK cells home to the lung in an MCP-1-dependent fashion (Morrison et al. 2003). It is likely that this, and other chemokines, such as CCL10, which is responsible for the recruitment of NK cells during viral or mycobacterial infection, are involved in the recruitment of NK cell to sites of fungal infection (Lande et al. 2003; Trifilo et al. 2004).

In contrast to the mechanisms of NK cell recruitment, the activation of NK cells by fungal organisms is well described. Fungi or fungal antigen possess the ability to activate NK cells both directly or indirectly. The mechanism of activation is not entirely understood; however, through the stimulation of IL-2, fungi can induce lymphokine-activated killer cells (LAK). The association of increased IL-2 production with fungal infection was reported in association with aspergillosis (Benedetto et al. 1988). In cryptococcal meningitis, defective NK cell function was reconstituted with IL-2, and NK cells responded to IL-2 with enhanced anti-cryptococcal activity (Gonzalez-Amaro, 1991; Horn and Washburn, 1995; Levitz and Dupont, 1993). The best evidence for the induction of LAK comes from studies on the effect of *Candida*. Early reports analyzing the effects of *C. albicans* inoculation indicated that splenic cell-mediated antifungal activity correlated with cytotoxicity against NK sensitive YAC-1 targets. (Baccarini et al. 1983; Wojdani and Ghoneum, 1987). It was later postulated that LAK cells were responsible for this activity (Marconi et al. 1985). Since then, it has been established that whole *Candida* organisms or components of the cell wall including mannoprotein and glucans stimulate the IL-2 dependent induction of LAK cells with enhanced cytotoxicity (Scaringi et al. 1988, 1990, 1992, 1994).

IFN-γ likely plays a role in fungal activation of NK cells. Although NK cell activity in cryptococcal meningitis was not reconstituted by IFN-γ, other reports suggest that IFN-γ can enhance the anticryptococcal activity of NK cells, and in systemic cryptococcosis, enhanced NK cell activity was IFN-γ dependent (Horn and Washburn, 1995; Salkowski and Balish, 1991). With this in mind, fungi may

directly activate NK cells, as *C. neoformans* and *C. albicans* have been reported to stimulate the production of IFN-γ from NK cells (Levitz and North, 1996).

NK cells activated in response to fungal challenge produce cytokines important in the regulation of the immune response and may display natural or opsonin-dependent antifungal activity. It follows that the relative contribution of NK cell effector mechanisms may vary in response to different fungal pathogens.

NK cells demonstrate inhibitory or cytotoxic activity against *P. braziliensis*, and *C. immitis*, further, the decreased cytotoxic activity of NK cells is associated with recurrent infection, but the regulatory function of NK cells in these infections is not known (Petkus et al. 1987; Jimenez and Murphy, 1984).

In histoplasmosis, perforin knockout mice displayed increased mortality and fungal burden, in response to *H. capsulatum* suggesting that direct antifungal activity may be important in defense against this pathogen (Zhou et al. 2001). Furthermore, NK cells have been demonstrated to exhibit both natural and antibody-dependent cell mediated cytotoxicity (ADCC) against *H. capsulatum*. In susceptible mice, NK cell cytotoxic function is impaired, suggesting the contribution of NK cells to the defense against *H. capsulatum* is largely due to cytotoxic effector function (Tewari et al. 2000).

A more comprehensive appreciation for the role of NK cells in fungal infection may be gained by examining the NK cell response to *C. neoformans* and *Candida*. The suggestion that NK cells have an important role in cryptococcal infection was first made in the mid-eighties (Murphy, 1985; Nabavi and Murphy, 1985). Host resistance to *C. neoformans* correlated with NK cell activity, and the adoptive transfer of splenocytes to mice depleted of NK cells and other natural effectors restored anti-cryptococcal activity, but only if NK cells were present (Hidore and Murphy, 1986[A]). It was then shown that specific antibody to *C. neoformans* could enhance the anti-cryptococcal activity, demonstrating ADCC (Nabavi and Murphy, 1986). Subsequently, the susceptibility of *bg/bg* mice was firmly linked to the presence of NK cell activity by more than one group (Hidore and Murphy 1986[B]; Salkowski and Balish, 1991). Other telling indications linking murine cryptococcal resistance to NK cell function was the demonstration that a population of Thy1+, T-cell-antigen-negative (NK) cells were responsible for pulmonary resistance to *C. neoformans* and in preventing disseminated infection (Hill and Dunn, 1993). More detailed examination of the interaction between murine NK cells and *C. neoformans* revealed that NK cell activity against *C. neoformans* is not simply growth inhibition, but a cytotoxic effector function (Hidore, 1991[A], 1991[B]) (Figure 2). The binding interaction between NK cells and cryptococcal organisms was demonstrated to share features with NK-tumor interactions, including a requirement for $Mg2^{+}$, and reorganization of the microtubule organization center towards the contact point. Conversely, in place of a broad contact surface employed during anti-tumor activity, NK cells contact *C. neoformans* with microvilli (Murphy and Hidore, 1991[A]).

There is also information to indicate the importance of NK cells in human disease. NK cells from patients with cryptococcal meningitis were demonstrated to have anti-cryptococcal activity, as did human NK cells (Gonzalez-Amaro et al. 1991;

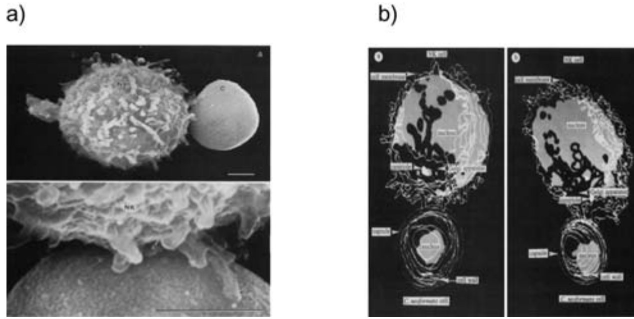

Figure 2. Interaction of NK cells with *Cryptococcus neoformans.* a) Scanning electron micrographs of NK cell-C. neoformans conjugates. (Murphy et al. 1991). b) 3-dimentional reconstruction from transmission electron micrographs of NK cell-C. neoformans conjugates demonstrating polarizatin of the golgi apparatus and centrioles towards the contact point. From a) (Murphy et al. 1991) "Binding interactions of murine natural killer cells with the fungal target *Cryptococcus neoformans*" and b) (Hidore et al. 1991) "Responses of murine natural killer cells to binding of the fungal target *Cryptococcus neoformans*". Both figures reprinted with permission from the American Society for Microbiology

Levitz and Dupont, 1993, Ma et al. 2004). Additional reports utilizing isolated human lymphocytes and the human NK cell line NK 3.3, supported these findings, and perforin was demonstrated to be the effector molecule responsible for human NK cell responses (Levitz et al. 1994; Murphy et al. 1993; Ma et al. 2004).

The production of cytokines and regulatory function of NK cells in the response to *C. neoformans* is also well documented. As indicated above, NK cells respond to direct stimulation by *C. neoformans* through the production of IFN-γ, independent of IL-2 or IL-12 (Levitz and North 1996). In addition the interaction between NK cells and *C. neoformans* was demonstrated to inhibit the production of TNF-α and GM-CSF (Murphy et al. 1997). Thus, NK cells may serve also to regulate the cytokine response, promoting a Th1 skewed T-cell response through the production of IFN-γ, and dampening non-protective or harmful Th2 inflammatory responses. The regulatory role of NK-derived IFN-γ has been pursued. One group has intensely investigated the role of IL-12 and IL-18 in the NK cell response to *C. neoformans*, and found that *in vitro* these cytokines synergistically induced fungicidal activity of peritoneal exudates cells, through the production of NK cell-derived IFN-γ and increased NO production (Zhang et al. 1997). These results were confirmed *in vivo*, with the additional finding that IL-4 production was reduced (Qureshi et al. 1999). Further studies, indicated that NK cell-derived IFN-γ induced TNF-α-dependent nitric oxide production and fungicidal activity in macrophages (Kawakami et al. 1999, 2000[A], 2000[B]). This experimental model helps to demonstrate a regulatory role for NK cells, similar to that observed for NKT cells in response to cryptococcal challenge. The regulatory and fungicidal activity of NK cells in response to *C. neoformans*; however, differs greatly from the host NK cell response to *Candida*.

C. albicans possesses marked immunomodulatory effects against NK cells (Bistoni et al. 1983, 1985). It was discovered that injections with inactivated

C. albicans induced a cytolytic effector population of cells against the NK cell sensitive target YAC-1 (Marconi et al. 1985). Splenic NK cell activity was demonstrated to increase in response to heat killed *C. albicans*, and NK cell-mediated anti-tumor activity was increased by stimulating with mannoprotein and beta-glucan preparations from *C. albicans* (Ausiello et al. 1987, 1987; Scaringi et al. 1988; Wojdani and Ghoneum, 1987). Further study with inactivated *C. albicans* demonstrated induction of IL-2 dependent cytotoxicity in LAK cells against both NK sensitive and insensitive targets (Scaringi et al. 1990). *C. albicans* cell wall preps or mannoprotein were found to act as a booster for this phenomenon (Scaringi et al. 1992, 1994).

The effector function of this population of LAK cells against fungi has also been investigated. LAK cells have effective antifungal activity against both the hyphal and yeast forms of *C. albicans*. Multiple injections of inactivated *C. albicans* stimulated the generation of cytotoxic peritoneal cells with phenotypic and functional characteristics of LAK cells generated *in vitro* with IL-2 (Scaringi et al. 1991). Furthermore, these cells demonstrated marked anti-hyphal activity, and pre-exposure of hyphae to stimulated peritoneal cells reduced the lethality of the fungus in immuno-depressed mice (Scarnigi et al. 1991). Splenocytes activated with IL-2 developed anti-fungal activity, and NK cells derived from human blood were demonstrated to mediate the direct growth inhibition of 6 different strains of *Candida* yeast, a function enhanced by IL-2 (Beno and Mathews, 1992; Gulay and Imir, 1996). IL-2 activated NK (LAK) cell anti-candidal activity was reported in one case in the presence of IFN-γ (Mathews and Witek-Janusek, 1998). Potentially conflicting reports illustrate the complexity as demonstrated by depletion of NK cells in the T cell defective environment of SCID mice. In these mice, NK depletion did not increase susceptibility to intraperitoneal infection with *C. albicans*, but could be explained by the absence of specific T cell activation signals (Greenfield et al. 1993). Although there is strong evidence that under certain conditions LAK cells may exhibit cytotoxic activity against *C. albicans*, requirements for cytotoxic activity for optimal host defense are unclear.

The regulatory role of NK cells in *C. albicans* infection is well established. These cells have the ability to regulate immune responses to this fungus through the production of cytokines and directly though interactions with other innate immune cells. Direct stimulation of NK cells with fungi results in the production of IFN-γ, TNF-α and GM-CSF (Blanchard et al. 1991; Djeu, 1991; Levitz and North, 1996; Arancia et al. 1998). The production of these cytokines appears to be important in the regulation of neutrophil activity against *C. albicans*. Large granular lymphocytes (LGL) or NK cells were first reported to regulate the anti-fungal activity of polymorphonuclear (PMN) cells through a soluble PMN activating factor (Djeu and Blanchard, 1987). The soluble PMN activator was likely TNF-α, although NK cell-derived GM-CSF might synergize with TNF-α to provide a potent recruitment and activation of PMN-mediated fungicidal activity (Blanchard et al. 1991; Djeu, 1991, 1992).

In addition to the regulation of neutrophils through cytokine production, LAK cells play an interesting role in the direct regulation of the monocyte/macrophage

system. One group has pursued this phenomenon in the response to *C. albicans*. The first report indicated that LAK suppressed the anti-fungal activity of GM-CSF and IL-3 cultured monocytes (Wei et al. 1991). The observed suppression; however, was alleviated by the presence of IFN-γ in monocyte culture (Wei et al. 1991). Monocyte antigen presenting ability, and IL-1 production, revealed a similar pattern of suppression following exposure to LAK cells. The evidence suggests that fungal activation of NK cells can lead to selective pressures on monocyte activation, and alter T-cell mediated responses accordingly (Wei et al. 1992).

The contribution of NK cells to immune responses against *C. albicans* appears to be significant. The regulatory role of NK cells in response to *Candida* is highly active and involves both cytokine and direct regulation, and there is substantial evidence for direct cytotoxic effector function.

16. SUMMARY

The contribution of non-neutrophilic granulocytes and innate cytotoxic lymphocytes to fungal immunology is considerable. Collectively, these cells participate in allergy, inflammation and fungal infection in various tissues, but most notably in the respiratory tract. Mast cells, basophils and eosinophils are responsible for the initiation of allergic responses to fungi. This reaction may be relatively harmless, as in mild rhinitis, or conversely, may contribute to damaging late phase and chronic reactions characterized by the infiltration of eosinophils, Th2-dominated inflammation, airway hyperreactivity and tissue remodeling typical of asthma, ABPM or AFS. Though allergy to fungi is potentially damaging, the cells responsible may also be contributing to defense against fungal pathogens. That role; however, will require additional experimental support. Innate cytotoxic lymphocytes have demonstrated importance in fungal infections such as pulmonary cryptococcosis. These lymphocytes participate through the secretion of cytokines helping to balance the Th1/Th2 response and by promoting clearance of fungi, or through direct cytotoxic effector function against fungal pathogens.

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CHAPTER 6

GENES AND GENE PATHWAYS IN *CANDIDA* INFECTION

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Abstract: Advances in genetic technology have promoted an explosive increase in our knowledge of genes relevant to *Candida* infection, and our understanding of their mode of action. Although the major influence on susceptibility to systemic infection is the presence or absence of complement C5, at least two other genes, as yet unidentified, influence the severity of tissue damage. Mice in which specific genes have been deleted (gene-knockout) mice are now readily available, and have been used both in the analysis of receptor interactions with *Candida*, and to study the role of T cell-derived cytokines in clearance of the infection and the development of host resistance, but results have not always been consistent. Gene profiling studies, in both humans and mice, will no doubt resolve some of the present anomalies

1. INTRODUCTION

At the most basic level, variation in susceptibility to infectious disease is determined by the genetic context in which it occurs. Although this statement is now intuitively obvious, progress in identifying genes relevant to human disease and determining their mode of action has been slow.

Of all the fungal diseases, candidiasis represents the most significant cause of morbidity and mortality. Most genetic studies have focussed on this organism, and it is here used as a paradigm for the genetic control of fungal disease. In recent years, it has been the subject of intensive research, nevertheless, the basic pathways that detemine susceptibility to infection have not yet been fully delineated. This can be attributed, at least in part, to the many different manifestations of infection in humans (oral, vaginal, systemic, cutaneous), and the likelihood that host responses to each involve different contributions from the innate and adaptive immune responses. In addition, the search for genetic associations with susceptibility to candidiasis in humans has been complicated by the commensal nature of many *Candida* spp., and their ability to exploit weaknesses in host defence mechanisms to cause infection.

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2. GENES IN HUMAN DISEASE

Chronic mucocutaneous candidiasis (CMC) has been classified into a number of sub-groups, depending on individual manifestations of the disease (Kirkpatrick 2001), but the common feature is typically an impairment of T lymphocyte function. It is also the syndrome for which there is most evidence for familial inheritance, and the *AIRE* (autoimmune regulator) gene has been linked to autoimmune polyendicrinopathy candidiasis ectodermal dystrophy (APCED) syndrome (Nagamine et al. 1997), in both a Finnish (Aaltonen et al. 1994) and an Iranian (Bjorses et al. 1996) pedigree. There is evidence for additional genes associated with CMC, but these have not yet been identified.

Systemic, or disseminated candidiasis, is most frequently encountered in a hospital setting, usually in patients with other severe medical conditions. Although familial transmission of susceptibility has not been documented in this form of the disease, a polymorphic allele in the promoter region of the IL-4 gene is associated with an increased prevalence of systemic candidasis among patients with acute leukemia (Choi et al. 2003). However, further studies of genes associated with the innate immune response, such as low-affinity Fcγ receptors (*FCGR2A, FCGR3A, FCGR3B*), chitotriosidase (*CHIT1*) p-22-phox NADPH oxidase (*CYBA*) and mannose-binding lectin (*MBL2*) failed to demonstrate any correlation with susceptibility to chronic dissemiated candidiasis in the context of acute leukemia (Choi et al. 2005).

These results are obviously only indicative of associations. As *Candida* is a commensal organism, individual defects in the host response may produce an environment that is conducive to infection; however, the affected pathway may not be directly involved in the normal host response against the yeast. Similar considerations are relevant to investigations of variables that control the host response against both oral and systemic infections in mice, although in this case, the use of natural mutations has more recently been superseded by the specific deletion of particular genes in gene knockout (GKO) mice.

3. MURINE MODELS OF THE DISEASE

Inbred mice are readily infected with *C. albicans*, with brain and kidney prime foci for infection (Louria 1985; Papadimitriou and Ashman 1986). There is considerable variation between strains in mortality after systemic challenge (Marquis et al. 1986; Marquis et al. 1988), but no systematic strain-dependent correlations could be demonstrated. Further studies (Ashman and Papadimitriou 1987) evaluated both mortality and colony counts in infected tissues, as well as the severity of lesions in the tissues.

Tissue damage showed a clear correlation with the genetic background of the mouse, and the phenotype segregated as a Mendelian co-dominant (Ashman and Papadimitriou 1992). Analysis in the AKXL recombinant inbred set (Ashman et al. 1997) demonstrated that the *Candida albicans* resistance gene (*Carg1*) was probably

located within a 17 cM segment of chromosome 14 (Ashman 1998). The functions of this gene in determining the expression of mild or severe tissue damage have not yet been defined, but as the phenotypes are expressed in nude mice (Fulurija et al. 1997), they are probably related to qualitative or quantitative differences in the effector functions of bone marrow-derived phagocytic cells. Nevertheless, mice that are genetically susceptible or resistant to tissue damage display substantial differences in both early immune and inflammatory responses, and in the development of immunological memory, suggesting that the gene may also have an impact on the host response by inducing some form of regulatory activity. The existence of a second gene, suggested by data from the AKXL recombinant inbred mice (Ashman et al. 1997), was later confirmed in [C57BL/6 × C57L] × C57L backcross mice (Ashman et al. 1998). This gene (*Carg2*) was also a Mendelian co-dominant, that affected the severity of tissue damage but not the magnitude of the fungal burden.

Less is known about mouse strain-dependent influences on oral or other mucosal infections. The severity of oral infection in BALB/c and CBA/CaH mice was consistent with the pattern of lesions after systemic infection (Farah et al. 2002), indicating that the effect of the *Carg1* gene was also demonstrable at the level of the oral mucosa. The only other reported mouse strain-dependent difference in the severity of oral infections was between BALB/c and DBA/2 mice, the latter being more infection-prone (Chakir et al. 1994; Elahi et al. 2000). The basis for this may be genetically complex, being attributed to γ/δ T cells in one model (Chakir et al. 1994), and to a reduced production of IL-4 in the other (Elahi et al. 2000). More recently, C57BL/6 and BALB/c mice, that are equally susceptible to gastric candidiasis, were shown to exhibit significantly different cytokine, chemokine, and β-defensin responses in the gastric tissues (Schofield et al.), with the BALB/c mice being markedly less responsive.

In contrast to the above, most inbred and outbred strains of mice were similarly susceptible to vaginal candidiasis (Calderon et al. 2003). The only exceptions were the outbred strain CD-1, that was markedly resistant, and CBA/J that was moderately resistant. The basis for the resistance of these strains is unknown, although CD-1 is known to be resistant to endocrine disruption by oestrogen, and thus may not respond to the oestrogen priming necessary to establish experimental infection.

3.1. Natural Mutations

The earliest applications of this approach in mice were studies of fungal infection in T cell-deficient 'nude' mice (Cutler 1976; Rogers et al. 1976). Nude mice were shown to be no more susceptible than euthymic controls to systemic infection with *C. albicans*, indicating that innate rather than adaptive immunity was a major component of the host response against this form of the disease. These conclusions were shown to hold for a thigh lesion model in nude mice (Tabeta et al. 1984), and were also confirmed in studies of systemic candidiasis in two strains of inbred

mice (Fulurija et al. 1997), and in both T and B cell-deficient SCID mice (Mahanty et al. 1988). Mice carrying the beige (*bg/bg*) mutation developed more extensive tissue damage than controls after systemic challenge (Ashman and Papadimitriou 1991), an effect attributed to defects in granulocyte production or function in these animals.

Resistance against mucocutaneous candidiasis in mice appears to be determined by both innate and cell-mediated immunity. Nude mice infected orally develop a severe infection that does not heal, and is dependent on $CD4^{+}$ cells for its resolution (Farah et al. 2002), but gastrointestinal candidiasis was shown to disseminate only in mice with defects in both innate and adaptive immunity. After intragastric or oral inoculation of *C. albicans*, multiply-immunodeficient (*bg/bg, nu/nu*) mice, deficient in both T cells and phagocytic cells, developed a persistent gastrointestinal infection (Balish et al. 1990), whereas mice that lacked only phagocytic cells (*bg/bg, nu/+*) cleared the infection efficiently (Cantorna and Balish 1990). The severity of vulvovaginal candidiasis in nude mice was comparable to that in euthymic controls (Black et al. 1999; Cantorna et al. 1990), again suggesting that innate and cell-mediated immunity play different roles in resistance against mucocutaneous candidiasis at distinct anatomical sites.

The different patterns of disease after systemic and mucosal infection in these mice are broadly consistent with the clinical data, but are indicative of the complexity of the host response against *Candida*, and of the caution required in generalisation of results from different experimental models.

3.1.1. Role of complement

The complement system is a complex cascade of proteins that forms one of the enzyme systems found in plasma. Complement produces a rapid, highly amplified response to a trigger stimulus. In the host immune system, complement functions by opsonising pathogens, facilitating lysis by phagocytes, and by directing phagocytes to the site of inflammation.

One of the dominant variables that influence susceptibility to *Candida* infection in mice is the presence or absence of the third component of complement (C5), although the effects differ depending on the route of infection and the strain of mouse. Some C5-deficient mice (A/J, DBA/2) are highly susceptible to *C. albicans* infection, showing a much higher early mortality, and a greater fungal burden in the kidneys than C5-sufficient mice (Ashman et al. 1993; Ashman et al. 1996; Hector et al. 1982). However, the effect of C5-deficiency, although still significant, was much less marked in mice bred on the B10 background (Lyon et al., 1986). After reconstitution with C5-sufficient serum, the fungal burden in the kidneys of DBA/2 mice was decreased, and survival was prolonged (Ashman et al. 2003).

Although C5-deficient mice can not generate complement-derived serum chemotactic factors, and were somewhat less efficient in clearing cutaneous candidiasis (Wilson and Sohnle 1988), their initial responses to the infection, and the accumulation of neutrophils in the *Candida*-infected skin of these animals were normal. As the candidacidal activity of phagocytic cells from normal and C5-deficient

mice is equivalent (Morelli and Rosenberg 1971), it is clear that the opsonising and/or chemotactic properties of C5 are critical factors in the early containment and elimination of the yeast. Interestingly, C5 deficiency did not affect the severity of mucosal colonisation (Ashman et al. 2003), suggesting that rapid recruitment of inflammatory cells may be vital for protection of susceptible organs, such as the kidney, from being overwhelmed by the infection. In other anatomical regions, such as the skin or oral cavity, the proliferative capacity of the yeast may be more limited, and different host resistance mechanisms may play a more important role.

3.2. Gene Knockout (GKO) Mice

Recent advances in molecular genetics have resulted in an explosive increase in our knowledge of the role of specific genes in infectious disease, and there is a considerable amount of information on responses to *Candida* infection in mice from which immunologically-relevant genes have been selectively deleted. However, direct deletion of genes has not yet provided unequivocal pointers to the most important pathways in the host response, and the analysis has not been rigorously applied to models of the different types of infection.

The functional dichotomy between responses to disseminated and mucosal infection evident in nude mice was reproduced in mice lacking the α and δ chains of the T cell receptor, and thus deficient in α/β and γ/δ T cells. These animals were found to be highly susceptible to orogastric candidiasis, but were resistant both to acute systemic candidiasis after intravenous inoculation, and also to disseminated candidiasis of endogenous origin (Jones-Carson et al. 2000). Conversely, B cell knockout mice showed no increased susceptibility to either orogastric or acute systemic candidiasis (Wagner et al. 1996). Given the concordance between the data from the immunodeficient and the α/δ GKO mice, it seems reasonable to interpret other findings in the context of either a predominantly innate response following systemic infection, or a predominantly T cell-mediated response to mucocutaneous challenge.

Although it might have been expected that deletion of genes for specific, immunologically relevant, cytokines would clarify the main pathways involved in the host response, this has generally proved not to be the case. Mice in which the gene for interferon-γ (IFN–γ) had been deleted showed either increased susceptibility to both gastric and systemic candidiasis (Balish et al. 1998), or no change in the severity of either form of the disease (Qian and Cutler 1997). Intraperitoneal, rather than intravenous infection resulted in a marked increase in mortality of the GKO mice (Kaposzta et al. 1998), although, paradoxically, the increased susceptibility was unrelated to the extent of organ colonization. After gastrointestinal colonisation, mice lacking the IFN-γ receptor (IFN-$\gamma R^{-/-}$) were more susceptible to infection (Cenci et al. 1998), showing increased fungal growth in the stomach compared to wild-type mice, and failing to develop Th1-mediated acquired immunity after

immunisation. The impaired resistance correlated with defective IL-12 responsiveness, but was independent of IL-12 production, suggesting that IFN-γ is required for development of IL-12-dependent Th1 immunity.

IL-18 as well as IL-12, is a strong stimulator of IFN-γ production, and treatment of normal mice with IL-18-specific antibodies was shown to increase the fungal burden in the kidneys (Stuyt et al. 2002), but since comparable treatment of IFN-γ GKO mice had no effect, it appeared that IL-18 had an indirect effect mediated through endogenous IFN-γ. These general results were reproduced in IL-18 GKO mice (Netea et al. 2003), but these investigators also demonstrated a reduced recruitment of monocytes to the sites of *Candida* infection, as well as a defect in MIP-2 production, and a dramatic down-modulation of IFN-γ production.

Conversely, after oral infection with *C. albicans*, mice deficient in IL-12 developed chronic oral infections that did not resolve (Ashman et al. 2004), and were also highly susceptible to gastrointestinal candidiasis (Mencacci et al. 1998a). However, the severity of systemic infection in IL-12 GKO mice was unaltered (Hu 2004), suggesting that IL-12 is important in promoting the development of the Th1-type responses required for recovery from mucosal infection, but that the pathways of innate immunity may dominate in systemic infection. Nevertheless, the cytokine interactions in infected mice appear complex, with evidence for a positive regulatory loop between IL-12 and IL-10 that promotes optimal responses of the Th1 cells (Mencacci et al. 1998a), even though it may compromise the efficiency of the innate immune response against the yeast.

This concept tended to be supported by investigations of systemic candidiasis in IL-10 GKO mice. These animals consistently demonstrated increased resistance against intravenous challenge (Del Sero et al. 1999), but showed no difference in susceptibility to orogastric candidiasis (Vazquez-Torres et al. 1999) or oral infection (Hu 2004), although Del Sero and colleagues (Del Sero et al. 1999) also reported a lower fungal burden in gastrointestinal infection, with reduced fungal-associated inflammatory responses. Unfortunately, the mechanisms of this increased resistance are still unclear, in one case being attributed to a greater efficiency of neutrophil-mediated killing (Vazquez-Torres et al. 1999), and in the other, to increased production of nitric oxide, as well as up-regulation of IL-12, and TNF-α, and the generation of Th1-type cell-mediated immunity (Del Sero et al. 1999).

IL-4 is another cytokine important in determining the nature of the T-helper reponse to *C. albicans*, and deletion of the gene for IL-4 was reported to decrease the severity of the early stages of systemic or gastrointestinal infections (Mencacci et al. 1998a). However, the mice later displayed defective IFN-γ and IL-12 production, but not IL-12 responsiveness, failing to mount a protective Th1 response, and succumbing to the infection. These data contrast with that of Vazquez-Torres et al (Vazquez-Torres et al. 1999), who reported an increased susceptibility to systemic, but not to orogastric candidiasis, and a recent study found no efffect of IL-4 deletion on either systemic or oral infections (Hu 2004).

At present, experiments that have investigated the role of T cell cytokines in GKO mice have not revealed definitive pathways of host responsiveness. It seems

clear that genetic pathways leading to the production of IFN-γ, IL-12 and IL-10 all interact in the generation of protective T cell responses against mucosal (oral, orogastric, and gastrointestinal) infections, but they are less clearly implicated in the resolution of primary systemic infections. Both clinical and experimental data have demonstrated a requirement for neutrophils in the resolution of systemic infection, and interference with recruitment of these cells markedly affects susceptibility to infection. IL-17A is a proinflammatory cytokine important in activation of the innate immune response, and deletion of this gene dramatically decreased survival, with substantial increases in the fungal burden in the kidneys (Huang et al. 2004). Conversely, expression of mIL-17A in vivo protected normal mice from lethal infection.

Tumour necrosis factor (TNF-α) is one of the more important of the cytokines that mediate pro-inflammatory activities in the early phases of the host response, and TNF-α GKO mice were found to demonstrate increased susceptibility to *C. albicans* infection (Marino et al. 1997). TNF-α GKO mice showed higher levels of infection in the kidney but not in the brain after systemic infection (Hu 2004), whereas there was a significant increase in the severity, but not the duration, of the infection after oral inoculation. Deletion of both TNF-α and lymphotoxin-α (LT) resulted in substantially increased growth of *Candida* in the organs, that was attributed to delayed recruitment of neutrophils and a reduced phagocytic capacity of these cells, although oxidative metabolic pathways and killing of yeasts appeared unaffected in these mice (Netea et al. 1999). Comparable experiments (Mencacci et al. 1998b) demonstrated increased susceptibility to both systemic and gastrointestinal infection, again attributed to the impaired effector function of neutrophils, although in this case, the effect was correlated with the induction of non-protective Th2 rather than protective Th1 responses. A similar explanation was invoked to explain the increased susceptibility to *Candida* sepsis and abscess formation after intra-abdominal infection of TNF-α/LT GKO mice (Vonk et al. 2002).

The crucial importance of phagocytic cells in the host response led to an examination of the role of both reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in the control of systemic and mucosal candidiasis (Balish et al. 2005). Mice doubly deficient in phagocyte oxidase (Phox) and nitric oxide synthase 2 (NOS2) were highly susceptible to infection, but the effector functions of the phagocytic cells from the peritoneal cavity of either single or double GKO mice were not different from those of the wild-type controls. This result is consistent with that above (Netea et al. 1999), and suggests that factors other than ROI or RNI play an important role in the killing of *Candida* and clearance of infection.

To this point, the focus of the discussion has been on the phenotypes expressed after *C. albicans* infection of various cytokine GKO mice, with the aim of detecting commonalities that might lead to predictions of important effector pathways. A reciprocal strategy has been to identify the receptors through which effector cells recognize *C. albicans*, and thus trace the downstream targets of the early effector response.

Toll-like receptors (TLRs) are currently a major focus for investigation, and mediate macrophage recognition of many different microbial ligands. However, the role and importance of the various TLRs in *Candida* infection are still contentious.

3.2.1. Studies in vivo

In one study (Villamon et al. 2004a), TLR2 GKO mice infected either intravenously or intraperitoneally demonstrated significantly increased mortality compared to controls. This impairment was associated with a decreased macrophage production of TNF-α and macrophage inhibitory protein-2 (MIP-2), although phagocytosis and production of reactive oxygen intermediates by these cells was unaltered, and the mice remained competent to develop specific humoral immune responses (Villamon et al. 2004b).

In contrast, Netea et al (Netea et al. 2004) found that the TLR2 GKO mice were more resistant to disseminated infection. Levels of pro-inflammatory cytokines were unchanged, but production of IL-10 was reduced. They concluded that signaling through TLR2 enhanced IL-10 production and survival of a population of regulatory T cells that tended to reduce the efficiency of the innate immune response. They further demonstrated that TLR4 GKO mice were also highly susceptible to *C. albicans* infection (Netea et al. 2002b), although production of pro-inflammatory cytokines by macrophages was unchanged, and the candidacidal potential of the neutrophils and macrophages was normal. In this model, the increased susceptibility appeared to be associated primarily with impaired chemokine expression and recruitment of neutrophils. These contradictory data have yet to be reconciled, although Netea (Netea et al. 2005) in a response to Gil (Gil et al. 2005) suggested that the discrepancies might be related either to genetic variability between mouse strains, or to differences between the experimental models used.

A more comprehensive study evaluated susceptibility to intravenous and mucosal infection with either low-virulence *Candida* yeasts, or highly virulent *Candida* hyphae, in mice with deletions in genes coding for various TLRs (Bellocchio et al. 2004). After intravenous infection of TLR2 GKO mice, survival was unchanged but the fungal burden in the organs was significantly decreased; in contrast, colonisation of the stomach was markedly increased following gastrointestinal challenge. These data again tend to suggest the elicitation of different pathways of host responsiveness in systemic versus mucosal infection. Deletion of TLR4 had no effect on the severity of systemic infection with either virulent or avirulent *Candida*, but also increased susceptibility to gastrointestinal colonisation, whereas mice lacking TLR9 demonstrated increased resistance against both systemic and gastrointestinal disease. Interestingly, MyD88 GKO mice, in which production of TNF-α following activation of TLR2 is impaired, show an increased fungal burden following systemic challenge with the avirulent, but not the virulent strain of *C. albicans*. A requirement for recruitment of the MyD88 adapter protein, leading to activation of NFkB and its downstream targets would also be consistent with the later development of protective Th1 immunity after infection with the avirulent, but not the virulent yeast.

The Th1-mediated promotion of host inflammatory responses was further demonstrated to be linked, at least in part, to interactions between CD40 and CD40 ligand, as deletion of the ligand resulted in increased fungal load in the kidneys, although this only took place late in the course of infection (Netea et al. 2002a). The increased susceptibility was associated with reduced concentrations of TNF-α in the plasma, and was attributed to decreased candidacidal activity of macrophages, as a consequence of lower nitric oxide production by the GKO mice. $CD40L^{-/-}$ mice also show an increased susceptibility to oral candidiasis (Farah, unpublished data), but the mechanisms have not yet been elucidated.

The mannose receptor has been shown to be implicated in *Candida* killing and cytokine secretion by mouse macrophages (Kaposzta et al. 1998), and it has also been reported that engagement of this receptor results in fungal degradation, the production of pro-inflammatory cytokines, and up-regulation of co-stimulatory molecules and MHC class II – a protective Th1-type response (Romani et al. 2002). However, when tested directly, mice lacking the mannose receptor were found to be no more susceptible than wild-type (Lee et al. 2003). Phagocytosis by peritoneal macrophages was equivalent in the two groups, and was inhibitable by glucan, but not by mannan. Both groups demonstrated competence in antibody production.

3.2.2. Studies in vitro

When macrophage cell lines generated from the bone marrow of TLR2- and MyD88-GKO mice, and TLR4-deficient mice were assessed for their functional activity against *C. albicans*, macrophages from TLR2 GKO mice were found to be more efficient in containing the yeast, compared to controls (Blasi et al. 2005), whereas the secretory activity was unchanged, suggesting that these two effector functions may be triggered by different recognition events. The functional activity of macrophages from the other two groups was unaltered. However, other studies, also using macrophages from MyD88 GKO mice, found that recognition (Roeder et al. 2004), as well as phagocytosis and intracellular killing of *C. albicans* was impaired compared to controls (Marr et al. 2003), and cytokine production substantially decreased. The apparent conflicts between data generated in the different laboratories have yet to be reconciled.

Other genes expressed in macrophages have been demonstrated to play a role in the host response against infectious disease, though their involvement in candidiasis has been less rigorously defined. The *Slc11a1* gene (previously named the natural resistance-associated macrophage protein 1, Nramp1, located on chromosome 1, controls natural resistance to several parasites, such as *Mycobacterium bovis, Leishmania donovani*, and *Salmonella typhimurium* through its pleiotropic effects on macrophage activation and function (Kovarova et al. 2001). In *Candida* infection, macrophage cell lines carrying the resistance allele of this gene acted more effectively against both morphogenic forms of the fungus, and produced more TNF-α in response to stimulation than did congenic cell lines from susceptible mice (Puliti et al. 1995).

The precise pathways that link recognition and effector activities have yet to be delineated, although the leucine zipper transcription factor C/EBP β is known to play a part, as demonstrated by the increased susceptibility to *C. albicans* infection of C/EBP β-deficient mice (Screpanti et al. 1995). Macrophage cell lines derived from the GKO mice showed impaired expression of the genes for TNF-α, IL-6, and iNOS, although activation of NFκB was normal (Gorgoni et al. 2002). Production of IL-12p40 was up-regulated, whereas expression of IL-12p35 was completely inhibited. This finding is provocative, in that it may be relevant to identification of the pathways that lead to the dramatically increased susceptibility to oral, but not systemic, candidiasis of IL-12 GKO mice (Ashman et al. 2004).

Apart from differences between experimental systems used by the research workers, discrepancies between results obtained using GKO mice may be caused by a) redundancy in cytokine function; b) differential expression of pathogen-associated molecular patterns (PAMPs) on different strains or isolates of the yeast, resulting in activation of different pathways depending on the combination of receptors to which the yeasts bind; c) failure to discriminate between experimental models of the oral (predominantly T cell) and systemic (predominantly innate) styles of response. The advent of microarray technology, that permits screening of the expression of thousands of genes simultaneously, now offers the opportunity to examine, in a much more rigorous manner, gene pathways that are activated by exposure to the yeast, and the ways in which these change with time. Use of these global expression profiling technologies have revealed common themes in the immune/inflammatory responses of both humans and mice.

Gene expression profiling of human peripheral blood monocytes was used to assemble a picture of the temporal sequence of of gene activation in these cells after exposure to viable *C. albicans* for periods up to 18hr (Kim et al. 2005). The cells remained viable, and 93% demonstrated phagocytosis. The monocytes showed early up-regulation of genes encoding pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, as well as increased expression of genes for chemokines including IL-8, macrophage inflammatory proteins (MIP) 1, 3, and 4, and macrophage chemoattractant protein 1 (MCP-1). Expression of these genes reached a peak at about 6hr, and gradually declined thereafter, whereas expression of genes that promoted monocyte survival were up-regulated at that time, and remained elevated. Somewhat surprisingly, given the emphasis on T cell responses in animal experiments, expression of genes encoding T cell regulatory molecules such as IL-12, IFN-γ and transforming growth factor-β (TGF-β) was not significantly altered. Furthermore, there was no evidence of differentiation along the lineage leading to antigen presentation, which would be consistent with a lack of lymphocyte involvement at this stage of the response. As it is known that TLR2 signaling through NFκB is sufficient for the differentiation of monocytes/dendritic cells towards the antigen-presentation pathway (Schjetne et al. 2003), this provides indirect evidence that TLR2/NfκB may not be the primary transcriptional target of pattern recognition receptors detecting yeast PAMPs.

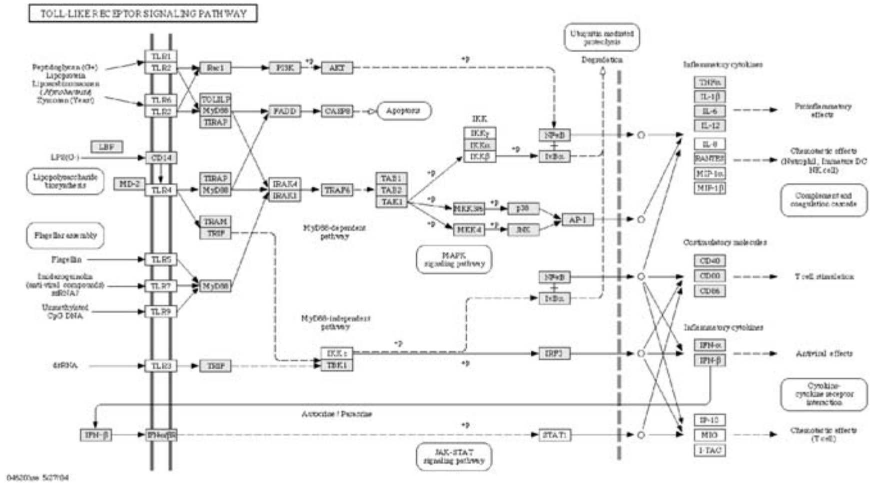

Figure 1. Toll-like receptor signaling pathway. Reproduced with permission from the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/dbget-bin/www_bget?path:dmmu04620)

A broadly similar response was seen in a human monocytic cell line after exposure to *C. albicans* for 3hr in vitro (Barker et al. 2005). These cells also responded to challenge by increased expression of *TNF*-α, *IL-8, CD83, MIP1A* and *MIP1B*, as well as a number of genes involved in signal transduction. This study also failed to demonstrate any significant involvement of T cell-related cytokines, although it could be argued that these might not necessarily be expressed at such an early time (3hr) after infection. Interestingly, the patterns of gene activation did not appear to differ markedly after exposure to opsonised (Kim et al. 2005) or unopsonised (Barker et al. 2005) yeasts. In mice, bone marrow macrophages from the tissue-susceptible CBA/CaH and tissue-resistant BALB/c strains were exposed to live *C. albicans* for 1hr, and expression profiles examined (Li, Wells and Ashman, unpublished data). CBA/CaH mice were much more reactive than BALB/c, showing regulation of over 800 genes compared to 300 in the latter strain. A comparison of genes regulated in the two strains was used to identify pathways common to both, that could be expected to represent the basic activation patterns in response to contact with the yeast. Three were prominent – TNF-α acting via TLR2 (Fig. 1), p38 MAP kinase (Fig. 2), and TGF-β. Also expressed in both mouse and human monocytes were chemokines and anti-apoptosis genes. As in humans, there was no evidence of activation of pathways leading to antigen presentation or production of T cell-related cytokines, which suggests that recognition of *C. albicans* does not elicit a classical TLR response, that tends to drive macrophages down the antigen-presentation/inflammatory pathway.

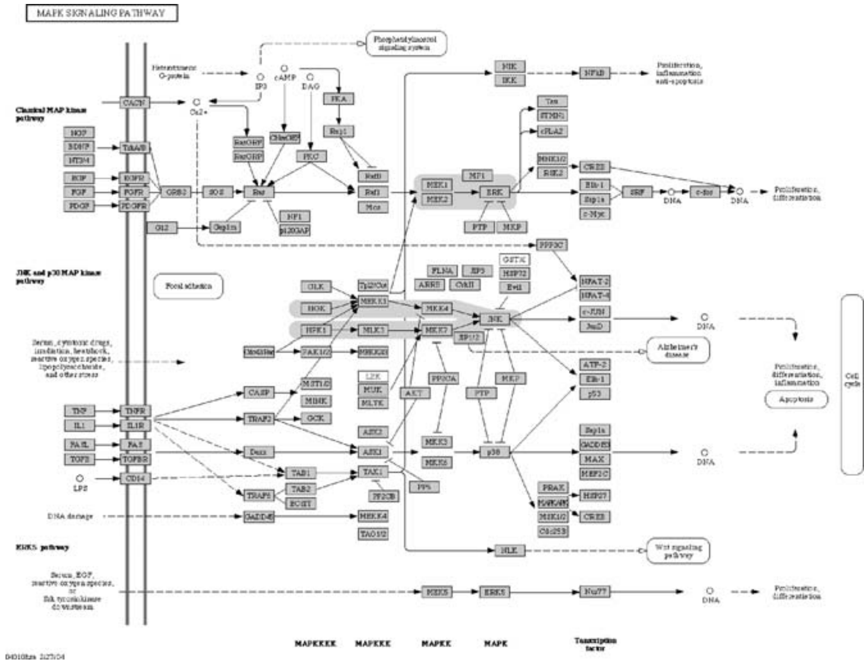

Figure 2. MAP kinase signaling pathway. Reproduced with permission from the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/dbget-bin/www_bget?path:dmmu04010)

4. CONCLUDING REMARKS

Gene profiling is a powerful tool, that has provided an important new perspective on cellular responses elicited by contact with *C. albicans*, although it has highlighted many inconsistencies between the biological and array data, that remain to be resolved. Unsurprisingly, there was a strong emphasis in both human and mouse arrays on the expression of genes that promoted cell survival, consistent with biological data demonstrating that both Fc- and complement-receptor mediated activation and phagocytosis induce macrophages to commence cell division (Luo et al. 2005), thus increasing the pool of effector cells available to combat the infection.

Neutrophils and other inflammatory cells are acknowledged to be crucial to the host response, and gene profiling data confirms expression of chemokines and other inflammatory mediators that function to recruit these cells. The initial stimulus can occur through a variety of receptors; however, a definitive picture of the relative roles of the different receptor/ligand interactions has yet to be obtained. Clearly, TLR2 plays a significant role in the early host response against *C. albicans*, although the fact that responses in TLR2 GKO mice are only partially, but not completely abrogated, confirms that there exist alternative receptors and pathways of activation.

The TLR pathway (Fig. 1) provides highly conserved, and presumably stereotyped, responses to infectious agents, although other pathogen receptors may confer more flexibility on the innate immune response than has previously been recognized (Gordon 2002). This flexibility may also be enhanced by cooperative interactions between TLRs and other receptors, as detailed studies of receptor/ligand interactions have revealed considerable versatility in mechanisms of activation. *Candida* is a large organism, and internalization and phagocytosis is assumed to be necessary for killing. However, all TLR ligands can be recognized as soluble proteins (Underhill and Gantner 2004), whereas it is not yet established that TLRs can bind ligands on microbial surfaces, and thus they may not function as phagocytic receptors, but as regulators of phagocytosis.

In this context, Dectin-1 has been shown to be a major macrophage receptor for β-glucans (Brown et al. 2002), and both DC-SIGN and other c-type lectins appear to be important for phagocytosis of non-opsonised *C. albicans* (Taylor et al. 2004). The microarray data are consistent with this, in that expression of TLR receptors is not transcriptionally regulated by exposure to the yeast, whereas expression of c-type lectin receptors is regulated. SIGNR1, a mannan-inhibitable receptor distinct from the mannose receptor cooperates with Dectin-1 in internalization of particles, but neither binding of Dectin-1 nor SIGNR1 appear to be essential for TNF-α production. Although B-glucan is a major component of yeast cell walls, its presentation depends on the form of the yeast, as it is exposed only at bud scars. Thus Dectin-1 recognizes the yeast, but not the hyphal form of *C. albicans* (Gantner et al. 2005).

Dectin-1acts synergistically with TLR2 to induce synthesis of TNF-α and IL-12, but can also activate alternate syk-dependent kinases, leading to production of IL-2 and IL-10 (Rogers et al. 2005). As these pathways are independent, it was postulated that they may represent a mechanism by which innate host responses might direct the adaptive response towards the development of T helper type 1 versus regulatory T cells. However, neither human nor mouse gene expression data support this interpretation, as there is no evidence for up-regulation of cytokines such as IL-12 or IL-10, that are representative of either the Th1 or Th2 style of adaptive immune response. However, this issue is far from simple, as binding through other receptors, such as the mannose receptor or Fc receptors, has been shown to modulate patterns of cytokine production (Underhill and Gantner 2004).

A second pathway activated in murine macrophages by exposure to *C. albicans* is p38 MAP kinase, and this pathway has two interesting associations: first, the yeast selectively upregulates Cox-2 but not Cox-1, the transcription of which is mediated through p38 MAP kinase and NFkB (Deva et al. 2003); and second, the AIRE gene, that has been identified as leading to susceptibility in humans, is also regulated by p38 MAP kinase (Nagafuchi et al. 2005).

Although the precise pathways involved in host responses must be speculative at present, the gene profiling data strongly indicate that early responses to *Candida* activate innate/inflammatory responses. This is consistent with animal experiments indicating a dominant role for innate immunity in the primary response against

systemic infection, but raises the question of the trigger for the development of the T cell response required for effective resistance against mucosal (oral and gastrointestinal) infection. This will probably await a better understanding of the complex interplay between the different receptor pathways and their targets.

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SECTION 2

SOLUBLE FACTORS

CHAPTER 7

COLLECTINS AND PENTRAXINS

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Abstract: Innate immunity is the first line of defense against pathogens. It plays a key role in the activation and orientation of adaptive immunity. Recognition and elimination of pathogens and of dying cells is mediated by pattern recognition receptors (PPRs) that have both cellular and humoral components. Cellular PPRs such as Toll-like receptors, scavenger and C-type lectin receptors are present on the antigen presenting cells. The humoral innate immune molecules are multifunctional and diverse and include soluble factors such as C1q, the collectins (mannose-binding lectin, surfactant protein A and D), the ficolins, and the pentraxins. This chapter discusses the roles of collectins and pentraxins in defense against fungal pathogens. Collectins have been shown to be involved in a range of immune functions including viral neutralisation, clearance of bacteria, fungi and apoptotic and necrotic cells, down regulation of allergic reactions and resolution of inflammation. Their basic structures include a triple-helical collagen region and a C-terminal homotrimeric lectin or carbohydrate recognition domain (CRD). The trimeric CRDs can recognise carbohydrate or charge patterns on microbes including fungi, allergens and dying cells, while the collagen region may interact with receptor molecules, present on a variety of immune cells, and thus initiate clearance mechanisms. The pentraxins are structurally unrelated to the collectins and include small pentraxins such as C-reactive proteins (CRP), serum amyloid protein (SAP) and long pentraxins such as PTX3. They belong to a superfamily of evolutionarily conserved proteins characterised by a structural motif, the pentraxin domain. The pentraxins are an essential component of humoral innate immunity, with CRP and SAP being acute phase reactants in human and mouse, respectively. CRP and SAP can function as opsonins for a range of pathogens. The protypical long pentraxin, PTX3, has been recently shown to be an important and non-redundant component of anti-fungal defense, especially against infection by *Aspergillus fumigatus*

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1. INTRODUCTION

Innate immunity is the first line of resistance against pathogens and it plays a key role in the activation and orientation of adaptive immunity. Recognition of pathogens and damaged tissues is mediated by pattern recognition receptors (PRRs) (Medzhitov 2001), consisting of cellular and humoral components. Cellular PRRs, including Toll-like receptors, scavenger and C-type lectin receptors, and G protein-coupled formyl peptide receptors form a formidable repertoire of innate immunity of antigen presenting cells (APCs) (Gordon 2002). The soluble wing of innate immunity is also multi-faceted and diverse; it includes C1q, collectins, ficolins, and pentraxins.

Human collectins, including mannose-binding lectin (MBL), surfactant proteins A (SP-A) and D (SP-D), are collagen-containing C-type (calcium dependent) lectins. These are large hydrophilic proteins and their basic structures include an N-terminal triple-helical collagen region and a homotrimeric ligand-recognition domain, called a C-type lectin or a carbohydrate recognition domain (CRD) (Kishore and Reid 2001; Holmskov et al. 2003). MBL is a serum protein synthesised in the liver and is the recognition molecule of the lectin complement pathway (Fugita et al. 2004). SP-A and SP-D are primarily synthesised by alveolar type II cells and considered important for pulmonay surfactant turn-over and homeostasis. SP-A can bind dipalmitophosphatidylcholine (DPPC, the major lipid component of lung surfactant) while SP-D preferentially binds to phosphatidylinositol (PI) as well as glucosylceramide, both minor components of surfactant that contain sugar moieties. In addition to their role in surfactant homeostasis, SP-A and SP-D are considered important host defence components against respiratory pathogens and allergens (Wright 2005. Kishore et al. 2005). The widespread expression of SP-D, at most sites of mucosal secretion within the body, is consistent with its possible role in innate immunity at many other locations besides the lungs.

Collectins are involved in a range of immune functions including viral neutralisation, clearance of bacteria, fungi, and apoptotic and necrotic cells, down regulation of allergic reactions and resolution of inflammation (Table 1). There is high affinity binding, in a Ca^{2+}-dependent, carbohydrate-specific manner, of the multimeric collectins to arrays of repetitive carbohydrate moieties, which are commonly found on the surface of viruses, bacteria, yeast and fungi. These interactions reduce the pathogens' ability to colonise, and cause agglutination and killing of the pathogen via opsonophagocytosis and the superoxidative burst by macrophages and neutrophils. In addition, binding of MBL to repetitive carbohydrate patterns on pathogen surfaces can activate the lectin complement pathway through the MBL-associated serine protease (MASP), designated as MASP-2, that leads to the activation of complement components C4, C2 and C3 (Figure 1). This is analogous to the classical complement pathway where binding of C1q to target ligands leads to association and activation of the C1r-C1s serine protease complex (reviewed in Kishore et al. 2004a). Of the four known MASPs (MASP-1, MASP-2, MASP-3 and sMASP), MASP-2 resembles C1s in its ability to cleave C4 and C2, and thus

Table 1. Proposed diverse functions of collectins[1]

Surfactant homeostasis and biophysical activities
Anti-viral properties
Anti-bacterial and anti-fungal properties
Direct microbial growth inhibition
Resistance to allergen challenge and pulmonary hypersensitivity
Helper T cell polarisation
Clearance of apoptotic and necrotic cells
Control of pulmonary inflammation
Suppression of lipid peroxidation
Extra-pulmonary non-specific immunity
Tissue remodeling
Activation of the lectin pathway by MBL

[1] Reviewed extensively in Fujita et al. 2004, Holmskov et al. 2003, Kishore and Reid 2001, Kishore et al. 2002, Kishore et al. 2005, Kishore et al. 2006, Lu et al. 2002, Weis et al. 1998, Wright 2005

generate a C3 convertase – which is a step common to both the classical and lectin pathways of complement activation (reviewed in Fugita et al. 2004). The eventual assembly of the membrane attack complex (MAC) and its insertion into the pathogens cell membrane leads to lysis of the pathogen.

Pentraxins are a superfamily of evolutionarily conserved proteins characterised by a structural motif, the pentraxin domain (Szalai et al. 1999, Pepys and Hirschfield 2003, Mantovani et al. 2003). The short pentraxins, C-reactive protein (CRP) and serum amyloid protein (SAP), are the main acute phase proteins in humans and mice, respectively, and have diverse and important functions in immunity through their opsonising properties. Pentraxin 3 (PTX3), a prototypical long pentraxin family member, is produced in response to pro-inflammatory cytokines, most abundantly by dendritic cells (DCs). Structural and functional studies, and experiments with transgenic and gene-deficient mice have reaffirmed the role of pentraxins in innate and adaptive immunity.

In this chapter, we discuss the roles of collectins and pentraxins in anti-fungal innate immunity. In order to give an overview of the area, we also summarise other ligands and functions of collectins and pentraxins, which are highly multifunctional and multifaceted innate immune molecules (Tables 1 and 2). We have mainly focused on MBL, SP-A and SP-D with respect to the collectins, and CRP, SAP and PTX3 as representatives of the pentraxin family, although there are several other well-characterised members in both families. Furthermore, given the emphasis of the book on the fungal immunology, we have concentrated on the roles of SP-A, SP-D and PTX3 in anti-fungal immunity, where some exciting developments have been made recently.

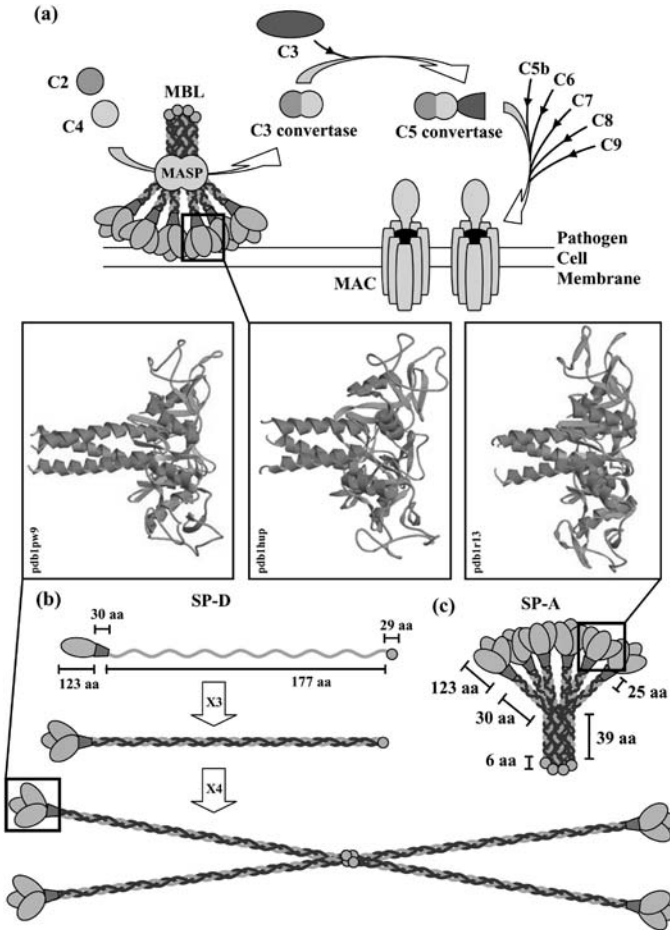

Figure 1. Structural organisation of human collectins. The basic polypeptide structure found in all the collectins is organised into four regions: a cysteine-containing N-terminus, a triple-helical collagen-like region composed of repeating Gly-X-Y triplets, followed by an α-helical coiled-coil neck region, and a globular CRD. This polypeptide chain undergoes trimerisation via the neck and collagen-like regions to form a trimeric structural subunit (b). Six of these trimeric subunits then undergo further assembly to yield hexameric structures in case of MBL and SP-A (a, c), although dimers, trimers, tetramers and pentamers are also found. The hexameric forms of MBL and SP-A resemble complement protein C1q in their overall organisation (C1q is only found as a hexamer of a structural subunit which is composed of three different polypeptide chains). SP-D has a tetrameric structure with four of the homotrimeric structural subunits linked via their N-terminal regions, but trimers, dimers and monomers also exist (a). Ribbon diagrams (inset) of the X-ray crystal structures of trimeric neck and CRDs of MBL, SP-A and SP-D show their predominantly β-sheet jellyroll three-dimensional structure. The primary ligand-binding sites (one per CRD) are located at the CRD surface opposite the neck region. The SP-A and SP-D illustrations are approximately to scale. MBL binding to the microbial surface via the CRDs activates the MASPs. MASP-2 cleaves C4 and C2 to generate C3 convertase (C4b2a), which cleaves C3. This leads to the complement lytic pathway, culminating in the formation of membrane attack complex (MAC) and pathogen killing (a) (See Color Section.)

Table 2. Proposed functions of pentraxins[1]

Pentraxin	Known ligands	Functions and mechanisms involved
CRP	Phosphorylcholine, *Streptococcus pneumoniae*, C1q, chromatin, histones, small nuclear ribonucleoprotein U1, glycans, phospholipids, poly-L-lysine, poly-L-arginine, and myelin basic protein, modified plasma lipoproteins, apoptotic cells, capsular components of bacteria and fungi	Enhancement of phagocytosis of apoptotic cells by macrophages; Activation of the classical complemet pathway; Protection against development of SLE; Pathogen clearance via C1q
SAP	Agarose (4,6-cyclin pyruvate acetal of β-D-galactose), *Streptococcus pyogens, Neisseria meningitides*, influenza virus, LPS, heparin, 6-phosphorylated mannose, 3-sulfated saccharides, laminin, type IV collagen, fibronectin, proteoglycans, C4b-binding protein, amyloid fibrils	Major DNA- and chromatin-binding protein in plasma; Stabilisation of amyloid deposits and thus participation in the pathogenesis of the systemic amyloidosis, Alzheimer's disease, and prion diseases; Protection against chromatin-induced autoimmunity; Protection against selected pathogens
PTX3	C1q, apoptotic cells, *Aspergillus fumigatus* (galactomannan), *Pseudomonas aeruginosa, Salmonella typhimurium, Paracoccidioides brasiliensis*, zymosan (mannan), outer membrane protein A, TNF-α-induced protein 6 (TSG-6), fibroblast growth factor 2 (FGF2)	Acivation of classical complement pathway by binding to surface anchored C1q; Inhibition of the classical complement pathway by binding to fluid phase C1q; TSG-6: PTX3 interaction acts as a nodal point for the assembly of hyaluronic acid-rich extracellular matrix, essential for female fertility; Binding to late apopototic cells and inhibition of recognition of apoptotic cells by DCs; Complement-mediated clearance of apoptotic and necrotic cells; Anti-Aspergillus immunity; Matrix deposition, tissue repair, and remodelling; Blockade of angiogenic activity of FGF2

[1] Reviewed elaborately by Mantovani et al. 2003, Nauta et al. 2003, Pepys and Hirschfield 2003, Szalai 2002.

2. STRUCTURAL ORGANISATION OF HUMAN COLLECTINS

The basic structure of collectins is organised into four regions (Kishore and Reid 2001, Lu et al. 2002, Holmskov et al. 2003): (i) a cysteine-containing N-terminus (required for disulfide-dependent oligomerisation) that is linked to (ii) a triple-helical collagen region composed of repeating Gly-X-Y triplets (associated with maintaining the molecular shape, stability and state of oligomerisation), followed by (iii) an α-helical, coiled-coil neck region (whose main function is protein trimerisation), and (iv) a globular structure at the C-terminus comprising a C-type lectin or CRD (that mediates calcium-dependent ligand-binding to entities such as pathogens via carbohydrates, or surfactant phospholipids, etc.) (Figure 1). The CRDs are arranged as trimers at the end of the triple-helical collagen region (Kishore and Reid, 2001). The CRDs can engage a range of pathogens and ligands, including fungi (Table 1) due to its carbohydrate or charge pattern recognition properties whereas the collagen region interacts with putative receptors present on alveolar type II cells, or immune cells such as macrophages and neutrophils in order to bring about effector functions such as phagocytosis and production of cytokines (Gardai et al. 2003).

MBL, SP-A and SP-D are large oligomeric structures, each assembled from multiple copies of either a single polypeptide chain, or in the case of human SP-A, two closely-related chains: SP-A1 and SP-A2 (Figure 1). The two transcribed SP-A genes (SFTPA1 and SFTPA2) and one pseudogene have been localised to chromosome 10q21-24, within a cluster that includes the SP-D and MBL genes (reviewed in Kishore et al. 2006). It is considered that each SP-A structural subunit normally has one SP-A1 chain and two SP-A2 chains, although oligomers composed of only one type of either polypetide chain can be expressed *in vitro*. SP-A has a basic hexameric structure in which six structural subunits, of 105 kDa each, associate to yield a molecule of 630 kDa (18 chains); smaller oligomers composed of 2 to 5 structural subunits are also found. Each structural subunit is composed of three 35-kDa-polypeptide chains that are held together by disulfide bonds located in the N-terminal halves of the chains. The overall shapes of SP-A and MBL are very similar to that of the serum complement protein C1q, with the hexamers of all three molecules appearing in the electron microscope as a bouquet-like structure with six globular heads linked by collagen-like strands to a fibril-like central core (Figure 1a, c; Kishore et al. 2004a).

SP-D is composed of oligomers of a 130-kDa subunit, each subunit containing three identical polypeptide chains of 43 kDa, with an N-linked oligosaccharide structure at Asn^{70}. Human SP-D assembles into a 520 kDa tetrameric structure with four of the 130 kDa homotrimeric subunits linked via their N-terminal regions (Figure 1b), but trimers, dimers and monomers of the 130 kDa subunit are also present in SP-D preparations. Up to eight of the 520 kDa tetrameric structures can undergo further oligomerisation to give SP-D multimers having a large array of up to 96 chains. The degree of subunit oligomerisation affects the recognition of and binding strength of collectins to the carbohydrate ligands on the surface of pathogens (Holmskov et al. 2003, Leth-Larsen et al. 2005).

The collagen-like region consists of repeating motifs of Gly-X-Y, where X and Y can be any amino acid (generally proline or hydroxyproline). In MBL and SP-A, the Gly-X-Y repeat is interrupted giving rise to a kink, and hence the C1q-like tulip bouquet shape (Figure 1a, c). The relatively large collagen-like region of SP-D may offer greater freedom to the distal CRDs to bind target pathogens and agglutinate them. Following the formation of the triple-helical collagen-like region, the N-terminal cysteine residues form disulfide bridges between monomers, which in turn dictates the degree of multimerisation of the single trimeric subunits of SP-A and SP-D (Brown-Augsburger et al. 1996; McCormack et al. 1999).

3. PATHOGEN RECOGNITION AND CLEARANCE BY COLLECTINS

Collectins are pattern recognition molecules that recognise terminal monosaccharide residues of glycoproteins and glycolipids present on the surface of many pulmonary pathogens. The broad selectivity of the monosaccharide binding site and the geometrical arrangement of the multiple CRDs allow MBL, SP-A and SP-D to bind tightly to arrays of non-self carbohydrate structures (Weis et al. 1998). Collectins bind mannose and glucose residues, which are components of pathogens cell surface glyccoconjugates, more avidly than galactose, fructose and sialic acid, that constitute higher eukaryotic glycoproteins (reviewed in Kishore and Reid, 2001; Holmskov et al. 2003). Collectins deal at several levels with a wide range of potentially pathogenic viruses, bacteria and fungi as summarised in Table 1. Agglutination restricts the spread and colonisation of the pathogen, while opsonisation by collectins enhances phagocytosis and killing via oxidative mechanisms via recruited phagocytes. SP-A and SP-D have been shown to have a direct inhibitory effect on the microbial growth that gives enough opportunity to the recruited phagocytic cells to clear the pathogens at the early stages of infection. In addition, SP-A and SP-D can also modulate release of cytokines and chemokines at the site of infection by macrophages and dendritic cells (DCs). Thus, the regulation of priming of an acquired immune response against pathogens is another important aspect of the multiple roles played by collectins in host defense.

4. INTERACTION OF MBL, SP-A AND SP-D WITH FUNGI

MBL has been shown to bind clinical isolates of *Candida albicans* and *Aspergillus fumigatus* and promote C4 deposition in a concentration-dependent manner (Neth et al. 2000). MBL enhances the uptake of serum-opsonised or immunoglobulin-opsonised *Cryptococcus neoformans* by macrophages (Levitz et al. 1993). In addition, MBL decreases the release of TNF-α by macrophages when challenged with a cryptococcal membrane glycoprotein (Chaka et al. 1997). Curiously, interaction of *C. albicans* with monocytes in the presence of MBL enhances TNF-α production (Kitz et al. 1992).

Role of MBL in host defense against allergic and invasive aspergillosis has recently been examined extensively (Kaur et al. 2006). MBL bound and agglutinated *A. fumigatus* conidia via the CRDs, resulting in activation of the lectin complement pathway. MBL enhanced the association of conidia with the polymorphonuclear cell (PMNs) independently of complement, which further increased in the presence of complement. However, MBL-mediated increase in the oxidative burst of PMNs and conidial killing was observed only in the presence of complement. The *in vivo* administration of a recombinant form of human MBL in a murine model of invasive pulmonary aspergillosis (IPA) led to a marked increase in the survival percentage, proinflammatory cytokines such as TNF-α and a significant decrease in the pulmonary fungal load and anti-inflammatory cytokines such as IL-10, in comparison with the untreated IPA mice. The results suggest that MBL acts as an immunoregulatory molecule and plays an important role in the first-line defense against *A. fumigatus* via its direct interaction with conidia.

The interaction of SP-A and SP-D with fungal pathogens has been more extensively studied. Both SP-A and SP-D contribute to the clustering of *Pneumocystis carinii in vivo* by interacting with gpA (glycoprotein 120), a mannose- and glucose-rich glycoprotein expressed on cysts and trophozoites, two developmental stages of the pathogen (Zimmerman et al. 1992). Increased bronchoalveolar lavage fluid (BALF) levels of SP-A in *P. carinii* pneumonia have been implicated in the pathogenesis. Binding of *P. carinii* to SP-A coated alveolar macrophages is significantly reduced and hence subsequent phagocytosis of the pathogen is also reduced (Koziel et al. 1998). The BALF levels of SP-D are also raised 4-fold in *P. carinii* patients. Dodecameric forms of SP-D have been shown to cause aggregation of the pathogen, however the SP-D mediated aggregation impairs the phagocytosis of the *P. carinii* by alveolar macrophages (Yong et al. 2003). Thus, SP-A and SP-D are probably used by *P. carinii* to its advantage in order to avoid phagocytosis and killing.

SP-D, but not SP-A, can agglutinate the pathogenic unencapsulated forms of *C. neoformans* (Schelenz et al. 1995), a yeast-like fungus (basidiomycetes) that can cause infection in the lungs and disseminate to the CNS to cause meningitis in immunocompromised subjects. The acapsular form enters the lung and the capsule, the principal virulent component, is synthesised within the host. SP-D appears to target the initial stages of infection by binding with high affinity and agglutinating acapsular forms. SP-D also binds the two capsular components, glucuronoxylomannan and mannoprotein-1 with high affinity. The binding and agglutination of acapsular *C. neoformans* by SP-D can be inhibited by glucuronoxylomannan, suggesting that infective forms can modulate SP-D, and thus, protect acapsular forms from SP-D mediated aggregation (van de Watering et al. 2004). On the contrary, SP-A binds both the acapsular and encapsulated forms of *C. neoformans* in a concentration, calcium and sugar dependent manner. It binds the acapsular form three times better than the capsular form. However, binding of SP-A to the acapsular form does not enhance its phagocytosis by a variety of phagocytic cells, suggesting that escape from SP-A mediated phagocytosis may help the pathogen in further dissemination (Walenkamp et al. 1999).

SP-D has been shown to bind *C. albicans* in a sugar and calcium dependent manner causing agglutination and inhibition of pseudohyphal/hyphal growth of the pathogen (Rozendaal et al. 2000). However, SP-D binding to *C. albicans* inhibits phagocytosis by alveolar macrophages, highlighting the fact that SP-D mediated agglutination and growth inhibition obviates the need for macrophage activation. SP-A also does not cause an increase in the phagocytosis of *C. albicans* by phagocytic cells. SP-A has been shown to suppress production of proinflammatory cytokines and chemokines by alveolar macrophages and monocytes (TNF-α, IL-1β, MIP-1α and MCP-1) without affecting the basal expression of the proinflammatory cytokines such as IL-6 (Rosseau et al. 1999).

SP-A and SP-D bind to *A. fumigatus* conidia and agglutinate them in a sugar and calcium dependent manner. *A. fumigatus* β-glucan has been found to be one of the ligands that interact with SP-D (Allen et al. 2001). The interaction of SP-A and SP-D with conidia also enhances phagocytosis, killing and production of superoxide radicals by alveolar macrophages and circulating neutrophils (Madan et al. 1997). Binding of SP-A, but not SP-D, is partly inhibitable by a mixture of surfactant phospholipids and SP-B and SP-C (Allen et al. 1999).

Intranasal administration of SP-D or rhSP-D in a murine model of IPA, where mice are immunosuppressed with hydrocortisone and challenged intranasally with *A. fumigatus* spores, has been shown to have a protective effect. Untreated IPA mice showed 100% mortality at seven days, whereas SP-D or rhSP-D treatment rescued about 80% of the IPA mice. SP-A did not have a significant effect on survival (Madan et al. 2001a). Subsequent studies have revealed that the protective effects of SP-D or rhSP-D in the IPA model coincides with reduced colony forming units, lack of hyphal growth, and raised levels of the protective cytokines, TNF-α and IFN-γ, in BALF. (U. Kishore, unpublished).

5. DIRECT FUNGISTATIC FUNCTIONS OF SP-A AND SP-D

Recently, SP-A and SP-D have been shown to have a direct anti-microbial effects on the growth and viability of Gram-negative bacteria (Wu et al. 2003) including strains of *E. coli, K. pneumoniae*, and *Enterobacter aerogenes*, probably via increasing the permeability of the bacterial membrane. This anti-microbial activity can be localised partly to the CRD region. Similar direct effects of SP-A and SP-D have been reported against intracellular yeast, *Histoplasma capsulatum*, consistent with the evidence that collectins cause altered membrane permeability and leakage (McCormack et al. 2003). SP-A has also been shown to attenuate the growth of *Mycoplasma pneumoniae* in culture, an activity inhibitable by DPPC, again suggesting involvement of the CRD region. Disaturated phosphatidylglycerols in the *M. pneuminiae* lipid extract appears to be the target for SP-A (Piboonpocanun et al. 2005).

A direct fungistatic effect of rhSP-D in terms of inhibition of conidia germination and hyphal growth has also been observed using about 50 clinical isolates of *Aspergillus* species including *A. fumigatus, A. niger, A. flavus* and *A. terreus* (Warn

et al. 2005). In the growth rate assays, all strains demonstrated both an overall reduction in growth rate (as measured by increasing optical density) in a dose dependent fashion (maximum 80%). Reduction in growth rate was most clearly seen by increased lag time (time until the optical density increased), which increased by 2.3 h. Thus, SP-D substantially reduces the growth rate of *Aspergillus in vitro* and this fungistatic activity can be localised to the CRDs. This is an additional mechanism by which SP-D provides protective immunity without involving agglutination and phagocytes, highlighting its role as a primordial agent of immune defense.

6. SUSCEPTIBILITY OF COLLECTIN GENE-DEFICIENT MICE TO FUNGAL PATHOGENS

SP-A gene-deficient or knock-out mice (SP-A$^{-/-}$) survive and breed normally, having normal SP-B, SP-C and SP-D levels, phospholipid composition, secretion and clearance, and incorporation of phospholipid precursors. Although there is a complete absence of tubular myelin in SP-A$^{-/-}$ mice, it does not appear to have a significant physiologic effect on surfactant homeostasis (Korfhagen et al. 1996). SP-D gene knock-out mice (SP-D$^{-/-}$) survive normally in the perinatal and postnatal periods but show remarkable abnormalities in surfactant homeostasis and alveolar cell morphology. The SP-D$^{-/-}$ mice exhibit a progressive accumulation of surfactant lipids and apoproteins in the alveolar space, hyperplasia of alveolar type II cells, massive enlargement of intracellular lamellar bodies, and an accumulation of foamy alveolar macrophages secreting ten-fold higher levels of hydrogen peroxide (Botas et al. 1998; Korfhagen et al. 1998; Wert et al. 2000). The SP-D$^{-/-}$ phenotypes are quite similar to those observed in the mice deficient in granulocyte-macrophage colony stimulating factor (GM-CSF) (Dranoff et al. 1994).

SP-A$^{-/-}$ mice show increased bacterial proliferation and systemic dissemination following intratracheal inoculation with Group B *Streptococci*, and defective clearance of *S. aureus, P. aeruginosa* and *K. pneumoniae* (Korfhagen et al. 1998). These mice also show increased susceptibility to respiratory syncytial virus, *M. pneumoniae* and *Pneumocystis* compared to the wild-type mice. These studies on SP-A$^{-/-}$ mice essentially reaffirm the role of SP-A as an important pulmonary immune molecule (Le Vine et al. 1998; 1999a; 1999b). SP-D$^{-/-}$ mice have also been shown to be susceptible to a few pathogens tested despite the existing inflammatory conditions in the lungs.

There are only a few studies that have examined the susceptibility of SP-A$^{-/-}$, or SP-D$^{-/-}$ mice to challenge with opportunistic fungal pathogens especially under conditions of immunosuppression or neutropenia. SP-A$^{-/-}$ mice, immunosuppressed with corticosteroids, have been shown to be more susceptible to *P. carinii* infection as evident from increased frequency and severity of *P. carinii* induced pneumonia in these mice (Linke et al. 2001). In a murine model of CD4^{+} depletion and *P. carinii* intratracheal infection, SP-A$^{-/-}$ mice show severe infection (Atochina et al. 2004a). Infection in SP-A$^{-/-}$ mice is also associated with peribronchial and

perivascular infiltration, and increased but attenuated BALF levels of SP-D, IFN-γ, IL-4, IL-5 and TNF-α, suggesting a dysregulation of cytokine balance following challenge with *P. carinii*. Thus, SP-A$^{-/-}$ mice remain susceptible to *P. carinii* infection despite an exaggerated inflammatory response, but attenuated generation of reactive oxygen-nitrogen species prevails in the lungs (Atochina et al. 2004a). Similarily, CD4^{+} depeleted SP-D$^{-/-}$ mice are more susceptible to *P. carinii* infection compared to CD4^{+} depeleted wild-type mice. The susceptibility is concomitant with increased pulmonary infiltration, inflammation and NO levels (Atochina et al. 2004b). These studies involving *P. carinii* infection of SP-A$^{-/-}$ and SP-D$^{-/-}$ mice appear to highlight the importance of SP-A and SP-D in defense against *P. carinii*.

Immunosuppressed SP-A$^{-/-}$ mice, when subjected to IPA, show greater resistance to *A. fumigatus* conidia challenge (40% mortality) than the WT hydrocortisone treated mice (100% mortality). Interestingly, intranasal delivery of SP-A to the SP-A$^{-/-}$ IPA mice increases the mortality to 60%. The overall mortality in immunosuppressed SP-D$^{-/-}$ IPA mice is similar to WT-IPA mice (~ 60%), however, SP-D$^{-/-}$ IPA mice tend to die significantly sooner, display a higher hyphal density and have tissue injury in the lungs. Intranasal treatment with SP-D, or rhSP-D, has been found to reduce the mortality of SP-D$^{-/-}$ IPA mice, from 100%, to 50% and 33%, respectively (Madan et al. unpublished). It is interesting to note that SP-A$^{-/-}$ and SP-D$^{-/-}$ mice show distinct immune responses to corticosteroid-induced immunosuppression. Immunosuppressed SP-A$^{-/-}$ mice had significantly lower levels of TNF-α, IL-4, IL-5 and IL-10, and an increased IFN-γ to IL-4 ratio (2.7 fold) in lung cell suspension, compared to immunosuppressed WT mice. Immunosuppressed SP-D$^{-/-}$ mice, however, showed a significant increase in IL-4 levels, but a decrease in IL-10 and IFN-γ to IL-4 ratios, when compared with immunosuppressed WT mice. SP-A treated SP-A$^{-/-}$ IPA mice show increased mortality with decreased TNF-α, IL-10, IL-13, and IFN-γ to IL-4 ratio than SP-A$^{-/-}$ IPA mice. The IFN-γ to IL-4 ratios in SP-D or rhSP-D treated SP-D$^{-/-}$ IPA mice are generally higher than SP-D$^{-/-}$ IPA mice. These observations appear to suggest that SP-A or SP-D deficiency may have differential effects on the host's ability to mount an immune response against *A. fumigatus* under immunocompromised conditions.

Studies involving infection of SP-A$^{-/-}$ and SP-D$^{-/-}$ mice with *P. carinii* and *A. fumigatus* have revealed that disease susceptibility due to these gene deficiencies is valid only when these mice are immunocompromised (Atochina et al. 2004a; Madan et al. 2005, unpublished). This point is amply highlighted by studies involving the challenge of MBL-A knock-out mice with *C. albicans* (Lee et al. 2002). In rodents, MBL is encoded by two genes- MBL-A and MBL-C. In a murine model of disseminated candidiasis, MBL-A$^{-/-}$ mice offer resistance to challenge with *C. albicans* in a way similar to the WT mice and both mice have comparable survival and fungal load. It appears that functional redundancy in the mechanisms involved in the fungal clearance seem to compensate for SP-A, SP-D or MBL-A defieciency in mice. Thus, studies with double knock-out mice (SP-A and SP-D; MBL-A and MBL-C) are likely to be interesting.

7. ROLES OF COLLECTINS IN RESISTING ALLERGIC INFLAMMATION AND HYPERSENSITIVITY INDUCED BY FUNGAL ALLERGENS AND ANTIGENS

A. fumigatus causes systemic infection via the lungs (IPA or IA) in the immunosuppressed subjects. However, in the immunocompetent individuals (probably with genetic susceptibility), it can cause an allergic disorder, called allergic bronchopulmonary aspergillosis (ABPA), which is different from other hypersensitivity responses to inhaled allergens in that the *A. fumigatus* spores grow in the respiratory tract and continually shed soluble and particulate antigens and allergens in the large subsegmental bronchi. Thus, the interaction of SP-A and SP-D with glycoprotein allergens of *A. fumigatus*, and the subsequent outcome of these interactions has been examined *in vitro* and *in vivo* (Madan et al. 1997b, 2001). SP-A and SP-D have been previously shown to bind allergens derived from pollen grains and dust mite (Kishore et al. 2002). SP-A, SP-D and rhSP-D can also bind to the three-week culture filtrate (3wcf) of *A. fumigatus* as well as purified glycoprotein allergens, gp55 and gp45, inhibit the ability of specific IgE to bind these allergens, and block histamine release from sensitised basophils isolated from ABPA patients (Madan et al. 1997b). Consistent with their roles in the modulation of allergic reactions, SP-A and SP-D have been reported to reduce the proliferation of PBMC isolated from dust mite-sensitive asthmatic children (Wang et al. 1998), and SP-D in particular has a suppressive effect on the secretions of IL-2 by PBMC (Borron et al. 1998). Furthermore, SP-A suppresses the production and release of IL-8 by ionomycin-stimulated eosinophils (Cheng et al. 1998). Since IgE cross-linking, histamine release and lymphocyte proliferation are essential immunologic steps in the development of *A. fumigatus* induced ABPA symptoms, SP-A and SP-D appear to be important in resisting allergenic challenge and dampening subsequent *A. fumigatus* induced hypersensitivity reactions in the lungs (Kishore et al. 2002, 2005).

The therapeutic effects of intranasal administration of SP-A, SP-D or rhSP-D in a murine model of ABPA induced by *A. fumigatus* 3wcf using BALB/c strain of mice have been examined (Madan et al. 2001b). ABPA is an *A. fumigatus*-induced allergic disorder which is clinically characterised by episodic bronchial obstruction, positive immediate skin reactivity, elevated *A. fumigatus*-specific IgG and *A. fumigatus*-specific IgE antibodies in serum, peripheral and pulmonary eosinophilia, central bronchiectasis, and expectoration of brown plugs or flecks. The murine model resembled the human disease immunologically, exhibiting high levels of specific IgG and IgE, peripheral blood and pulmonary eosinophilia, and a Th2 cytokine response. Intranasal administration of SP-A, SP-D or rhSP-D (3 doses on consecutive days) significantly lowered eosinophilia and specific antibody levels. This therapeutic effect persisted up to 4 days in the SP-A treated ABPA mice, and up to 16 days in the SP-D or rhSP-D treated ABPA mice. Lung sections of the ABPA mice showed extensive infiltration of lymphocytes and eosinophils, which were considerably reduced following treatment. The levels of IL-2, IL-4 and IL-5 were decreased, while that of IFN-γ was raised in supernatants of the cultured spleen

cells, indicating a marked shift from pathogenic Th2 to a protective Th1 polarisation of helper T cell (Th) immune response.

In view of the proposed role of SP-D in regulation of *A. fumigatus* mediated allergic hypersensitivity, Atochina et al. (2003) hypothesised that an elevated SP-D production is associated with the impaired ability of C57BL/6 mice to develop airway hyper-responsiveness (AHR) to *A. fumigatus* challenge. The allergen challenge to sensitised C57BL/6 mice induced a markedly increased SP-D protein expression in the surfactant fraction ($1,894 \pm 170\%$ of naïve controls) that was 1.5 fold greater than the increase in Balb/c mice ($1,234 \pm 121\%$). In addition, sensitised and exposed C57BL/6 mice had significantly lower IL-4 and IL-5 in the BALF than Balb/c mice ($p < 0.05$), suggesting that enhanced SP-D production in the lung of C57BL/6 mice may contribute to an attenuated AHR in response to allergic airway sensitisation. Thus, SP-D may act by inhibiting production of Th2 cytokines.

It is evident that SP-A and SP-D appear to offer protection against allergenic challenge at various levels, suggesting a hierarchical role for these two molecules of innate immunity. These protective mechanisms seem to involve allergen scavenging that leads to inhibition of allergen-IgE cross-linking and histamine release, suppression of the activation of sensitised basophils, mast cells or eosinophils, suppression of B and T cell proliferation, modulation of DCs and macrophages, and Th cell polarisation (reviewed in Kishore et al. 2002; Sonar et al. 2006). The ability of SP-A and SP-D to suppress proliferation of specific B-lymphocytes may account for the lowering of specific IgG and IgE levels in the treated group of allergic mice; this effect may well be amplified by a decrease in IL-2 levels since IL-2 is central to lymphocyte growth and differentiation. IL-5 is a differentiation factor for eosinophils whereas IL-4, together with IL-13, is an important factor for isotype switching of B-lymphocytes, leading to the secretion of IgG1 and IgE. These cytokine profiles mark a characteristic Th2 response in the allergic immune reaction that is characterised by secretion of IL-4, IL-5, IL-10 and IL-13 and generation of humoral immune responses. Shifting of cellular responses from a predominantly Th2 to a Th1 cytokine profile, following treatment with SP-A, SP-D or rhSP-D, appears central to the protective mechanism since IFN-γ, a Th1 cytokine, promotes cellular immunity and normally inhibits Th2 differentiation in response to IL-4. Among the factors that have been shown to influence the Th1-Th2 balance, IL-12 is dominant in directing the development of Th1 cells that produce high amounts of IFN-γ. Thus, SP-A and SP-D have been shown to modulate DCs differentially. The SP-D mediated binding and uptake of *E. coli* by bone-marrow derived mouse DCs has been shown to increase antigen presentation of *E. coli* expressed proteins to T-cell hybridoma (Brinker et al. 2001)). Curiously, pre-treatment of immature DCs with SP-A (or C1q) has been shown to inhibit LPS-mediated surface expression of maturation markers: MHC class II and CD86. Stimulation of immature DCs by SP-A also inhibits the allostimulation of T cells and enhances dextran endocytosis (Brinker et al. 2003). These results appear to suggest an immune balancing role for SP-A and SP-D during pulmonary inflammation (Kishore et al. 2006).

Since complement activation contributes to the pathogenesis of allergy, the role of MBL in ABPA pathogenesis has recently been assessed (Kaur et al. 2006). Significantly higher MBL levels and activity were observed in the allergic patients as compared to the controls (44 patients of bronchial asthma with allergic rhinitis, 11 ABPA patients, and 40 unrelated, age-matched controls of Indian origin). High MBL activity showed a positive correlation with peripheral blood eosinophil counts in the allergic patients ($r = 0.75$). The levels of the two mouse MBLs (MBL-A and MBL-C) were evaluated in mice before and after sensitisation with allergens and antigens of *A. fumigatus*. The MBL-A levels were significantly higher in the mice after their sensitisation with fungal allergens ($p < 0.05$). In view of genetic polymorphisms in the collagen region of MBL gene contributing to varied plasma MBL levels and activity, single nucleotide polymorphisms (SNPs) in exon 1 and intron 1 (encoding the collagen region) of *MBL* in these two patient groups were examined. One of the intronic SNP G1011A showed significant association with both categories of allergic patients in comparison to the controls. The intronic SNP also showed a significant association with elevated peripheral blood eosinophil counts and a decreased percent-predicted FEV1 of the patients, the two commonly used markers of allergic airway diseases (Kaur et al. 2006). It appears that allergic patients with '1011A' allele and high plasma MBL levels and complement activity may be susceptible to a severe form of respiratory allergic disease.

8. SUSCEPTIBILITY OF SP-A$^{-/-}$ OR SP-D$^{-/-}$ MICE TO FUNGAL ANTIGENS AND ALLERGENS

The susceptibility of SP-A$^{-/-}$ or SP-D$^{-/-}$ mice to the *A. fumigatus* allergen challenge, as compared to the wild-type mice has been examined recently (Madan et al. 2005). Both SP-A$^{-/-}$ or SP-D$^{-/-}$ mice show intrinsic hyper-eosinophilia and several fold increase in lung levels of IL-5 and IL-13, with a lowering of the IFN-γ to IL-4 ratio in the lungs, suggesting an inherent bias to a Th2 immune response in the gene-deficient phenotype as compared to the wild type mice. Treating SP-A$^{-/-}$, or SP-D$^{-/-}$, mice with SP-A or SP-D, respectively, reduces this hyper-eosinophilia and Th2 predominance. The SP-A$^{-/-}$, and SP-D$^{-/-}$ mice show distinct immune responses to *A. fumigatus* sensitisation, SP-D$^{-/-}$ mice being more susceptible than wild-type mice to pulmonary hypersensitivity induced by being *A. fumigatus* allergens. Interestingly, SP-A$^{-/-}$ mice have been found to be nearly resistant to *A. fumigatus* sensitisation. Intranasal treatment with SP-D or rhSP-D can rescue the *A. fumigatus* sensitised SP-D$^{-/-}$ mice, while SP-A treated *A. fumigatus* sensitised SP-A$^{-/-}$ mice show several fold elevated levels of IL-13 and IL-5, resulting in increased pulmonary eosinophilia and damaged lung tissue. This validates important roles for SP-A and SP-D in the modulation of pulmonary hypersensitivity and suggests differential mechanisms involved in SP-A and SP-D mediated resistance to allergen challenge. Hyper-eosinophilia exhibited by both SP-A$^{-/-}$ and SP-D$^{-/-}$ mice, probably due to significantly raised levels of IL-5 and IL-13 in these mice, suggests that SP-A and SP-D have a role in regulating eosinophil infiltration and modulation in the lung in response to environmental stimuli. It is interesting to note that similar to SP-D$^{-/-}$

mice, IL-13 over-expressing mice have characteristic foamy macrophages, type II cell hypertrophy, fibrosis, massive inflammation involving eosinophilia, protease-dependent acquired emphysema, and airway hyperresponsiveness (AHR) (Homer et al, 2002). Given the involvement of IL-13 in processes such as mucus production and AHR, as well as eosinophil survival, activation and recruitment, it is likely that certain physiological effects in SP-$A^{-/-}$ as well as SP-$D^{-/-}$ mice arise due to over-expression of IL-13 (Homer et al. 2002, Madan et al. 2005). It is also evident that SP-A and SP-D inhibit allergen-mediated eosinophilia in the lungs through down-regulation of IL-5. SP-A and SP-D have important roles in the regulation of the cytokine milieu and eosinophilia in the lungs, and the inherent hypersensitivity due to their deficiency in the SP-$A^{-/-}$ and SP-$D^{-/-}$ mice argues for it.

9. PENTRAXINS AS ADAPTORS BETWEEN INNATE AND ADAPTIVE IMMUNITY

Pentraxins are characterised by the presence, in their carboxy-terminus, of a 200 amino acid pentraxin domain, with an 8 amino acid long conserved pentraxin signature sequence (HxCxS/TWxS, where x is any amino acid) (Figure 2). CRP and SAP are classic short pentraxins produced in the liver. CRP is produced as a non-specific acute phase reactant to inflammation, infection and tissue injury. CRP levels in the plasma of healthy adults are barely detectable but can potentially increase as much as 10,000-fold following an acute phase stimulus as a result of accelerated rates of transcription in the liver (Pepys and Hirschfield 2003). Circulating CRP is produced only by hepatocytes, mainly in response to the proinflammatory cytokine IL-6, but lymphocytes and monocytes/macrophages are also able to synthesise CRP. SAP, a basement membrane component, is the main acute phase protein in mice, whereas in human serum it is constitutively present at 30–50 μg/ml. Long pentraxins are expressed in a variety of tissues including CNS. PTX3, a prototypical long pentraxin, is produced in response to pro-inflammatory cytokines, most abundantly by DCs. As shown in Table 2, CRP, SAP and PTX3 have diverse and important functions in immunity. Recently, PTX3 has been shown to have a nonredundant role in resistance against *A. fumigatus*.

10. ANTI-MICROBIAL FUNCTIONS OF SHORT PENTRAXINS: CRP AND SAP

The physiological functions of CRP and SAP involve calcium dependent binding to a range of ligands (Table 2). CRP binds to phosphorylcholine (PC), a major constituent of C-type capsule polysaccharides of *Streptococcus pneumoniae*, as well as various pathogens including bacteria, yeasts and fungi (Szalai 2002). CRP has been shown to act as an opsonin for the phagocytosis of attenuated strains of *C. albicans* blastopores and thus may offer protection against candidosis independent of complement (Richardson et al. 1991a). However, virulent strains appear to be less sensitive to CRP-mediated phagocytosis and subsequent

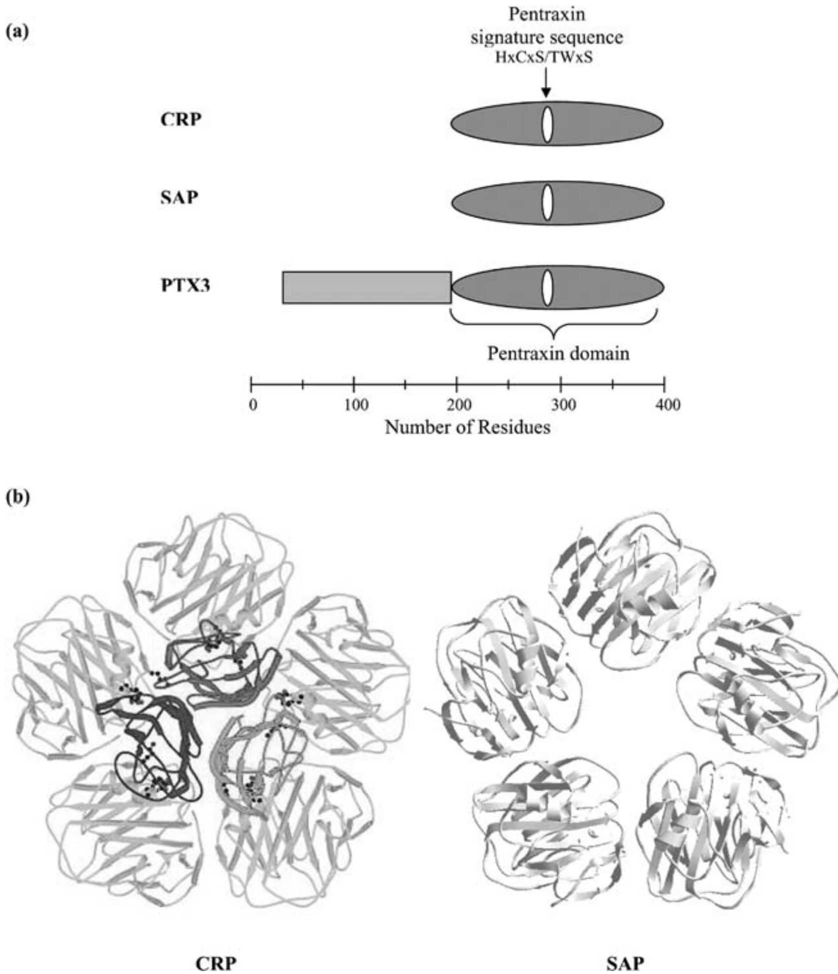

Figure 2. (a) Organisation of short and long penraxins. Pentraxins are characterised by the presence in their carboxy-terminal of a 200 amino acids pentraxin domain, with an 8 amino acid long conserved pentraxin signature (HxCxS/TWxS, where x is any amino acid). The human CRP and SAP genes are located on chromosome 1q23 and are organised in two exons, the second exon encoding for the pentraxin domain. The long pentraxin, human PTX3 gene, localised on human chromosome 3 band q25, is organised in three exons separated by two introns, the third exon codes for the pentraxin domain. The mature SAP protomer is 204 amino acid long (25,462 Da) and has a pentameric structure in the presence of physiological levels of calcium (127,310 Da). In the absence of calcium, SAP consists of both pentameric and decameric forms. Each SAP protomer is glycosylated with a single N-linked biantennary oligosaccharide at Asn^{32}. Human CRP is composed of five identical nonglycosylated protomers. The PTX3 protein (40,165 Da) consists of a C-terminal 203 amino acids pentraxin-like domain (containing an N-linked glycosylation site in the C-terminal domain at Asn^{220}) and an additional N-terminal region (178 aa) unrelated to other known proteins. PTX3 protomers can assemble as decamers and higher oligomers upto 900 kDa. (b) Crystal structures of CRP and SAP. Each CRP protomer has a characteristic lectin fold composed of two layered ß sheets with a flattened jellyroll topology; five protomers are noncovalently

killing by human neutrophils (only 10% killing). CRP has also been shown to bind *A. fumigatus* conidia and enhance phagocytosis by neutrophils without requiring participation of complement components (Richardson et al. 1991b). In an earlier study (Jensen et al. 1986), CRP was found to bind hydrophobic fractions containing phosphorylcholine from *A. fumigatus* hyphal homogenate in a calcium-dependent manner, suggesting that CRP may modulate immune response to antigen and allergens of *A. fumigatus*.

Despite extensive biochemical, structural and diagnostic characterisation of CRP and SAP, their *in vivo* functions are still debated. Transgenic and gene-deficient mice have also been used to identify biological functions. However, the two proteins are regulated differently in human and mouse. Administration of human CRP to the transgenic mice increases survival extent and time of mice infected with *Streptococcus pneumoniae* (Szalai et al. 1995). Similar protective effects of CRP overexpression have been noted against *Haemophilus influenzae* and *Salmonella enterica* (Weiser et al. 1998, Szalai et al. 2000). Studies using $SAP^{-/-}$ mice have revealed that SAP plays a dual role in bacterial infections (Noursadeghi et al. 2000). SAP binds to *Streptococcus pyogenes*, *Neisseria meningitidis* and the rough variant of *E. coli*, and shows anti-opsonic effect, thus reducing phagocytosis and killing by neutrophils. $SAP^{-/-}$ mice survive an otherwise fatal challenge with *S. pyogenes* and rough *E. coli* J5. However, $SAP^{-/-}$ mice are more susceptible to a non-binding smooth strain of *E. coli*. Thus, SAP offers protection against non-binder pathogens such as the smooth variant *E. coli*, while a strong anti-opsonic effect is observed when SAP binds to bacteria, resulting in enhanced virulence of the infectious agent. Such interesting studies have not been carried out using fungal pathogens.

11. PRO-INFLAMMATORY NATURE OF PTX3

A variety of cell types can produce PTX3 *in vitro* upon exposure to primary inflammatory signals. These include endothelial cells, smooth muscle cells, adipocytes, fibroblasts, and mononuclear phagocytes. Myeloid DCs, however, are the highest

Figure 2. associated to form a pentamer (115,135 Da)(Shrive et al. 1994). Ligand bound CRP or SAP can bind to C1q and activate the classical complement pathway (Nauta et al. 2003), which may be one of the mechanisms involved in enhanced phagocytosis of pathogens by phagocytic cells. The interaction between one pentameric molecule of CRP and the heterotrimeric globular domain of C1q has been shown (Kishore et al. 2004a, 2004b). The three chains of C1q (in color) are docked within the CRP pentameric structure. SAP is composed of 5 or 10 identical subunits noncovalently associated in pentameric rings interacting face to face (Emsley et al. 1994). Human SAP has a tertiary fold, which resembles that of the legume lectins like Concanavalin A. SAP protomers have a flattened ß-jelly roll topology with a single long helix folded on the top of the ß-sheet. The five subunits are arranged in a ring around a hole and are held together by hydrogen bonds and salt bridges. The decamer is stabilised by ionic interactions between the two pentamers. Each SAP subunit can bind two calcium ions, and residues involved in calcium binding are conserved. Based on molecular modelling, the PTX3 pentraxin domain has a simlar structural fold to SAP, since most of the ß-strands and the α-helical regions are conserved (See Color Section.)

producer of PTX3 (Doni et al. 2003), which facilitates pathogen recognition and activation of an appropriate adaptive immune response. PTX3 production is induced by pro-inflammatory signals such as IL-1, TNF-α, LPS, lipoarabinomannans and TLR agonists (Vouret-Craviari et al. 1997). But, IFN-γ, a pro-inflammatory cytokine, inhibits PTX3 expression and production differentially (Polentarutti et al. 1998) while IL-10, an anti-inflammatory cytokine, induces PTX3 expression in DCs and monocytes (Perrier et al. 2004). Following exposure to inflammatory signals (LPS, IL-1, and TNF), or infectious agents (*C. albicans, C. neoformans*), PTX3 is has also found to be expressed in the CNS (Polentarutti et al. 2000).

12. A MAJOR ROLE OF PTX3 IN ANTI-*ASPERGILLUS FUMIGATUS* IMMUNITY

PTX3 binds selected pathogens, including *A. fumigatus* conidia, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Paracoccidioides brasiliensis*, and zymosan, but not *Escherichia coli*, *Burkholderia cepacia*, *Listeria monocytogenes*, or *C. albicans* (Garlanda et al. 2002, Diniz et al. 2004). PTX3 binds neither LPS nor the classical short pentraxin ligands such as PC, phosphoethanolamine (PE), or high pyruvate agarose. Binding of PTX3 to *A. fumigatus* conidia (as well as *A. flavus* and *A. niger*) can be inhibited by galactomannan, a major constituent of the conidial wall, but not by dextran, galactose, fucose or mannose (Garlanda et al. 2002). However, PTX3 does not bind hyphae or mycelium of *A. fumigatus*. PTX3 also enhances phagocytosis of conidia by macrophages, consistent with an increase in MCP-1 production by DCs. *A. fumigatus* conidia in fact rapidly induces PTX3 production in human and murine mononuclear phagocytes and DCs.

PTX3$^{-/-}$ mice are viable and display a normal life span in a conventional mouse facility. The only apparent abnormality is a severe deficiency in female fertility. PTX3$^{-/-}$ mice, when challenged with *A. fumigatus* conidia, die within 3 days while all wild type mice survive. The susceptibility of PTX3$^{-/-}$ mice to IPA is associated with a marked increase in lung and brain colonisation, a massive inflammatory response, the presence of hyphae and extracellular conidia, compared to only few intracellular conidia and minimal inflammation in the wild type controls (Garlanda et al. 2002). Alveolar macrophages and DCs derived from PTX3$^{-/-}$ mice have defective recognition of conidia. Reconstitution with purified PTX3 protein can restore phagocytic as well as conidiocidal properties of these phagocytes *in vitro*. Similarly, addition of PTX3 also restores the ability of DCs from PTX3$^{-/-}$ mice to produce IL-12. In addition, reduced BALF levels of IFN-γ and IL-12 and increased IL-4 levels in PTX3$^{-/-}$ mice can be reversed by PTX3 treatment. Thus, PTX3 shifts the balance of the immune response from a pathogenic Th2 to a protectie Th1 bias. Since PTX3 binds to C1q and activates the classical complement cascade (Nauta et al. 2003), the susceptibility of C1q$^{-/-}$ mice to IPA has also been examined (Garlanda et al. 2002). C1q$^{-/-}$ mice show an increased susceptibility to IPA that can be reversed by PTX3 treatment, suggesting that the ability of PTX3 to resist *A. fumigatus* challenge is C1q-independent. On the pathological aspects, infection

of mice with *A. fumigatus* results in the induction of PTX3 in BALF and plasma (128.2 ng/ml vs. 9.92 ng/ml control mice) irrespective of neutropenia. Furthermore, PTX3 plasma levels have been found to rise significantly (9.56 ± 3.15 ng/ml vs. 1.08 ± 0.13 ng/ml in control subjects) in patients with haematological malignancies and *A. fumigatus* systemic infection. These results implicate PTX3 as a soluble pattern-recognition molecule, having a non-redundant role in resistance against the fungal pathogen *A. fumigatus*.

In murine models of bacterial infections, such as *P. aeruginosa* lung infection (a pathogen recognised by PTX3), PTX3$^{-/-}$ mice show a partial increase in mortality and lung colonisation. In contrast, susceptibility to *L. monocytogenes* and to intra-abdominal sepsis caused by caecal ligation and puncture were not affected by PTX3 deficiency (Garlanda et al. 2002). Thus, PTX3 deficiency does not cause a generalised impairment of host resistance to microbial pathogens.

13. THERAPEUTIC POTENTIAL OF PTX3 FOR INVASIVE ASPERGILLOSIS (IA)

In view of the importance of PTX3 in host resistance to IPA, the protective effects of administration of PTX3, with and without Amphotericin B (AmB) or its liposomal formulation, has been assessed against IA using a murine model of allogeneic bone marrow transplantation (Gaziano et al. 2004). The prophylactic or therapeutic administration of PTX3 alone, or in combination regimens was given intranasally or intraperitoneally in mice challenged with *A. fumigatus* conidia. PTX3-treated mice showed resistance to infection and the protective effects were comparable to AmB or liposomal AmB treatment. PTX3, at doses of 1.0 and 0.2 mg/kg given prophylactically as well as concomitantly, induced complete resistance to IA, as evident from increased survival (> 60 days) of all mice in the treated group, reduced fungal burden in the lung and the brain. The therapeutic administration of PTX3 (1 mg/kg PTX3) post-infection significantly increased survival. This increased survival of mice was only seen at the higher dose, together with a reduced fungal burden in their lungs and brain. Treatment with PTX3 also significantly increased resistance to reinfection, as shown by decreased fungal growth in the kidneys of reinfected mice. Furthermore, lung sections from infected mice showed the presence of numerous *Aspergillu*s hyphae in the lung parenchyma, bronchial wall damage, necrosis, and few inflammatory cells. PTX3-treated mice exhibited infiltrates, no fungal growth or bronchial wall destruction.

In mice with IA, resistance to infection correlates with the activation of IFN-γ-producing Th1 cells. The numbers of CD4$^+$ and CD8$^+$, and Gr-1$^+$ neutrophils were significantly increased in the lungs of mice upon treatment with PTX3. Treatment with PTX3 also enhanced IL-12 production, and reduced the production of IL-10. Consistent with this, PTX3 treatment increased the frequency of Th1 and lowered IL-4-producing CD4$^+$ cells in the spleens, suggesting immunomodulatory functions of PTX3 *in vivo*.

It is also important to note that PTX3 in combinaton therapy greatly enhances the potency of suboptimal dose of AmB or liposomal AmB. When BM-transplanted mice were given prophylactic or therapeutic treatment with PTX3 alone, or together with AmB, or liposomal AmB, at suboptimal doses, none of them, except prophylactic PTX3, altered mortality. However, combination therapy with PTX3 and liposomal AmB rescued the mice from infection. Interestingly, addition of PTX3 to AmB formulation lowered BALF level of TNF-α and enhanced production of IFN-γ by stimulated splenocytes, suggesting that PTX3 may be involved in striking a balance between pro-inflammatory and anti-inflammatory responses at the site of infection. IL-4 levels in the spleen cell supernatant were also lowered siginificantly in combination therapy. Therefore, PTX3 appears to work synergistically with liposomal AmB and is potentially a novel adjunctive therapy in *A. fumigatus* infections.

The protective mechanisms that involve PTX3, in the IA model, probably involve its ability to bind *A. fumigaus* conidia and to enhance the phagocytosis of conidia by alveolar macrophages. Thus, it promotes the conidiocidal activities of these cells, upregulates the Th1 immune response, and downregulates the Th2 response (Garlanda et al. 2002, Gaziano et al. 2004). Unlike SP-D, PTX3 does not have a direct fungicidal effect on *A. fumigatus* conidia (Garlanda et al. 2002). The fact that PTX3 activates myeloid DCs to produce IL-12 and that the activated DCs themselves produce PTX3 appears to highlight a feedback loop that amplifies the protective Th1 response.

14. INVOLVEMENT OF THE DECTIN-1 RECEPTOR IN PTX3 MEDIATED PHAGOCYTOSIS

In order to further understand the role of PTX3 in the regulation of inflammation and resistance to infection, transgenic (Tg) mice overexpressing the murine PTX3 gene under the control of its own promoter have been generated. PTX3 Tg mice are more resistant to the systemic administration of LPS and to sepsis, and their peritoneal macrophages produce larger amounts of nitric oxide (NO) than wild-type cells (Dias et al. 2001). But, PTX3 Tg mice have an increased death rate with an exacerbated inflammatory response induced by ischemia and reperfusion (Souza et al. 2002), consistent with the notion that PTX3 upregulates the pro-inflammatory response.

Macrophages from PTX3 Tg mice exhibit opsonin-independent phagocytosis of zymosan particles and of the yeast form of the fungus *Paracoccidioides brasiliensis*, consistent with the fact that PTX3 can bind directly to zymosan and *P. brasiliensis*. In the case of *P. brasiliensis*, an enhanced microbicidal activity is matched with a higher production of NO by macrophages derived from PTX3 Tg mice. The basal level of TLR-6 and zymosan-induced dectin-1 expression is found to be significantly higher in PTX3 Tg macrophages compared to the wild type control. Thus, blockade of dectin-1 receptor by an anti-dectin-1 antibody, or by laminarin

(a short ß-glucan) has been found to inhibit the phagocytosis of zymosan by wild type and PTX3 Tg macrophages (Diniz et al. 2004).

Zymosan is a yeast-derived polysaccharide particle composed mainly of ß-glucan and mannan. It can potentiate the phagocytic and microbicidal ability of macrophages (Brown and Gordon 2003). Zymosan interacts with macrophages mainly via dectin-1 (Brown and Gordon 2001, Brown et al. 2002). Zymosan can also activate TLR2, eventually triggering NF-κB generation (Brown et al. 2003). Thus, zymosan induced upregulation of PTX3 in macrophages is likely to be mediated by NF-κB. This could be an additional mechanism through which PTX3 can mediate its immune response during fungal infection.

15. CONCLUSIONS AND PERSPECTIVES

Collectins appear to play important roles in controlling infection, lung allergy and inflammation when encounterd with fungal challenge. They act against fungal pathogens through their ability to agglutinate conidia, recruit and activate neutrophils and macrophages thereby inducing phagocytosis and/or production of superoxide radicals, and have direct effect on fungal growth. MBL recruits an additional mechanism of innate immunity whereby it can bind to fungal pathogens and activate the lectin complement pathway, causing direct lysis of the fungal pathogens. SP-A and SP-D also offer resistance to hypersensitivity caused by *A. fumigatus* allergens and antigens, by interfering with the allergen-IgE interaction, mast cell/basophil degranulation, cellular infiltration and helper T cell polarisation. They are also involved in manipulating cytokine and chemokine profiles during inflammation caused by fungal infection.

Studies involving MBL$^{-/-}$, SP-A$^{-/-}$ and SP-D$^{-/-}$ mice have highlighted the importance of these innate immune molecules in the clearance of fungal pathogen in an immunosuppressed context. The genetic deficiency of individual collectin genes in mice does not significantly alter the ability of host to deal with fungal pathogens compared to their wild type counterparts. When these knock-out mice are immunosuppressed via steroids, neutropenia or T- cell depletion, they become more vulnerable to the pathogen. Thus, the regulatory mechanisms that control collectin expression during fungal infection under immunocompromised conditions need to be fully understood. Another area of exploration is the regulatory roles of SP-A and SP-D in acquired immune response to various fungal pathogens mediated by their direct interactions with DCs and T cells. Furthermore, the importance of the microenvironment that would affect distinct immune mechanisms triggered by SP-A and SP-D, involving pro-inflammatory and anti-inflammatory events, are far from clear. In addition, the mechanisms and target pathways that govern the direct anti-fungal effects of SP-A and SP-D are poorly defined. PTX3, however, seems to play a non-redundant role in innate resistance against *A. fumigatus* and its deficiency in mice severely immunocompromises the hosts' ability to clear Afu infection.

Recent studies have also highlighted the therapeutic potential of SP-D and PTX3 against IA, which is the leading cause of mortality in immunocompromised patients

through neutropenia, transplantaion, aggressive chemotherapy in cancer and HIV (Kishore et al. 2005, 2006). Despite advances in early diagnosis and new antifungal agents, the majority of cases of IA remain undiagnosed and untreated at death. Clearly, defects in the innate and adaptive immune response are central to the IA pathogenesis. Thus, therapy aimed at strengthening the host immune response offers a promising new approach in the treatment of this infection. SP-D and PTX3 by virtue of their ability to enhance clearance of *A. fumigatus in vivo* appear attractive as new therapeutic candidates as part of a combination therapy. This issue assumes a greater significance since treatment with anti-fungal drugs has associated nephrotoxicity and heptotoxicity. For example, the AmB toxicity has also been associated with its ability to raise TNF-α levels through TLR activation. PTX3 in combination therapy appears to downregulate TNF-α production and at the same time, the Th1 immune response is strengthened. The effect of SP-D as an adjunct therapy is currently being tested. Thus, of the collectins and the pentraxins detailed here, SP-D and PTX3 offer novel ways of restoring innate and adaptive response to fungal infections.

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CHAPTER 8

COMPLEMENT IN FUNGAL INFECTIONS AND COMPLEMENT EVASION STRATEGIES

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Abstract: Fungal infections are of particular importance in modern medicine. This is mainly due to an optimised iatrogenic immunosuppression, which, for example, enables a longer survival of organ transplantation recipients, or due to better treatment options for chronic infections or neoplastic diseases. These improved therapeutic approaches will keep more patients for longer periods in immunocompromised states, where they are in particular susceptible to fungal infections. As a consequence, invasive fungal infections are "on the march". Therefore a deeper understanding of the interaction between the various fungi and the host's innate and adaptive immune defence system is warranted. This understanding is the essential prerequisite for a successful therapeutic approach. The present article reviews the current knowledge of the role of complement, as a central part of innate immunity, its activation by fungi, and its role as a fine tuner of adaptive immunity in the fungus-induced pathogeneses on the one hand, and the strategies of the fungi to evade complement attack on the other hand, with particular emphasis on candidiasis, aspergillosis, cryptococcosis, blastomycosis, histoplasmosis, paracoccidioidomycosis, and *Malassezia*-associated skin diseases

1. INTRODUCTION

Most fungal species are no threat to humans, since they have special growth requirements and/or limited ecological niches. Furthermore, effective barriers and a broad spectrum of protective host defence mechanisms have evolved in humans which strictly limit the possibility of a fungal infection. As a consequence, serious

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fungal diseases in immunocompetent humans are relatively rare, despite the presence of more than 100.000 known species of fungi on earth.

However, the prevalence of severe fungal infections has increased markedly due to the increasing number of severely immunocompromised patients, such as organ recipients, patients with haematological neoplasia, with immunosuppressive therapy in autoimmune diseases, or individuals with HIV infection. Among hospitalised patients fungal aetiology of pneumonia, urinary tract infection, surgical wound infection and blood stream infections was reported to have increased between 50% and 400% from 1980 to 1990 (Jarvis, 1995).

Invasive fungal infections are a critical cause of morbidity and mortality in predisposed patients, since they are hard to diagnose with certainty and difficult to treat with a limited number of clinically available drugs. An effective therapy remains a difficult task especially in advanced invasive fungal infections, therefore therapy is mostly given as prophylaxis. However, the extended spectrum of fungal pathogens creates uncertainty about the most appropriate prophylactic strategy (Martino and Subira, 2002).

Specific immunity against invading micro-organisms may be divided into an innate and an acquired part and it is the former which is of special importance in the antifungal reaction, since it allows an immediate reaction and recognizes a broad variety of fungal pathogens. Within the innate immunity the complement system with its multitude of soluble factors, restrictive regulator proteins and cellular receptors plays a dominant role. The complexity of this system facilitates discrimination between self and non-self with an effective but controlled attack on non-self structures (for reviews see Speth et al. 1999, Prodinger et al. 2003).

1.1. Complement

Complement is an inducible cascade of sequential activation steps consisting of different soluble complement factors, receptors and regulator molecules (reviewed in Speth et al. 1999, Prodinger et al. 2003) (Fig. 1).

Three different pathways lead to the formation of C3 convertases as the central prerequisite step for the final activation of the complement cascade. The classical pathway is initiated by binding of complement factor C1q to an antigen-antibody complex or directly to a target molecule/surface. Attachment of other complement factors and their proteolytic cleavage generate the classical pathway C3 convertase. The same convertase is also formed antibody-independently via the lectin pathway with binding of mannan-binding lectin (MBL) to carbohydrates on the surface of pathogens as initiating step. The alternative pathway starts by spontaneous modification of complement factor C3 or cleavage by inflammatory proteases, thus forming an alternative C3 convertase with the same enzymatic activity.

Independent of the starting mechanism, the further cleavage of C3 into the small C3a and the large C3b by the C3 convertases is the central step of the complement

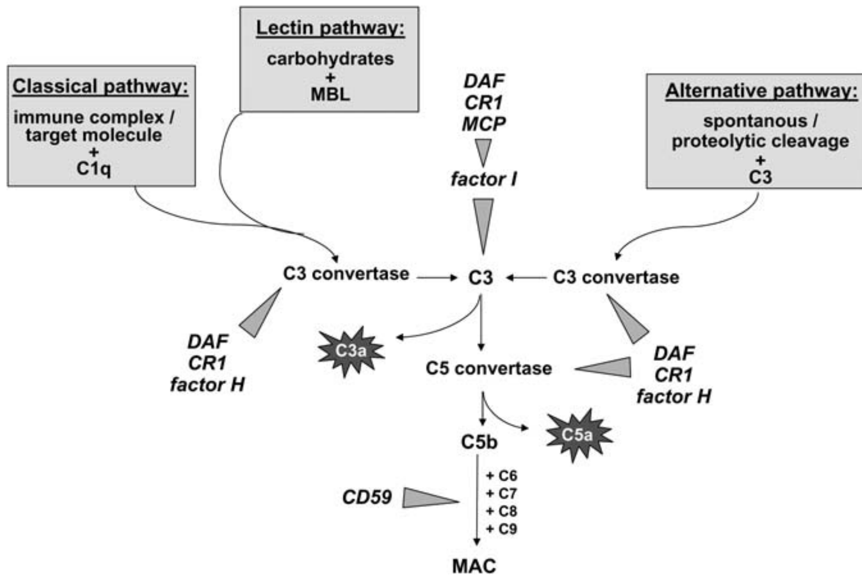

Figure 1. Activation pathways and regulation of the complement system. Complement is activated via three different pathways; A number of regulator proteins tightly control the cascade (Δ)

cascade. Whereas C3a harbours numerous biological activities as an anaphylatoxin, C3b takes further part in the complement cascade. It attaches to the C3 convertases and thus forms the C5 convertases with cleavage of C5 into the even more powerful anaphylatoxin C5a and the usually membrane-associated C5b. The following C5b-dependent assembly of C6, C7, C8 and C9 leads to the formation of the lytic membrane-attack complex (MAC) with C9 as the structural element of the pore in the target membrane.

1.2. Complement Regulators

The complement machinery is under strict control of a battery of membrane-bound and fluid-phase regulator proteins (reviewed in Speth et al. 1999, Prodinger et al. 2003), which prevent an exceeded inflammatory reaction and protect uninfected host cells against the complement attack. The regulator molecules act by three different mechanisms: (i) promotion of the cleavage of C3b into iC3b by factor I; that inactivated C3b is unable to further participate in the cascade; the fluid-phase regulator factor H (fH) and the membrane-bound CD46 (MCP) and CR1 are effective and essential co-factors for that factor I-mediated cleavage; (ii) prevention of C3/C5 convertases assembly and acceleration of their decay; the regulators CD55 (DAF), CR1 and factor H act by this mechanism; (iii) modulators of MAC formation, e.g. CD59 inhibits the incorporation of C9 into MAC.

1.3. Complement Receptors

The spectrum of complement-associated molecules is broadened by cellular receptors for different complement proteins (reviewed in Speth et al. 1999, Prodinger et al. 2003). The receptors CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) bind C3b and fragments derived thereof. The anaphylatoxin receptors C3aR and C5aR (CD88) bind the cleavage products C3a and C5a and mediate their inflammatory reaction.

1.4. Complement Functions

Formation of the lytic MAC that integrates into the membrane of the pathogen forming a pore and thus inducing osmotic lysis is not the only function of the complement system, as the complex network of complement factors, regulators and receptors is used for a variety of other immunological processes (reviewed in Speth et al. 1999, Prodinger et al. 2003); (1) although C3b after cleavage by factor I can not participate any more in the direct cascade, the product iC3b – if bound on pathogens or infected cells – is a potent opsonizer; binding of opsonising iC3b to its receptors on phagocytes induces phagocytosis by granulocytes and macrophages and killing by NK-cells; (2) solubilization and clearance of immune complexes from the blood, mainly by interaction of opsonising C3 fragments on the surface of the complexes with CR1 on erythrocytes; (3) the anaphylatoxins C3a and C5a have pro-inflammatory activity and are involved in the stimulation and chemotaxis of myeloid cells bearing the specific receptors C3aR and C5aR; (4) activation of lymphocytes by C3a, C5a, C3b and fragments derived thereof, via their specific receptors and intracellular signal transduction pathways, thus influencing or even intensifying an adaptive immune response; similarly signal transduction pathways are activated in astrocytes and microglia (reviewed in Speth et al. 2002); (5) complement receptors, especially CR3 and CR4 can also mediate homotypic or heterotypic adhesion of cells and are involved in cell migration to the site of inflammation via binding to blood vessel endothelium.

1.5. Complement Evasion Mechanisms

When complement activation and/or stimulation of phagocytes are insufficient for an effective antifungal defence, fungi may establish a foothold in the host, especially when evasion mechanisms are employed by the fungal invaders.

The widespread evolution of anti-complement mechanisms in a large variety of pathogens, including viruses and bacteria, underlines the general importance of complement in infectious diseases and indicates a close relationship between selection for complement evasion and virulence.

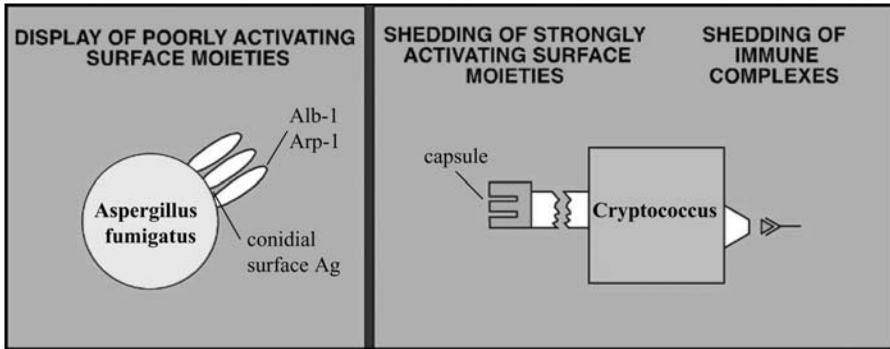

Figure 2. Fungi avoid recognition by complement by displaying poorly activating surface moieties, such as *Aspergillus fumigatus* Alb-1 or Arp-1, which mask the strongly activating conidial surface antigens, or by shedding strongly activating capsules or immune complexes, as executed by *Cryptococcus*

Two main strategies are employed by fungi to evade complement attack (Würzner, 2003): the most successful principle is probably the one of disguise, i.e. complement does not recognise the invasion of the pathogen. Fungi, as other pathogens, best achieve this by displaying non- or poorly activating surface moieties or by getting rid of highly activating ones (Fig. 2). Once detected by complement, fungi still have a potent arsenal to avoid their eradication. This includes removing, consuming or destroying complement (Fig. 3). Some fungi are even able to employ complement for their own protection, e.g. by attaching inhibitors onto their cell surface (Fig. 4).

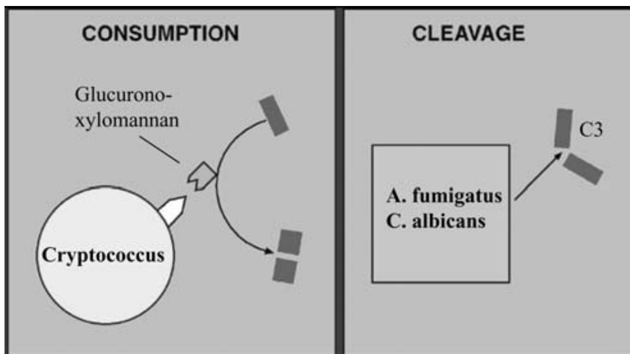

Figure 3. Fungi avoid eradication by complement by consuming complement a bit away from its cell membrane, as done by *Cryptococcus* shedding glucuronoxylomannan, or by cleaving complement, as performed by *Candida albicans* and *Aspergillus fumigatus*

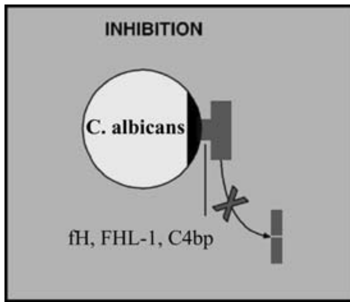

Figure 4. Fungi avoid eradication by complement by employing complement inhibitors by binding them to their membrane, such as factor H or FHL-1 by *Candida albicans*

2. COMPLEMENT AND INVASIVE CANDIDIASIS

Virtually the entire oro-gastrointestinal tract, from mouth to anus with the exception of the stomach, is colonised by *Candida spp*., detectable in more than 70% of mostly immunocompetent human beings. Another location predominantly colonised by these frequent saprophytes of mucous membranes is the vulvovaginal cavity (Odds, 1988). From the affected locations it becomes evident that both endogenic and exogenic infections may occur. At present *Candida spp*. represent a major cause of opportunistic fungal infection in locally or systemically immunocompromised hosts, ranging from superficial thrush to life-threatening systemic infections (Eisenstein, 1990). *Candida albicans* is still the most frequently isolated yeast (> 60%), although other subspecies, which are in part more resistant against the currently applied antifungal agents, appear to be on the rise.

As a first line of defence, the vulnerable physiological flora of the mucous membrane constitutes an important barrier, inhibiting efforts by *Candida* to establish a foothold in the host. It can be destroyed or altered systemically by antibiotics, or locally through mechanical forces or lack of nutrients (Odds, 1988).

The innate, phylogenetically older, 'unspecific' immunity, consisting among others of polymorphonuclear leukocytes, macrophages and complement, constitutes the second line of defence and likely plays a more important role than the acquired 'specific' immune response (Fidel, 2002). This is best illustrated by the fact that patients with a 'specific' immune defect, such as a CD4 depletion caused by HIV infection, often suffer from an 'only' mucocutaneous form of candidiasis, whereas patients with a defect in the phagocytic system, which usually does not occur in early HIV disease, develop systemic disease (Fidel, 2002).

2.1. Complement Activation by *Candida*

By facilitating chemotaxis and opsonisation (Morelli and Rosenberg, 1971, Kozel, 1996), but not lysis – as the thick and complex cell wall will not allow this (Kozel, 1996), complement plays an important part of innate immunity against

fungal infection. Activation is triggered by the galactomannan/β-glucan cell surface mainly via the alternative pathway (Ray and Wuepper, 1976, Thong and Ferrante, 1978, Kozel, 1996). That this pathway probably plays the major role is confirmed by animal studies showing that experimental candidiasis takes a similar course in C4 (i.e. classical pathway) deficient guinea pigs when compared to normal animals. Furthermore, Kozel and coworkers (1996a) have indicated that the lectin pathway probably also plays only a minor role; interestingly, activation via the alternative pathway is characterised by a significant lag phase – classical pathway activation instead leads to a more immediate synchronous binding of C3 over the entire cell surface, indicating a significant role for naturally occurring anti-*Candida* antibodies, especially for the immediate response. The latter is corroborated by data from Han and Cutler (1995), who have reported that passive immunisation of mice with antimannan antibodies protects against disseminated candidiasis. Mannose-binding lectin itself appears to play a dual role: as trigger of the lectin pathway and as complement-independent growth-inhibitor of yeasts (Ip and Lau, 2004).

Neutrophils and monocytes (Diamond and Haudenschild, 1981) have been shown to phagocytose unopsonised *Candida*, but recent studies have shown that properly opsonised *Candida* is phagocytosed to a much greater extent (Gruber et al. 1998, Triebel et al. 2003). Interestingly, cellular infection with human cytomegalovirus downregulates phagocytic CR3 and thereby decreases phagocytosis of yeasts, such as *Candida albicans* (Gafa et al. 2005).

Although it is well accepted that lysis is not a prerequisite for efficient antifungal action, the presence of membrane attack complex on *Candida* does play a role, as it induces a higher mitochondrial activation in *Candida* and augments its phagocytosis (Triebel et al. 2003). It is however not clear how the surface bound membrane attack complex induces these effects.

Mice depleted by complement using cobra-venom factor (involving the whole alternative pathway) show an increased susceptibility to fungal complement infections (Gelfand et al. 1978), which is probably not due to an impaired chemotaxis as there was no difference of the total amount of infectious micro-organisms in genetically C5 deficient mice (Fulurija et al. 1996). However, the latter appear to be more susceptible to cutaneous candidiasis than normal mice (Wilson and Sohnle, 1988), possibly due to a dysregulated inflammatory response (Mullick et al. 2004). This increased susceptibility may involve temperature-dependent expressed fungal surface molecules as detailed below, and also show a decreased resistance to disseminated candidiasis (Morelli and Rosenberg, 1971). In humans there appears to be no association of *Candida* infections and single complement deficiencies (Figueroa and Densen, 1991).

2.2. Complement Evasion Strategies by *Candida*

Although an impaired immune status of the host is probably the most impor-tant factor leading to disease, virulence factors of the yeast also play a role (Odds, 1988).

These may allow evasion from destruction by complement and can be divided into two categories (Würzner, 2003).

The first comprises factors by which *Candida* can protect itself from complement attack either by membrane bound molecules or by released enzymes. As early as 1980 Diamond and co-workers realised that released cell wall mannoproteins were able to interfere with chemotaxis by blocking their chemotactic response to activated complement (Diamond et al. 1980). Another example is the degradation of complement C3 by proteolytic cleavage of C3 (Fig. 3), brought about by an extracellular protease and resulting in a decreased opsonisation and thus diminished phagocytosis (Kaminishi et al. 1995).

The second category includes factors by which *Candida* can actively attach to cells followed by invasion into them. Both C3d-binding by CR2-like molecules (Calderone et al. 1988), as well as iC3b-binding moieties – which resemble CR3-like molecules (Heidenreich and Dierich 1985, Edwards et al. 1986, Eigentler et al. 1989, Alaei et al. 1993, Gale et al. 1996) - have been described. Whereas CR2-like molecules do not appear to have a strong antigenetic relationship to human CR2, for CR3 at least a few of the anti-human α-subunit (or CD11b) binding antibodies also bind to the yeast CR3, although this relationship is also not too strong. The observation that this molecule is differentially expressed, i.e. preferably on (pseudo)hyphal forms (Heidenreich and Dierich, 1985), which are known to display a greater adhesive and invading potential and a better phagocyte-resisting capacity than the yeast form (Bernhardt et al. 2001) has led to the hypothesis that this structure may serve as an important virulence factor.

It is of interest that the CR3 analogue is preferably expressed at 30° C (Eigentler et al. 1989, Würzner et al. 1996) rather than at body temperature, suggesting that it is more important for the early steps of initial colonisation when the yeast comes from outside the body (exogenic infection) rather than for systemic disease. It has been proposed that via such a complement receptor like molecule *Candida* can attach to human erythrocytes to acquire the iron which is essential for its growth (Moors et al. 1992). Hostetter and coworkers have cloned a CR3-like molecule (Gale et al. 1996) and have shown that its gene is important for adhesion and hyphal morphogenesis (Gale et al. 1998). However, it appears that a different CR3-like molecule exist (Mayerl and Würzner, unpublished observation); this molecule, like human CR3, is binding to HIV (Würzner et al. 1997). This may be of clinical relevance as binding of HIV to *Candida* leads to an increased release of secreted aspartic proteases and diminished phagocytosis, and thus may promote candidiasis (Gruber et al. 1998). Interestingly, the corresponding binding regions on HIV envelope proteins show a strong similarity to regions of human C3, representing a case of "concerted molecular mimicry" (Würzner, 1999). From the data it appears that *Candida* may benefit from this interaction, but it is as yet not clear, whether this also applies to HIV. A possible, however still theoretical, mechanism would be that HIV attached to *Candida* may escape the destructive action of the immune system simply by hiding within the complex fungal cell wall; also lined up on *Candida*, HIV may be more infectious for its proper target cells.

As several other pathogenic micro-organisms, *Candida* can also acquire the complement regulators factor H and FHL-1 from the host - which retain their complement regulatory activities when attached to the fungal surface (Meri et al. 2002) – and this may both facilitate adhesion to cells and inhibit the complement cascade in the vicinity of the yeast (Fig. 4). It appears that two different receptors are involved, one reacting with SCRs 6 and 7 of both factor H and FHL-1, and one binding to both SCRs 6 and 7 and SCR 19 (Meri et al. 2002).

Classical pathway regulators, such as C4BP can also be employed by *C. albicans* to specifically inhibit the classical pathway of complement (Meri et al. 2004).

3. COMPLEMENT AND INVASIVE ASPERGILLOSIS

Aspergillus species are ubiquitous saprophytes in soil and on dead organic substrates, but some of them can also cause infections in mammals. *A. fumigatus* accounts for approximately 90% of cases of invasive aspergillosis, followed by the species *A. flavus, A. terreus* and *A. niger* (Denning, 1998). Portals of entry for the small asexual conidia include damaged skin, operative wounds, cornea, and ear. The predominant way into the human host, however, is via inhalation. The normal daily dosage of inhaled conidia does not represent any danger for immunocompetent individuals and the fact that about every second individual is colonised has no clinical consequence. In patients with severe and profound immunosuppression conidia are able to germinate and to form hyphae, which penetrate tissue barriers, thus turning colonization of the respiratory tract into invasive aspergillosis. Invasive pulmonary aspergillosis is the predominant form with 80–90%, and in most cases the lung represents the only focus of *Aspergillus* infection (Denning 1998, Khoo et al. 1994). Dissemination can affect other organs with the brain being the most common extrapulmonal site.

3.1. Complement Activation by *Aspergillus*

Complement affects the pathogenesis of invasive aspergillosis by two different ways. The first mechanism involves the early processes of the complement cascade with the initial activation pathways and subsequent deposition of C3 on the fungal surface. *Aspergillus* conidia and hyphae were indeed proven to activate complement (Kozel et al. 1989, Sturtevant et al. 1992). Complement activation by resting conidia is mediated by the alternative pathway, whereas the classical pathway is involved in swelling conidia and hyphae. Thus, the way of complement activation changes with the maturation of conidia to hyphae (Kozel et al. 1989). Beside C3 deposition on the fungal surface, complement activation also results in the degradation of bound C3b into fragments like the opsonising and CR3-binding molecule iC3b (Sturtevant et al. 1992). Opsonization is a prerequisite for the phagocytosis by monocytes, bronchoalveolar macrophages and polymorphonuclear cells (Sturtevant et al. 1992). Therefore, C3b deposition and degradation on *Aspergillus* is a central interaction between the fungus and the human host; a direct inverse correlation

between C3 deposition and virulence has been proven. Highly pathogenic species like *A. fumigatus* and *A. flavus* bound fewer C3 molecules and were thus less well recognized by phagocytic cells than the less pathogenic species *A. glaucus* or *A. nidulans* (Henwick et al. 1993).

Recently it was found that liver cells respond to *A. fumigatus* with an increase in C3 secretion, C3 gene expression and expression in TLR2 and TLR4 without help of Kupffer cells (Wright et al. 2004).

The second mechanism relevant to control invasive aspergillosis involves late steps of the complement cascade. Mice deficient in the C5 complement component are unable to fulfill the complete complement cascade and show a decreased resistance to dissemination by *A. fumigatus* (Hector et al. 1990). The 50% lethal dose values for deficient mice were 10- to 1,000-fold lower than those for C5-competent mice. This enhanced susceptibility for aspergillosis is not due to the lack of terminal membrane attack complexes, as fungi show resistance to direct lysis by MAC, yet as a result of their thick cell wall (Levitz, 1992). The inability to form the chemoattractant C5a might be the central deficit in the mice (Laxalt et al. 1979, Waldorf et al. 1985). By binding to its specific receptor C5aR C5a induces a wide range of pro-inflammatory effects (Ember et al. 1998). C5a recruits inflammatory cells to the site of infection, enhances cellular adhesion, stimulates oxidative metabolism and liberates lysosomal enzymes and numerous inflammatory mediators (Ames et al. 1996, Gerard et al. 1991). The anaphylatoxin C5a and its metabolite $C5a_{desArg}$ were also identified as inductors of TNF-α and IL-6 secretion (Kacani et al. 2000). Low doses of C5b-C9 complexes might be also relevant for inflammatory processes evolving around *Aspergillus* foci as they bind to membranes, trigger activation and prostaglandin secretion (Daniels et al. 1990, Schonermark et al. 1991).

3.2. Complement Evasion Strategies by *Aspergillus*

The central role of complement in the pathogenesis of *Aspergillus* infections is underlined by the diverse evasion strategies of the fungus. The surface of fungal conidia is optimized to limit complement activation. Disruption of the fungal gene alb1, which encodes a pigmentation factor for conidia results in a more efficient binding of C3 on the conidial surface and a better ingestion by human neutrophils (Tsai et al. 1998). Similarly, inactivation of the gene for the pigmentation protein arp1 also increased the deposition of C3 on conidia (Tsai et al. 1997). Thus, both alb1 and arp1 are able to mask complement activating surface antigens (Fig. 2).

Furthermore, *A. fumigatus* secretes a soluble factor which inhibits complement activation and opsonization of the fungus itself (Washburn et al. 1986, Washburn et al. 1990). The complement inhibitor (CI), which is also produced by *A. flavus*, was found to selectively inhibit activation of the alternative complement pathway and to interfere with C3b-dependent phagocytosis and killing (Washburn et al. 1986). Further biochemical characterization suggested that phospholipids co-migrating with CI contribute to its functional activity (Washburn et al. 1990). Later studies by Sturtevant revealed the synthesis of a proteolytic enzyme, unrelated to the

complement inhibitory factor, that was able to degrade C3 (Sturtevant et al. 1992, Tomee et al. 2000), thereby interfering with complement activation (Fig. 3).

In the brain the interaction of complement and *Aspergillus* is of special importance. Cerebral aspergillosis occurs in 10%–20% of all cases of invasive aspergillosis, being a dangerous complication with a mortality rate up to 100% (Denning, 1998). Therapy of cerebral aspergillosis is of limited efficiency due to the restricted penetration of various antifungal agents through the blood-brain barrier. The local complement system displays a major opportunity to attack the fungus as lack of T-cells and antibodies in CNS. All brain cells have the capacity to synthesize complement proteins and work together to form a complete cascade (Morgan et al. 1996). Recently it was found that *Aspergillus*-induced complement activation in the brain resulted in low local complement synthesis and activation (Rambach et al. 2005). Beside opsonization of the fungus, phagocytosis by microglia, and infiltrating macrophages, complement might also stimulate microglia and astrocytes via induction of pro-inflammatory cytokines and formation of chemotactic C5a and C3a (reviewed by Speth et al. 2002). However, cerebral complement activation might also contribute to the fungus-induced tissue damage. Disturbance of brain homeostasis and inflammation have severe consequences for the functionality of the brain. Chronic complement activation is associated with neurodegeneration as shown in a variety of neuropathological conditions like Alzheimer's disease and multiple sclerosis. Beside a harmful inflammatory reaction, complement-induced brain damage might also include opsonization of surrounding "self" cells with subsequent phagocytosis and bystander lysis by generation of the membrane attack complex.

4. COMPLEMENT AND INVASIVE CRYPTOCOCCOSIS

Cryptococcosis is a worldwide disease, mainly affecting individuals with impaired cellular immunity. The etiologic agent, the encapsulated yeast *Cryptococcus neoformans* is transmitted by inhalation of the encapsulated yeasts or basidiospores into the alveolar spaces. Having a strong tropism to CNS, Cryptococcus is the most common cause of fungal meningoencephalitis, but it can also occur in localized forms or as disseminated disease. Up to 5–10% of patients with AIDS develop cryptococcosis (Good and Coax, 1990).

The polysaccharide capsule of cryptococci, the/a major virulence factor, is composed mainly of glucuronoxylomannan (GXM) and two minor constituents, galactoxylomannan (GalXM) and mannoprotein (MP). The capsule interacts with the complement system by activation of the complement cascade and by stimulation of synthesis. Encapsulated cryptococci are very powerful activators of the complement cascade; complement activation occurs predominantly via the alternative pathway (Kozel, 1998) and leads to the deposition of up to 10^7–10^8 C3 fragments per yeast cell at the capsular surface and in its interior (Kozel et al. 1988; Kozel et al. 1989; Young and Kozel 1993; Kozel et al. 1996b). To meet the requirement of enhanced complement synthesis for antifungal defence, cells can

respond to cryptococcal infection with increased local complement C3 synthesis. GXM was identified as the corresponding stimulus to induce C3 production e.g. in peritoneal cells (Blackstock and Murphy, 1997).

Complement activation intervenes in important mechanisms of the antifungal host defence. Initial control of cryptococcosis is highly dependent on phagocytosis of the fungus and phagocytic cells require opsonization by complement or antibodies to ingest encapsulated *C. neoformans* cells. Efficient binding and phagocytosis of complement-opsonized cryptococci occur via the complement receptors CR1, CR3 and CR4 on macrophages, and these receptors act independently of each other in their binding (Davies et al. 1982; Levitz and Tabuni 1991; Levitz et al. 1997). The complement receptors CR3 and CR4 are also involved in the complement-independent antibody-mediated ingestion of *C. neoformans*. IgM- and IgG-mediated phagocytosis of the fungus was proportional to CR3 expression and was reduced dramatically in macrophages of CD18-deficient mice (Taborda and Casadevall 2002). Dendritic cells (DC) also have the capacity for phagocytosis and killing of microbes. Similar to macrophages, binding of unopsonized C. neoformans to human DCs was negligible, and opsonization with human serum increased the binding (Kelly et al. 2005).

The second intervention of complement with cryptococcal infection involves proliferation of immune cells. Complement-mediated phagocytosis of live cryptococci by human macrophages induced progression to S phase and cell division (Luo et al. 2005). This phenomenon might represent a potential mechanism for increasing the number of effector cells after microbial ingestion. However, increased proliferation of phagocytes harbouring intracellular fungi can also promote spread of the fungal infection.

Third, complement also modulates the cytokine synthesis during cryptococcal disease. In the presence of complement, human polymorphonuclear cells respond to the contact with capsular material of *C. neoformans* with the secretion of interleukin-8 (IL-8) (Vecchierelli et al. 1998). The cytokine IL-8 is an important promoter of inflammatory immune responses with induction of chemotaxis and respiratory burst, and consequently the support for microbial killing as main biological activities (Matsushima and Oppenheim 1989; Liles and Van Hoorhis 1995). Further experiments revealed that the anaphylatoxins C3a and C5a generated during complement activation are the mediators for the induction of IL-8 by cryptococci (Vecchierelli et al. 1998). Patients with late-stage HIV infection have reduced responsiveness to C3a and C5a, decreased expression of the C5a receptor and thus an impaired IL-8 production (Monari et al. 1999). This might be one reason for the high incidence of cryptococcosis in HIV-infected patients.

Complement is also involved in the induction of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α). Whole cryptococcal cells as well as the isolated capsular components GXM, GalXM and MP are capable of inducing TNF-α synthesis in blood cells, provided that opsonizing C3 fragments are available (Delfino et al. 1996; Levitz et al. 1994). Also human DC release significant amounts of the TNF-α when stimulated with complement-opsonized cryptococci

(Kelly et al. 2005). The produced TNF-α efficiently enhances ingestion of opsonized encapsulated *C. neoformans* by macrophages with CR3 as the corresponding receptor (Cross et al. 1997). Thus, the stimulation of phagocytosis by complement occurs both via opsonization of cryptococci and via induction of stimulatory TNF-α.

C. neoformans however, has developed strategies to counteract the complement-mediated antifungal defence. Cryptococci shed their capsule to evade recognition (Fig. 2), and large amounts of GXM from their surface to induce complement activation away from the surface at a safe distance (Figure. 3). Since GXM has been shown to bind to CD18, the β-chain of the two complement receptors CR3 and CR4 (Dong and Murphy, 1997), it might be hypothesized that the released GXM interferes with the CR3-dependent phagocytosis. Furthermore, the putative blocking of CR-mediated adhesion of phagocytes to endothelium by soluble GXM might inhibit their migration from the blood vessels to the sites of inflammation in the surrounding tissue (Dong and Murphy, 1997).

A further defence mechanism of the fungus against immune attack is the downmodulation of the C5aR expression on human neutrophils. Encapsulated cryptococci and isolated GMX from the cryptococcal capsule markedly suppress the expression of C5aR. As a consequence, neutrophil binding of C5a and their subsequent chemotactic response are decreased (Monari et al. 2002). Thus, cryptococci interfere with complement-dependent migration of phagocytes to the site of infection as well as with the phagocytotic process driven by complement receptors.

5. COMPLEMENT AND INVASIVE BLASTOMYCOSIS

The ascomycete *Blastomyces dermatididis* is a dimorphic fungus that exists in nature as a sporulating mould and converts to a large thick-walled yeast form at body temperature. *Blastomyces* may produce epidemics following a point source of infection or induce sporadic endemic infections. After inhalation of conidia into the lung of humans or animals, these swell and undergo a temperature-induced phase transition to the yeast form required for proliferation and pathogenicity. Being mostly an asymptomatic infection blastomycosis may also manifest as severe pneumonia and, in immunosuppressed patients, potentially disseminate to other sites like bone, skin or brain. Infections that go undiagnosed or untreated often progress and become fatal even in immunocompetent hosts (Bradsher et al. 2003).

B. dermatitiis activates complement by both classical and alternative pathway resulting in an opsonization of the fungus with C3 fragments (Zhang and Klein, 1997). The main surface component responsible for complement activation and deposition is β-glucan; a glucan-deficient mutant yeast showed reduced and delayed C3-binding capacity (Zhang and Klein 1997; Zhang et al. 2001).

Blastomyces has developed an efficient evasion strategy to avoid complement attack. The glucan-induced complement activation is modulated by the fungal protein BAD1 (Blastomyces adhesion 1; formerly called WI-1), that is both displayed on yeast and released during infection. Lacking itself any intrinsic ability to mediate complement activation, BAD1 masks C3-binding sites on glucans.

In BAD1 knockout yeast the C3-binding capacity of *Blastomyces* is almost doubled compared to that in wild type, presumably by providing additional binding sites for C3 molecules (Zhang et al. 2001).

Beside evasion of complement attack *Blastomyces* also exploits complement molecules to suppress other immune defence mechanism and even to promote spreading and dissemination. The adhesion factor BAD1 binds to macrophages and tissues via interaction with CR3 (Newman et al. 1995; Brandhorst et al. 2004; Finkel-Jimenez et al. 2002). Yeast lacking BAD1 lack the capacity to enter lung macrophages and are easily killed by neutrophils (Finkel-Jimenez et al. 2002). The knockout *Blastomyces* also bind poorly to the lung tissue and thus might be unable to establish an effective infection of the lower respiratory tract. Furthermore, binding of soluble or membrane-bound BAD1 to CR3 on macrophages results in suppression of synthesis of TNF-α, a crucial defence cytokine against intracellular microbes. Macrophages of CR3 ($^{-/-}$) mice resisted both infection and TNF-α suppression by soluble BAD1 in vivo (Brandhorst et al. 2004). Thus *Blastomyces* is a perfect example for a fungus that developed both a mechanism for avoidance of complement recognition and a mechanism of complement employment to enhance infection and suppress antimicrobial cytokine induction.

6. COMPLEMENT AND INVASIVE HISTOPLASMOSIS

Histoplasma capsulatum is a dimorphic fungal pathogen that infects the host by inhalation and deposition of microconidia into the terminal bronchioles and alveoli of the lung. The inhaled microconidia convert to yeasts which are responsible for the pathogenesis of histoplasmosis. Although most infections by *H. capsulatum* are asymptomatic, histoplasmosis can be severe or even fatal in patients who have experienced a heavy exposure, have underlying immune defects, or develop a progressive disease that is not recognized and treated.

Similar to *Blastomyces*, *Histoplasma* uses complement receptors for spreading and dissemination. During the early phase of infection alveolar macrophages recognize and phagocytose *Histoplasma* microconidia and yeasts via the complement receptors CR3 and CR4 (Bullock and Wright 1987; Ignatov and Keath 2002). Heat shock protein 60 was identified as the ligand on the surface of *H. capsulatum* that interacts with the complement receptors on human macrophages (Long et al. 2003). Upon ingestion the yeasts survive the hostile intraphagosomal environment and proliferate intracellularly. Replicating yeasts destroy the alveolar macrophages and are phagocytosed by neighboring and recruited macrophages. Repetition of this cycle results in dissemination of the yeasts from the lung to other organs. Therefore complement receptor-dependent internalization of unopsonized and opsonized *Histoplasma* is a crucial step in the development of histoplasmosis (Long et al. 2003).

Another similarity of defence against immune attack with *Blastomyces* comprises the modulation of cytokine synthesis. The interaction of *Histoplasma* with CR3 initiates a negative signal to the monocytes and consequently the suppression of

IL-12 synthesis (Marth and Kelsall, 1997). The cytokine IL-12 is a key mediator of the cell-mediated immunity against microbial infection and IL-12-induced cell-mediated immunity is required for resistance to intracellular infections. Production of IL-12 by macrophages led to the polarization of naive T cells to produce a T_H1-cell response (Hunter, 2005). In addition to inducing IFN-γ, IL-12 stimulates effector CD4(+) T cells to express adhesion molecules and homing receptors that facilitate their migration to sites of inflammation (King and Segal, 2005). The CR3-mediated decrease of IL-12 levels during infection strongly favors the pathogen and an unfavourable pathogenesis. Mice infected with *H. capsulatum* and treated with neutralizing antibodies to IL-12 experienced accelerated mortality; in contrast, mice treated with IL-12 at the initiation of infection had a severalfold decrease in the colony counts of *H. capsulatum* and a substantially diminished mortality (Zhou et al. 1995).

7. COMPLEMENT AND INVASIVE PARACOCCIDIOIDOMYCOSIS

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the fungus *Paracoccidioides brasiliensis*. It is the most prevalent systemic mycosis of Latin America and 80% of the reported cases are from Brazil (Mackinnon, 1970). *P. brasiliensis* is a thermally dimorphic fungus that exists in a saprobic mycelial form at environmental temperature; conidia are inhaled into the lungs where they transform into the pathogenic yeast form at the temperature of the mammalian host (Borges-Walmsley et al. 2002). Two main clinical forms of PCM are described: the acute or subacute form (juvenile type) and the chronic form (adult type). The juvenile type develops fast within weeks to months with high rates of mortality due to severe hypertrophy of the reticuloendothelial system (spleen, liver, bone marrow) and progressive fungal dissemination. The adult form is the most common presentation (> 90%), develops more slowly over months to years and primarily afflicts the lungs with subsequent impairment of lung function. A restricted dissemination can occur to other organs with secondary lesions in skin, mucosa and lymph nodes. Recovery from PCM leaves fibrotic sequelae in all affected organs which can permanently interfere with organ functionality.

Complement activation by *Paracoccidioides brasiliensis* via classical and alternative pathway was revealed in vitro and in infected patients (Calich et al. 1979; Munk et al. 1992; De Messias and Mohren 1994). An alkali-insoluble polysaccharide fraction F1 derived from the fungal cell wall could be identified as complement-activating component (Crott et al. 1993, 1997). The role of complement and complement activation in the course of paracoccidioidomycosis is shown by the fact that null-alleles of the C4 chains A and B are significantly more frequent in PCM patients in comparison to control individuals (De Messias et al. 1991).

After opsonization the fraction F1 stimulated neutrophil functions in a complement-dependent manner (Crott et al. 1993). But complement might also indirectly affect the pathogenesis of PCM. PCM is frequently associated with high serum levels of immune complexes (IC) in the affected patients (Teixera et al. 2001).

These high levels correlate to low numbers of CR1 on erythrocytes which is responsible for binding to opsonized antigen and for a safe clearance of ICs in the liver. The low expression of CR1 on erythrocytes permits IC to remain longer in the blood stream and thus favour the appearance of clones of suppressor T lymphocytes in the circulation, provoking a depression on the cell immune response and a stimulated synthesis of anti-inflammatory IL-10. Indeed, there was a strong correlation between circulating IC and depressed cell-mediated immunity, and patients with PCM presented a lower CD4/CD8 ratio in the peripheral blood (Teixeira et al. 2001; Cherquer-Bou-Habib et al. 1989). Treatment of the patients resulted in a significant increase in the mean number of CR1 on erythrocytes, a reduction in IC levels and a simultaneous increase in CD4/CD8 T-cell count.

One possible explanation for the acquired reduction of CR1 on erythrocytes in PCM is the overload of the IC transport system by high amounts of circulating IC. As a consequence erythrocytes transferring IC to phagocytic cells in the liver loose a segment of the receptor and return to the circulation, the first step of a vicious circle (Cosio et al. 1990; Nardin et al. 1999). Alternatively, the high levels of the acute phase protein CRP observed in the serum of PCM patients may activate complement and bind to CR1 via C3b fragments. Similar to the clearance of IC, the clearance of CRP might involve loss of CR1 on erythrocytes (Mold et al. 1996).

The pathogenicity of *Paracoccidioides* is closely related to its capacity to evade complement attack. F1 fractions derived from low-virulent isolates activated the complement cascade more efficiently than highly virulent isolates (Crott et al. 1997).

8. COMPLEMENT AND *MALASSEZIA*-ASSOCIATED SKIN DISEASES

Yeasts of the genus *Malassezia (Pityrosporum)* are a normal part of the skin flora, and they are most often found in sebum-rich areas of the skin such as trunk, back, face and scalp. However, *Malassezia* is also thought to be connected to several common dermatologic conditions like pityriasis versicolor, *Malassezia* folliculitis and seborrheic dermatitis (SD); its role in atopic dermatitis and psoriasis is less well defined (Gupta et al. 2004). SD is a superficial fungal disease presenting clinically as scaling and inflammation on the areas of the body rich in sebaceous glands with patches of red, flaking greasy skin. The pathogenesis might involve an abnormal or inflammatory immune response to these yeasts, the presence of uncommon *Malassezia* species or toxin production by the fungus (Gupta et al. 2004); antifungal treatment reduces the number of yeasts on the skin, leading to an improvement in seborrheic dermatitis. *Malassezia* made up 46% of the microbial flora in normal subjects but 83% of the flora in patients in SD (Ashbee and Evans, 2002). In patients with AIDS, who are known to have a diminished T-cell function, a high incidence of SD has been found; whereas incidence of SD in normal population is around 1–3%, about 30–83% of AIDS patients suffer from this skin disease (Bergbrant, 1995; Ashbee and Evans, 2002). *Malassezia* has also been reported to cause sometimes fatal fungemia in premature neonates with a serious underlying disease. These rare cases of systemic infection are caused by the

infection of parenterally-received nutrition containing lipid emulsions (Redline et al., 1985, Long and Keyserling 1985).

The parallels between *Malassezia* and other fungal inducers of systemic mycoses concerning the connection with the complement system are striking. Complement proteins are expressed in the skin (Dovezenski et al. 1992), and *Malassezia* can activate the complement cascade via the direct and alternative pathways (Bergbrant et al. 1991; Suzuki et al. 1998a). The main complement-triggering molecule of the *Malassezia* is unknown, but might include (similar to *Blastomyces*) β-glucan in the cell wall (Ashbee and Evans, 2002). Since complement-mediated inflammation is associated with many dermatoses, it might also play a role in the pathogenesis of SD. Immunohistochemical studies of SD detected deposits of C3 in the lesions, localized solely around the collections of *Malassezia* cells and absent from uninvolved skin (Pierard-Franchimont et al., 1995; Ashbee and Evans, 2002). Also C1q showed an increased cellular and intercellular staining in patients compared with healthy controls and the intercellular staining was often more intense in lesions compared with non-lesional skin (Faergemann et al. 2001).

Complement also mediates the internalization of *Malassezia* by macrophages via complement receptor type 3 (Suzuki et al. 1998b). However, after 2h of internalization only 5% of the cells are killed, probably due to the production of azelaic acid by *Malassezia* (Akamatsu et al. 1991), indicating that, similar to *Histoplasma* and *Blastomyces, Malassezia* might use complement-mediated internalization by phagocytes to evade from immune attack.

There is also another striking similarity to *Blastomyces: Malassezia* significantly depressed the production TNF-α production by peripheral blood mononuclear cells (Kesavan et al. 1998). The precise mechanism of decreased TNF-α production is unknown but might (in analogy to *Blastomyces*) include binding of a fungal protein to human CR3.

9. CONCLUSION

The complement system is an important prerequisite for an effective antifungal immune response and all fungal pathogens – more or less efficiently – activate at least one of the three pathways. The spectrum of complement-associated immune reactions include assistance of phagocytosis by opsonization of the pathogen as well as production of chemotactic molecules which help to focus the defence of the host.

These roles of complement as first line of defence are reflected by the diverse strategies of the fungi to evade complement attack. In some cases like *Histoplasma* and *Blastomyces*, complement receptors are even used as fungus-binding molecules to support adhesion. Complement-related molecules on *Candida* are supposed to contribute to pathogenicity. Thus, new therapeutic options, taking complement evasion strategies into account, are warranted to combat fungal infections.

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CHAPTER 9

CYTOKINES

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Abstract: Cytokines are a family of soluble secreted mediators produced by cells that effect the functioning of the same or other cells. Aspergillus fumigatus is a potent stimulator of cytokine and chemokine production by a variety of cells present in the inflamed lung. Substantial research interest has focused upon the cytokine responses to fungal colonization of the lung. Models of fungal asthma and allergic bronchopulmonary aspergillosis (ABPA) strongly implicate Th2 cells and cytokines as the main orchestrators of these immune disorders. Manipulation of cytokine and chemokine function can vastly impact upon the course of invasive pulmonary aspergillosis (IPA) in humans and experimental models

1. AN INTRODUCTION TO CYTOKINE BIOLOGY

The cytokines are a superfamily of soluble secreted mediators produced by cells that direct the function of cells. Cytokines are effectively signaling molecules communicating between cells to direct their function by binding cell surface receptors. Cytokine receptor activation and signal transduction can instigate a range of cell activities including their differentiation, activation, maturation, trafficking, production and release of soluble mediators as well as apoptosis. Bar a few exceptions, the cytokines are defined by their rapid secretion following de novo synthesis. This characteristic distinguishes them from their stored counterparts such as prostaglandins and other lipid mediators. Secreted cytokines can work in an autocrine or paracrine fashion and are active in a diverse array of physiological processes including embryogenesis, tumorigenesis, angiogenesis, and the initiation, maintenance and modulation of host response mechanisms to invading microorganisms, and allogeneic or cancerous cells.

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1.1. The Cytokine Families

The cytokines are grouped by their structure into several families. The haematopoietins, interferons (IFN), tumor necrosis factor (TNF) and chemokines constitute the four main families. Three further families feature the transforming growth factor (TGF)-, interleukin (IL)-1/fibroblast growth factor (FGF) and IL-10-like cytokines, respectively. Cytokines signal via interaction with cytokine receptors, which are also grouped into families corresponding with their ligand(s) and conserved homology with the haematopoietin, TNF-α, IFN-γ or chemokine receptors (Arai et al., 1990; Kotenko et al., 2004; Lynch et al., 2003; Ware, 2003). Cytokines are important for the functioning of the immune system and dysregulated cytokine production is a feature of many pathophysiological conditions.

1.1.1. Cytokine receptors

The Class I cytokine receptor family (a.k.a the haematopoietin-receptor family) encorporates three subgroups, with each receptor formed of a complex of an α, β and γ chain, and featuring a conserved WSXWS sequence. The subfamilies are I) the receptors for erythropoietin, growth hormone and IL-13, II) the β_c receptors that share a common β chain, and III) the γ_c receptors that have a common γ chain. The Class II cytokine receptor family includes the IL-10 and interferon (IFN) receptors. The TNF receptor (TNFR) family includes the TNF receptors and also CD27, CD30, CD40, Fas and nerve growth factor receptor (NGFR). The fourth family encompasses the chemokine receptors, which have 7-transmembrane spanning regions and signal via G protein coupling.

1.1.2. Interleukins

Many cytokines are known as interleukins (IL), and are given a designated numerical suffix, due to the initial description of their function as soluble mediators of communication between leukocytes. However, cytokines are produced by many cell types other than those of the immune system and are not exclusively chemotactic, pro-inflammatory or restricted to modulating the activity of leukocytes. As key mediators in the immune system, the role of specific interleukins will arise time and again in discussion of immunity to fungal infection. The names, familial relationship, receptors and main functions of the interleukins are summarized in Table 1.

1.1.3. TNF/TNFR superfamily

The TNF and TNFR superfamily includes, of course, TNF a well known pro-inflammatory cytokine responsible for host damaging effects in conditions such as sepsis and arthritis. In the clinical setting high levels of TNF are frequently associated with detriment to the patient. Lymphotoxin, known for inducing apoptosis, is another established member of this family, which includes many pro- or anti-apoptotic factors. The TNF/TNFR family encompasses a number of cytokines whose functions have come to light more recently including OX40 ligand(L),

Table 1. Interleukin Vital Statistics

Cytokine	Source	Receptor	Target Cells	Function
IL-1 family				
IL-1α	Mono, MΦ, *B, Keratinocytes, fibroblasts	IL-1R I & II	B, T, DC, mono	Pleiotropic factor, acute phase endogenous pyrogen: pro-inflammatory, stim. IL-2 induction by T, sepsis, wound healing, angiogenesis
IL-1β	Mono, MΦ	IL-1R I & II	B, T, mono	As IL-1α
IL-1RA [sIL-RA, icIL-RA-1,-2,-3]	MΦ, mono, NØ, fibroblasts. [Soluble & intracellular isoforms]	–	–	Antagonist of IL-1α & IL-1β Inhibits IL-1, IL-2 and IL-2R
IL1F5–9	Selective plasma cells, MΦ, *keratinocytes,	sIL-18R, ?	?	Predicted roles in modulating inflammatory responses
IL-9	*Th2, Hodgkin's lymphoma	CD129 [&soluble R]	T, erythrocytes, airway s.m. cells	Promotes MC and B cell growth, CCL11 production by airway s.m. & EØ recruitment
IL-18	*MΦ, EC, epithelial cells	IL-18Rα/CD21 8, Il-18Rβ, IL-18BP	T	Th1 type cytokine: induces IFN-γ by T & NK, angiogenic, ↑VCAM-1, contributes to autoimmune and allergic conditions
IL-33	s.m. cells, *MΦ, *DC, airway epithelial cells,	ST2	Naïve Th2, MC,	Drives Th2-type response inc. IL-4, IL-5, IL-13 and Ig production, eosinophilia, splenomegaly, inhibits IFN-γ release, role in mucosal immunity
IL-2 family				
IL-2	Activated lymphocytes	IL-2R	T, B, NK, mono, oligodendrocytes	Lymphocyte expansion, tumour surveillance

(Continued)

Table 1. *(Continued)*

Cytokine	Source	Receptor	Target Cells	Function
IL-7	b.m. and thymus stromal cells, keratinocytes	Soluble CD127+γ_c	CLP, megakaryocytes	B & T cell development, T and megakaryocyte maturation
IL-15	Stimulated human fetal astrocytes & microglia	Il-15Rα, CD122, CD132	NK, mono (esp. PHA-activated PBMCs)	NK maturation, role in immune responses in CNS, generation of cytolytic cells.
IL-21	*CD4 T	IL-21R/NILR, γ_c	NK, T, B, DC	Costimulates B proliferation with CD40, stim. NK & T, stim. HSC proliferation
CSFs				
IL-3	*T, mono, NK, MC, EC, keratinocytes	IL-3R	HSC, EØ	Haematopoiesis, EØ chemoattractant, pre-B cell development, neutrotrophic
IL-5	T, MC, EØ	IL-5R	EØ, BØ	Haematopoiesis, B cell activation, IgM & IgA production, regulates eosinophilic inflammation
Chemokine family				
IL-8/CXCL8	*mono, fibroblasts, EC	CXCR1 CXCR2	Lymphocytes, NØ	NØ activation & chemotaxis
Type II cytokine receptor & ligand superfamily > type I interferons subfamily				
IL-28A, IL-28B, IL-29	pDC	IL-28Rα/IFN-λR1+IL-10Rβ	Ub except brain	Inhibits viral replication, upregulates MHC I
Type II cytokine receptor & ligand superfamily > IL-10 subfamily				
IL-10	Th2, B1, *CD4, *CD8, keratinocytes	CD210	MΦ, MC, B, fibroblasts, granulocytes, EØ	Inhibits inflammatory responses, CD8 chemotractant, costimulator for thymocytes & MC

IL-19	*mono, B	IL-20R1, IL-20R2	Mono, *T	Induces IL-6, TNF-α & O_2 production & apoptosis in mono, stim. cytokine production by *Th2, IL-13 in asthmatic mice
IL-20	Mono, keratinocytes	IL-20R1/2 or IL-22R1/2	keratinocytes	Regulates keratinocyte function, implicated in psoriasis
IL-22	NK, Th1, IL-9-stimulated T, MC	CRF2-4, IL-10R2	keratinocytes	Upregulates pro-inflammatory molecules inc. PDGF-A, CXCL5 & MMP3
IL-24	NK, B, naïve T, mono, melanocytes, vascular s.m., cancerous breast epithelial cells, Th2 (m), fibroblasts,	IL-20R1/2, IL-22R1/2	Mono, MEP,	Induces selective apoptosis of epithelial cells in human melonoma, ovarian & breast carcinoma, stim. Il-6 & TNF-α production by mono
IL-26	*$CD4^+$, NK, Th1	IL-10R2 + IL-20R1	Epithelial cells, keratinocytes	Role in herpes virus saimiri infection, induces Il-8, IL-10 & CD54 expression, mucosal immunity
IL-12 family				
IL-12p70 [IL-12A+B]	MΦ, mono, DC	CD212	T, NK	Key cytokine of cell-mediated immune responses: stim. Th1 response, IFN-γ production, NK- & T-mediated cytotoxity & proliferation
IL-12p40 [B only]	MΦ, mono, DC	IL-12R B1	MΦ	MΦ chemoattractant, antagonizes IL-12p70
IL-23	*DC, psoriatic cells	IL-23R, IL-12Rβ	Memory T (m), T(h)	Induces proliferation of memory T cells, induces IFN-γ in naïve & memory T (h), induces IL-17 in memory T(m), role in psoriasis

(Continued)

Table 1. *(Continued)*

Cytokine	Source	Receptor	Target Cells	Function
IL-17 family				
IL-17	$CD4^+$ memory T cells $CD4^+$ T helper subset	CD217/HVS13R, IL-17R family	Stromal cells, EC, epithelial cells, fibroblasts	Angiogenesis, regulates chemokine, IL-6 & IL-8 production, neutrophil maturation, role in collagen production
IL-25	polarized Th2, b.m. stromal cells,	TSA-1	double negative thymocytes, alveolar MΦ	Th2-type cytokine: stim. production IL-4, IL-5, IL-13 by double negative lymphocyte progenitors, lymphocyte proliferation, ↑IgG & IgE, ↑EØ number & EØ-mediated inflammation & chemokine release
Others				
IL-6	T, B, stim. Mono, fibroblasts, EC, BM stromal cells	CD126+CD 130/gp 130 [&soluble R]	T,*B, mono	Immunoregulatory esp. acute phase reaction, T differentiation, neuron growth, B maturation to plasma cells, trophoblast differentiation
IL-4	Th, MC, BØ, myoblast subset	IL-4R sIL-4R	B, T, mono, EC, MC	Th2-type cytokine, B cell help, ↑IgG & IgE production, Th regulation, imp. in allergy
IL-4δ-2	Th, MC, BØ	IL-4R sIL-4R	B, T, mono, EC	IL-4 antagonist
IL-11	b.m. stromal cells, mesenchymal cells	NR1+CD130	HSC	Haematopoietic, collaborates with IL-3 to induce megakaryocytopoiesis, inhibits pre-adipocyte differentiation

IL-13	Th2, MC, NK, *CD4, *CD8	IL-13Rα + CD132/γ_c	MΦ, mono, B, epithelial cells	Important in Th2 responses, inhibits MΦ cytotoxic activity, ↑IL-1RA, IgE & IgG synthesis, inhibits type 1 cytokine production, stim. expression of CD23, CD72 & MHC II gene products, mediates hyperreactivity, mucous overproduction and eosinophilia in asthma
IL-14	T, EC	?	B,	R expressed on * B cells, mitogen for * B, stim. Prostaglandin E & inhibits Ig production,
IL-16	*CD8, EØ, lung epithelium, fibroblasts,	CD4, CD9	T, EØ, MΦ, MC	Weak chemoattractant for EØ, CD4, MΦ, MC, suppresses HIV replication, indicated in allergy
IL-27 + IL-30	Mature DC	WSX-1/TCCR, gp130	NK, naïve $CD4^+$ T	Induces proliferation of naïve T, early initiator of Th1 responses synergizing with IL-12 & IFN-γ
IL-31	*T (esp. *Th2)	IL-31A+OSMR	*mono, epithelial cells	Promotes allergic & non-allergic dermatitis, may regulate allergic asthma
IL-32	*T, *NK, epithelial cells		MΦ, RAW & A459 cell lines	Induces copious TNF-α, induces IL-8 & MIP-2

Abbreviations: * =activated populations; APC, antigen presenting cells; B, B cell; b.m., bone marrow; BØ, basophil; CLP, common lymphoid progenitors, CNS, central nervous system; DC, dendritic cell; EC, endothelial cell; EØ, eosinophil; esp., especially; HSC, haematopoietic stem cells, inc., including; Ig, immunoglobulin; imp., important; MC, Mast cell; MEP, megakaryocyte/erythroid progenitor; Mono, monocyte; MΦ, macrophage; NK, natural killer cell; OSMR, oncostatin M receptor; PBMCs, peripheral blood mononuclear cells; R, receptor; s.m., smooth muscle; stim., stimulates; T, T cell; Ub, expressed by a multitude of cell types.

NB: most of the IL-Rs consist of one or more subunits that have a CD classification, e.g. IL-5R is CD125 + β_c. This table is only a summation of the current knowledge of interleukin function, the details of recently identified ligands have been omitted for the benefit of portraying enduring information. For more detailed reviews include {Renauld, 2003 #189; Kotenko, 2004 #5}.

CD27L, CD30L, CD40L, receptor activator of NF-κB (RANKL), glucocorticoid-induced TNF related protein (GITRL) and TNF-related apoptosis inducing ligand receptor 1 (TRAIL) (Hehlgans et al., 2005). Receptors of TNF/TNFR family are Type I Transmembrane proteins characterized by the presence of one or more cysteine-rich domains (CRD). These receptors bind either TRAF adapters or cytoplasmic death domains. There are currently in excess of 40 identified ligands of this family. Their effects span a wide variety of biological activities in immunity, inflammation, apoptosis, autoimmunity and organogenesis. Members of the TNF/TNFR superfamily currently represent important therapeutic targets in autoimmune disorders including Chrohns disease, IBD, osteoporosis and cancer.

1.1.4. IL-1/FGF family

The interleukin (IL)1-1/fibroblast growth factor (FGF) family includes FGF1-19 and IL-1α, IL-1β, IL-1RA, IL-18 and the more recently identified IL1F5-9. The IL-1/FGF family members share a common β-barrel structure consisting of 12 β-strands, however the two different factions of this family, the ILs and FGFs, share no significant sequence homology. Further the FGF receptors initiate tyrosine kinase activation leading to cell proliferation, which is important in growth and repair mechanisms. The diversity in biological function of the ligands in this family reflects distinct differences in the intracellular regions of their respective receptors.

The IL-1 family of cytokines are a group of highly pro-inflammatory molecules that are known to alter the host response to infectious or inflammatory challenges. The most notorius members of this family are IL-1α/β and IL-18. The functions of these two cytokines are highly regulated by the release of endogenous antagonists such as the IL-18 binding protein, and soluble receptors including the Type 2 IL-1 receptor and soluble ST2. The IL-1 cytokines signal via IL-1-like receptors a family of Toll domain-containing receptors belonging to a superfamily of receptors that also includes the Toll-like receptors (TLRs). The IL-1 receptors are able to recruit two serine/threonine protein kinases, the interleukin-1 receptor-associated kinases, through motifs shared with the TLRs. These stimulate intracellular signaling pathways, leading to the activation of transcription factors NF-κB and AP-1. By activation of gene transcription through their respective receptors, IL-1 and IL-18 stimulate the production of cytokines, chemokines and adhesion molecules that aid leukocyte recruitment to sites of infection in the initiation of an inflammatory response. Several of the IL-1 receptor family are as yet orphan receptors, and the receptors for ligands including IL1F5,-7, -8, -9 of the IL-1 cytokine family have only recently been identified thus the full impact of this group of structurally related proteins in the defence against pathogen invasion is not known but is likely to be immense.

1.1.5. The chemokine family

The chemokines, derived from their activity as '*chemo*tactic cyto*kines*', are a family of small, 8–14kDa, structurally related secreted peptides that stimulate migration

and activation primarily of leukocytes. In addition to their function as regulators of leukocyte recruitment, chemokines have a diverse range of biological functions in processes of haematopoiesis, lymphocyte development and trafficking, embryogenesis, angiogenesis, tumor growth and metastasis (Baggiolini, 2001; Rossi et al., 2000). Chemokines are sub-classified according to the positioning of conserved cysteine (C) residues in their N-terminal region into families CXC, CC, CX_3C and C (2003; Zlotnik et al., 2000). Chemokines mediate their effects via binding to a family of G protein coupled receptors (GPCRs), the chemokine receptors, which are named by the subfamily of chemokines that they bind with the suffix R as CXCR, CCR, CX_3CR and CR (Murphy, 2002). Receptor-binding is determined by the N-terminal region of chemokines, which contains the conserved cysteine motifs. Some chemokine receptor ligands are better known by their original names, as opposed to their more recent standardized nomenclature, which will be used here (2003; Zlotnik et al., 2000). In excess of 50 human chemokines have been described and homologous murine ligands have been identified for each. The cellular distribution and respective ligands for each of the chemokine receptors has been reviewed extensively elsewhere and thus will not be detailed here (Baggiolini, 2001; Elsner et al., 2004; Luster, 1998).

1.2. Cytokine Physiology

Although the function of cytokines are often listed separately, it is important to remember that cytokines often act in sequence, in concert or in conflict in a given immune response; e.g. IL-1 can induce the secretion of IL-2, while IL-2, IL-4, and IL-6 can synergize in generating cytotoxic T lymphocytes. Also IL-4 and IFN-γ can counteract each other's effects in the induction of MHC II expression on B cells and the induction of IgE production.

Many of the cytokine receptors are formed of subunit chains common to one or more other receptors. This frequently gives rise to simultaneous orchestration of several different downstream events depending on the particular subunit interaction. Taking the IL-2 receptor as an example, three formations of receptor expression have been observed. Consisting α, β and γ subunits or just a pair or single subunit chain, each giving rise to a different level of affinity for IL-2. The γ chain of the IL-2 receptor has been found to be common to receptor complexes for IL-4, IL-7, IL-9 and IL-15. Further regulation of the ligand for IL-2 receptor is achieved in vivo by production of soluble IL-2Rα. Another example is the considerable redundancy in the function of IL-4 and IL-13 which is almost certainly due to their shared ability to bind IL-13RA1. IL-4R can complex with γc to make a Type I receptor or with IL-13RA1 to make a Type II receptor. The former is a receptor specific for IL-4 while the latter complex can bind either IL-4 or IL-13. However, IL-13 can also bind with higher affinity to IL-13RA2, a decoy receptor that does not signal and has some functions that are distinct from IL-4 (Grunig et al., 1998; Kelly-Welch et al., 2003). Likewise the haematopoietic cytokines IL-3, IL-5 and GM-CSF, share an identical β chain in their respective receptors IL-3, IL-5 and CSFR2, and they

have highly overlapping functions in promoting eosinophilic inflammation (Robb et al., 1995). The absence or antagonism of a chemokine receptor frequently leads to a reciprocal increase in concentrations of its ligand, for example CCL2 in the BAL is raised following blockade or deletion of CCR2 (Maus et al., 2005).

1.3. Cytokine Production Following Fungal Stimulation

An inflammatory response involving cytokine and chemokine release, is initiated by the recognition of antigen or allergen. Indeed soluble Aspergillus antigens can directly stimulate cytokine and chemokine production by many cells. *A. fumigatus* antigens stimulate the release of IL-8/CXCL8 by human neutrophils (Braedel et al., 2004). *A. fumigatus* antigens stimulate production of IL-12 and TNF-α by monocyte-derived human immature dendritic cells (iDCs) inducing their maturation (Braedel et al., 2004). *A. fumigatus* stimulation also induces production of TNF-α, IL-1, IL-10 and chemokines, CCL3, CCL4 and CCL2, by murine macrophages (Geske et al., 2002; Pylkkanen et al., 2004; Taramelli et al., 1996), and of IL-12 and IL-6 by bone marrow-derived murine DCs (Braedel et al., 2004). Activation of both these murine cell types by *A. fumigatus* is mediated via TLR2 and TLR4 although only TLR2 is essential for activation by cellular extracts of *A. fumigatus* as opposed to culture supernatant (Braedel et al., 2004; Meier et al., 2003). Similarly, TLR2 and the TLR signaling adaptor protein MyD88 but not TLR4 are required for production of TNF-α by murine macrophages subjected to an alternative fungal stimulus, *Cryptococcus neoformans* (*C. Neoformans*) (Biondo et al., 2005). Comparison of several Aspergillus species found that *A. fumigatus* and *A. flavus* were more potent stimulants of macrophage-cytokine secretion than *A. terreus* and *A. niger* (Taramelli et al., 1996). This aggressive response to *A. fumigatus* seems surprising given that *A. fumigatus* is the major *Aspergillus* species causing invasive disease, and its ability to aggravate asthmatic responses despite enhanced induction of typically anti-inflammatory cytokines. Many tissue cells are capable of substantial chemokine production, including endothelial, epithelial, smooth muscle, mast cells and fibroblasts. It is currently unknown whether fibroblasts can be directly stimulated by *A. fumigatus* but fibroblasts are capable of producing a number of chemokines including CXCL1 and this may be upregulated by persistence of fungal material in the lung environment (Armstrong et al., 2004). *A. fumigatus* can also directly stimulate cytokine production, including IL-6, IL-8/CXCL8 and CCL2, by respiratory epithelial cells (Borger et al., 1999; Tomee et al., 1997).

1.4. Cytokines in Immunity to Fungal Disease

Upon infection with A. fumigatus the innate immune response is characterized by IFN-γ and IL-12 production with a robust cellular response. This is later superceded by a Th2-associated response that is most likely responsible for much of the immunopathology seen in chronic allergic disease. An adaptive immune response is required for the development of allergic disease. For an adaptive immune response

to fungal antigens to develop DC's must undergo a maturation step to become competent to activate naïve Th cells. Maturation can be induced by local IFN-α, TNF-α and CD40 ligand(L) or by pathogen-derived events such as initiation of TLR signaling. The matured DCs become professional antigen presenting cells (APCs) and can now activate T cells. Following activation by APCs, $CD4^+$ T helper cells (Th) differentiate into effector cells specialized in cytokine production and function.

It is well established that there are two subsets of T helper lymphocytes and that the predominance of one or other subtype shapes the immune response. As these subsets share the same profile of receptor expression it was a very important finding that the two subsets could be distinguished (and defined by) their repertoire of cytokine production upon activation. Th1 cells are defined by their production of IL-2 and IFN-γ, cytokines important in cell-mediated immunity, and are usually thought of as major effectors of the adaptive immune response to infectious pathogens. Th2 cells are defined by secretion of IL-4, IL-5 and IL-13, they also secrete IL-6 and are efficient at providing B cell help and driving immunoglobulin production. Th2 cells are also effectors of the adaptive immune response but are associated with allergic responses to antigen.

In general, Th1-type responses offer protection against development of invasive fungal disease while Th2 cells are associated with disease progression. T cells from healthy individuals produce TNF-α and IFN-γ upon stimulation with A. fumigatus antigens (Hebart et al., 2002). IFN-γ production induces a Th1 response, which favours resistance to fungal disease. IFN-γ augments the synthesis of anti-microbial peptides in macrophages and neutrophils by regulating the expression of NAPDH oxidase components required for biocidal activities such as respiratory burst (Cassatella et al., 1990). In contrast IL-10 inhibits this process in human neutrophils (Chaves et al., 1996). Production of IL-12 by macrophages and DCs occurs early and is an important determinant of a successful Th1-type response. Production of IFN-γ and IL-12 by $CD4^+$ interstitial T lymphocytes is associated with resistance to invasive fungal disease upon *A. fumigatus* infection in the lung; whilst IL-4 and IL-10 production by $CD4^+$ T lymphocytes are correlated with morbidity. The theory that the Th1/Th2 balance determines survival is further supported in mice where administration of IL-4 or IL-10 antagonists is protective, while mice treated with antibodies to IL-12, TNF-α or IFN-γ succumb rapidly to disease (Mehrad et al., 1999b).

1.4.1. Regulation of the inflammatory response

Cytokines are an important descriptor of another subset of T lymphocytes, regulatory T cells (Treg). Several Treg populations have been described. Since definitive markers have proved elusive Tregs are defined by their cytokine secretion including IL-10- or TGF-β-secreting Tregs, and their suppressive function. The magnitude of Treg function is thought to be an important determinant of the susceptibility to development of allergic responses. It is not yet clear whether Tregs are cause or effector of allergic inflammation but therapy to enhance their suppressive activities

may prove useful in fungal allergy. One caveat however, is that a direct link has been described between Treg proliferation and TLR2 activation in vivo.

1.4.2. Fungal recognition

Activation of T lymphocytes requires antigen presentation by APCs, cytokine production and the presence of co-stimulatory molecules. TLR on APCs initiate the signalling cascades that lead to production of co-stimulatory molecules, such as CD80 and CD86, and cytokines, such as IL-1β and IL-6, by activation of genes with NFκB binding sites (Visintin et al., 2001). The innate immune system is thus able to differentially instruct lymphocytes directing the adaptive immune response by coordinating expression of co-stimulatory molecules, depending on the type of pathogen insult encountered (Hoffmann et al., 1999). The adaptive immune response can be further directed by differential cytokine production following fungal recognition.

Dendritic cells (DCs), regarded as the most professional APCs, are able to discriminate between infections of aspergillus conidia versus hyphae by differential cytokine production (Bozza et al., 2002b). Phagocytosis of conidia induces production of TNF-α and IL-12, while phagocytosis of hypae induces TNF-α, IL-4 and IL-10 production (Bozza et al., 2002b). The different cytokine production profile by DC upon exposure to conidia or hyphae may reflect selective involvement of distinct recognition receptors e.g. TLR2 vs. TLR4 or TREM-1 (Carpenter et al., 2005a; Meier et al., 2003). Expression of both TLR2 and TLR4 is required for optimal activation of murine macrophages. In murine macrophages TNF-α, IL-6 and NO are produced mainly in a TLR4-dependent manner, while production of the chemokine MIP-2 was more dependent on TLR2 expression.

2. CYTOKINE RESPONSES IN THE LUNG ENVIRONMENT

2.1. Fungal Infections of the Respiratory System

Cytokine and chemokine responses are known to be involved in the pathogenesis of multiple fungal associated human diseases. These include invasive pulmonary aspergillosis (IPA), aspergilloma, allergic aspergillus sinusitis and hypersensitivity diseases. Further, IPA is a frequent opportunistic infection in patients receiving allogeneic stem cell transplantation (SCT) with an incidence of nearly 10%. Cytokine and chemokine responses are expected to have the greatest impact in diseases of an allergic nature. Their role in fungal asthma and allergic bronchopulmonary aspergillosis (ABPA) is of particular interest. Hypersensitivity pneumonitis is a less common allergic condition resulting from sensitization to *A. fumigatus* antigens. In contrast to fungal asthma and ABPA, IgE and eosinophilia are not prominent features in this allergic condition, which results from a Th1-polarised immune response to fungal exposure involving neutrophilic influx in the acute phases and T cells and macrophages in the chronic condition. Th1-type cytokines are expected to be central to the immunopathology occurring in hypersensitivity

pneumonitis but little research has focused on the mechanics of this lesser known allergic condition (Fink et al., 2005). This review will focus primarily on the role of cytokines and chemokines in the diseases in which their function has been more thoroughly investigated, IPA and ABPA.

Aspergillus pneumonia and systemic aspergillosis, i.e. IPA are not allergic conditions and occur primarily in immuno-compromised patients experiencing defective T cell or phagocyte function. These conditions are also complications often encountered by individuals with cystic fibrosis. In contrast, relatively normal individuals may present with allergic responses to fungus such as *A. fumigatus*. This relationship is paralleled in animal models. Animals with a significant impairment in phagocyte/neutrophil function succumb to a growth of fungus akin to invasive aspergillosis while mice with a full complement of normal immune responses are protected from invasive disease in these models (Hogaboam et al., 2003; Mehrad et al., 1999b). Normal mice can however develop significant and lasting allergic responses to the same fungal material (Hogaboam et al., 2000; Kurup et al., 1992). *A. fumigatus*-induced allergic airways disease models have been developed for pre-clinical research that provide insight upon the mechanics of fungal asthma and ABPA (Hogaboam et al., 2000; Kurup et al., 1994). Other models using alternative fungal species e.g. Alternaria or Cladosporium have been developed but a more thorough discussion of the role of cytokines in these disease processes can be achieved by considering the research carried out with *A. fumigatus* models (Havaux et al., 2005).

2.2. Invasive Pulmonary Aspergillosis (IPA)

IPA is characterized by hyphal invasion and destruction of pulmonary tissue resulting in devastating reduction in function. IPA is common amongst severely immunosuppressed individuals, due either to disease, (e.g. AIDS), or disease treatment (e.g. transplant recipients). Neutropenia, intensive immunosuppression and graft-versus-host disease (GVHD) are prominent risk factors for development of IPA (Latge, 1999; Shaukat et al., 2005). Aspergillus species are the most common type of invasive infection in allogeneic bone marrow transplant recipients (Marr et al., 2002). In these immunocompromised patients IPA is the most common presentation of infection with aspergillus fumigatus and associated with a morbidity of 35–60%. The treatment of invasive fungal infections is frequently unrewarding with mortality rates at 30–50% of patients with invasive aspergillosis or zygomycosis and 80–100% for those with disseminated fusariosis, invasive trichosporonosis or infection with *Scedosporium prolificans* (Hope et al., 2004; Walsh et al., 2004). Prophylactic antifungal therapy in SCT patients is also relatively ineffective. Current research is directed at the development of novel therapeutics but this will be greatly aided by basic research to better understand the pathophysiology of invasive fungal disease and the parameters affecting disease susceptibility. It is possible that the latter is dictated by their specific cytokine responses when facing a conidial challenge.

2.2.1. *Cellular responses to fungal invasion*

A neutrophil deficit or decrease in their functionality is known to be a major predictor of aspergillus fumigatus germination and growth within the lung compartment (Latge, 1999). There are two lines of defence against aspergillus in the lung. The foremost is phagocytosis of conidia by resident pulmonary macrophages. Inside the phagolysosome germination is inhibited and macrophages are extremely efficient at killing engulfed conidia. However, if Aspergillus conidia escape phagocytosis the second defence mechanism is enacted by recruited neutrophils, which can kill the invasive hyphae by oxidative mechanisms. Both mechanisms are important in the prevention of IPA. Recent evidence points to a significant contribution also of natural killer (NK), Th1 lymphocytes and dendritic cell recruitment and or activation in the defence against fungal growth (Bozza et al., 2002a; Bozza et al., 2002b; Bozza et al., 2003; Morrison et al., 2003). In each case cytokines or chemokines have been found to be key orchestrators of the cellular response. NK cells kill infected target cells by inducing apoptosis of the target cell and/or by releasing molecules such as perforin, which damage the cell membrane, leading to cell death. NK cells can produce substantial amounts of TNF-α, which may be involved in this process.

Acquired immunity against A. fumigatus has also been demonstrated. Significant protection against invasive disease was achieved by adoptive transfer of splenocytes from sensitized animals in a murine model of invasive fungal infection (de Repentigny et al., 1993). The authors concluded that the adaptive immune response was mediated by splenic macrophages. The predominating immune cell in the lung environment is the macrophage. Macrophages are an important source of many cytokines. M-CSF is known to enhance proliferation and differentiation of monocyte progenitors (Nemunaitis, 1998). M-CSF treatment increased anti-fungal activity of monocyte/macrophages in vitro and improved survival, while reducing fungal load in several animal models of invasive fungal infection (Gonzalez et al., 2001; Roilides et al., 2000). Vascular endothelial growth factor(VEGF) promotes myelopoiesis upstream of GM-CSF to enhance accumulation of myeloid cells in the bone marrow (Larrivee et al., 2005).

2.2.2. *Th1/Th2 cytokine balance in resistance to* A. fumigatus *infection*

Since IFN-γ and IL-10 act in opposition during an inflammatory response, a high IFN-γ:IL-10 production ratio by peripheral blood T cells is positively associated with response to antifungal therapy in early-stages of IPA (Hebart et al., 2002). The pro-inflammatory Th1-type cytokines IFN-γ, IL-12 and TNF-α work individually and in concert to enhance clearance of fungal material. In vitro TNF-α increases the capacity of neutrophils to damage *A. fumigatus* hyphae and enhances macrophage conidial phagocytosis. Both genetic deletion and antibody neutralization of IL-10 have been shown to reduce susceptibility of C57BL/6 mice to infection with *A. fumigatus* and *C. albicans*. The reduced fungal burden and increased survival of IL-10-deficient mice was attributed to an increased production of type 1 cytokines including IFN-γ, TNF-α and IL-12, and associated with increased NO production

(Del Sero et al., 1999). Conversely, deletion of TLR2 substantially reduced the type 1 cytokine response in the lungs with reduced IFN-γ, TNF-α and IL-12 production. The TLR2$^{-/-}$ mice had increased fungal burden, respiratory distress and their survival was reduced compared to wild type animals further supporting the importance of a type 1 cytokine response in resistance to fungal infection (Balloy et al., 2005b). Transient overexpression of IFN-γ augmented fungal clearance in a model of IPA (Shao et al., 2005a). Adoptive transfer of DCs transfected with an IL-12 adenovirus vector into *Aspergillus* infected mice augmented IFN-γ production to enhance fungal clearance (Shao et al., 2005b). Gliotoxin inhibits NFκB, a major cytokine transcription factor, thus inhibiting production of many pro-inflammatory cytokines including IFN-γ, IL-12 and TNF-α (Pahl et al., 1996). A series of genetic deletion experiments has shown that IL-4 suppresses protective Th1-type responses to *candida* infection while IL-6 and IL-12 are required for survival. The role of IL-10 in susceptibility to fungal infection post-transplantation is more complex, with a detrimental effect in the presence of a full complement of Th1 cytokines but a protective role in the absence of IL-12(Romani, 2004). Treatment of bone marrow transplant-recipient mice with sIL-4R or anti-IL-10 antibody increased their survival when subjected to a *candida albicans* infection (Mencacci et al., 2001). These experiments demonstrated that an altered Th-cytokine response following SCT was responsible for susceptibility to fungal infection and proof of concept for therapeutic blockade of Th2-type cytokines to prevent invasive fungal infection following SCT. Evidence exists that IL-10 is associated with an increased risk of fungal infection in humans as an ACC polymorphism in the promoter region of the IL-10 gene, which is thought to decrease IL-10 production confers protection against IPA in bone marrow-recipients (Seo et al., 2005).

2.2.3. Chemokines are intricately involved in the pathogenesis of IPA

Th2 cytokines can drive the production of CCL17, which is known to enhance Th2-driven diseases such as asthma and atopic dermatitis. Mice deficient of CCR4, the receptor for CCL17, were significantly protected from mortality in a neutropenic model of IPA (Carpenter et al., 2005b). The increased survival of CCR4$^{-/-}$ mice was associated with increases in IL-12 and CCL2 production in the lung.

Levels of chemokines CCL17 and CCL22 are significantly upregulated in the lungs and BAL fluid of mice in the first few days following infection with *A. fumigatus* conidia. Neutralization of CCL17 upregulated the level of CCL2 in the lung of mice and this was associated with increased survival in a model of IPA (Carpenter et al., 2005b). Conversely, neutralization of CCL2 resulted in reduced NK cell recruitment to the lung, increased fungal burden and doubled mortality in a murine model of IPA (Morrison et al., 2003). Together these experiments demonstrate that CCL2 is required for effective defence against *A. fumigatus* in immunocompromised mice. Using adoptive transfer of cells from CCR2$^{-/-}$ mice it has also been shown that CCR2, the receptor for CCL2 is required for the effective recruitment of NK cells into the lung compartment during the course of *A. fumigatus* infection.

The requirement for neutrophils to prevent invasive fungal disease was efficiently demonstrated by blockade of CXCR2, an important receptor for neutrophil chemotaxis. In both humans and mice the ELR^+ subset of CXC chemokines are known to be critical for neutrophil recruitment. Following intratracheal challenge with A. fumigatus conidia in mice two ELR^+ chemokines MIP-2 and KC (homologous to hCXCL1-3), were found to be produced in substantial amounts in the lung. All the murine ELR^+ chemokines are able to bind CXCR2 a receptor known to be important in neutrophil recruitment. Antibody neutralization of CXCR2 activity in a murine model of aspergillosis severely ablated the recruitment of neutrophils to the lung compartment. This resulted in an invasive disease in control animals similar to that of neutrophil-depleted animals, when each group was given an intracheal bolus of A. fumigatus conidia (Mehrad et al., 1999a). Furthermore transient expression of the CXCR ligand KC in the lung of mice experiencing IPA significantly improved their survival (Mehrad et al., 2002). The human CXCR2 is highly homologous to the mouse CXCR2 receptor thus this may prove a useful therapeutic target in treatment of defective neutrophil trafficking in humans.

2.2.4. Haematopoietic cytokines enhance neutrophil function to aid defence against fungal infection

Invasive aspergillosis is common in neutropenic individuals due to a deficit in functional phagocytic cells at the site of infection. This deficit could be secondary to a lack of production of granulocyte-colony stimulating factor(G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF) and/or macrophage-colony stimulating factor (M-CSF) (Roilides et al., 2001). Production and activation of mature phagocytes from haematopoietic progenitors is stimulated by GM-CSF, G-CSF and M-CSF. G-CSF stimulates the proliferation and differentiation of myeloid progenitor cells to neutrophils. Further, G-CSF enhances the phagocytic activity of neutrophils stimulated with *Candida, Aspergillus* and *Fusarium* species in vitro and increases the ex vivo fungicidal activity of neutrophils isolated from transplant patients (Gaviria et al., 1999; Natarajan et al., 1997; Pursell et al., 2003; Roilides et al., 1993). However G-CSF also has immunomodulatory function through receptors expressed on T cells and monocytes (Boneberg et al., 2000; Franzke et al., 2003). G-CSF treatment can suppress production of pro-inflammatory cytokines including IFN-γ and TNF-α, while also increasing IL-10 production (Valente et al., 2002). These suppressive immune functions may counteract the beneficial effects of G-CSF of increasing neutrophil fungicidal activities.

Both GM-CSF and G-CSF upregulate expression of TLR2, an important pattern recognition receptor (PRR) for fungi, and membrane CD14 expression on neutrophils. GM-CSF is a better candidate for immunotherapy as GM-CSF also enhanced the IL-8/CXCL8 secretion and superoxide production by neutrophils following stimulation with TLR2 ligands (Kurt-Jones et al., 2002). Furthermore, GM-CSF increases expression of Dectin-1 in murine macrophages (Willment et al., 2003). Dectin-1 is a major receptor for β-glucans of the fungal cell wall. Treatment with GM-CSF in vitro upregulates chemotaxis, LTB_4 sysnthesis, arichidonic acid

release, superoxide release and phagocytosis of fungal organisms by neutrophils. Therefore GM-CSF is able to substantially enhance the ability of neutrophils and macrophages to recognize and respond to fungal pathogens.

GM-CSF also accelerates haemopoiesis, in combination with erythropoietin and IL-3 to increase production and numbers in circulation of monocytes and granulocytes; and inhibits neutrophil apoptosis(Armitage, 1998; Epling-Burnette et al., 2001). Evidence of a role of GM-CSF, like G-CSF, in polarizing the immune response toward a Th1 or a Th2-type is currently unclear.

IFN-γ and GM-CSF augment the fungicidal activities of neutrophils in vitro towards selected fungal pathogens(Gil-Lamaignere et al., 2005; Roilides et al., 1993). Both cytokines increase the production of superoxide anions by neutrophils, though interestingly the extent of O_2^- production stimulated differed between the cytokines for different fungal stimuli. The antifungal phagocytic activity of neutrophils can also be increased by opsonins such as IgG, the release of which is upregulated by Th2-type cytokines. Treatment with recombinant GM-CSF in vivo proved beneficial in murine models of candidiasis, aspergillosis and histoplasmosis, and protected against fungal infection-induced mortality in a pilot study in leukaemia patients undergoing chemotherapy (Armitage, 1998).

A summary of the immune mechanisms dictating resistance versus susceptibility to IPA is illustrated in Figure 1.

2.3. ABPA

The greatest amount of data regarding the involvement of cytokines in fungal allergic disease in humans relates to ABPA. *Aspergillus fumigatus (A. fumigatus)* sensitization is common among asthmatics, atopics and cystic fibrosis patients. Inhalation of *A. fumigatus* spores triggers IgE-mediated inflammatory responses in the airways of allergic asthmatics, which may cause fatal obstruction of airflow (Denning et al., 2006).

ABPA in humans is associated with peripheral blood eosinophilia and a strong eosinophilic inflammatory response, which is accompanied by elevated total circulating IgE and *A. fumigatus*-specific IgE and IgG. An increased serum level of the soluble IL-2 receptor (sIL-2R) is also a characteristic feature of ABPA in humans. Raised sIL-2R is a crude indicator of the increased T cell activation occurring in ABPA and is enhanced even further during asthmatic exacerbations as compared to patients with remissive status (Brown et al., 1995). This increased T cell activation is indicative of the importance of T cell cytokines. These features together with a reduction in lung function and a marked remodeling pathology in ABPA patients mirror that of asthmatics but with the addition of pulmonary fungal colonization providing a continual antigen assault. Thus, ABPA patients usually present with advanced allergic airways disease. The immunopathology of ABPA follows that of allergic asthma with both diseases having early- and late-phase allergic responses featuring a strong Th2-type immunological response. Since both are frequently described as Th2-driven allergic conditions, Th2 cytokines

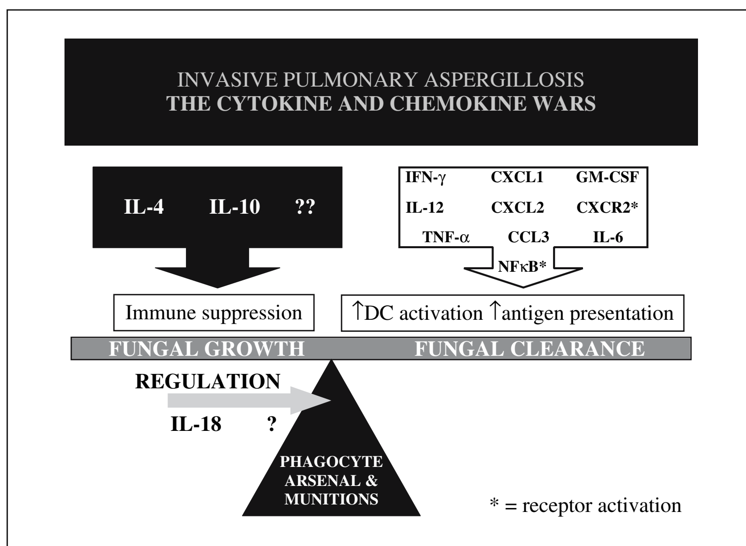

Figure 1. The balance between destruction of fungal conidia or spores and fungal colonization in the lung is dominated by Th1 cytokines and chemokines that promote fungal clearance by phagocytes (right). In healthy individuals Th1-type cytokines and chemokines drive innate immune mechanisms to ensure clearance of fungal material. A deficit in neutrophil or macrophage function allows the balance to swing in favour of fungal growth while the suppressive actions of Th2-type cytokines upon pro-inflammatory cytokines IFN-γ and IL-12 exacerbate the situation (left)

IL-4, IL-5 and IL-13 are expected to be central to the disease processes. ABPA occurs in 1–15% of CF patients and 1–2% of asthmatics. However, *A. fumigatus* allergy is much more prevalent than clinically defined ABPA. Since 10–25% of asthmatics are *A. fumigatus* allergic, as measured by skin-prick sensitivity, *A. fumigatus* exposure is likely to be a common exacerbating factor in allergic asthmatics.

Initially, production of IL-4, IL-5 and GM-CSF by infiltrating Th2 cells and the corollary eosinophilia and elevated IgE were thought to be major drivers of *A. fumigatus*-induced airway inflammation (Chu et al., 1996). However, although IgE and eosinophilia are constant features of allergy modeled in mice, neither is essential for initiation of *A. fumigatus*-allergic asthma (Corry et al., 1998; Grunig et al., 2003; Sandhu et al., 1978). It may be their role can only be appreciated when effects of manipulation can be studied over extended periods of time.

Th2-type allergic inflammation is characterized by the increased presence of eosinophils, lymphocytes and mast cells at the inflammatory site. Knowledge of the effects of IL-5 on eosinophils combined with evidence of both correlation of disease severity with the number and activation state of eosinophils, and elevated IL-5 levels in the broncho-alveolar lavage (BAL) and lung tissue of asthmatics highlighted IL-5 as a potent target for development of allergic disease therapy

(Bentley et al., 1993; Kay, 1991; Sur et al., 1995). Specifically in animals exposed to *A. fumigatus*, IL-5 is critical in eliciting blood, bone marrow and lung eosinophilia (Kurup et al., 1997; Murali et al., 1993). However, experiments using IL-5-deficient (IL-$5^{-/-}$) animals or treatment with anti-IL-5 neutralizing antibodies relinquished both IL-5 and eosinophils from responsibility for the allergic response to soluble *A. fumigatus* antigens. In each study, the hallmarks of the asthma phenotype were as apparent as in control mice, including AHR, goblet cell hyperplasia, mucous production and inflammatory cell influx. Eosinophils were indeed absent from the lungs but the magnitude and spectrum of inflammation appeared to be met by increased numbers of infiltrating mononuclear cells (Corry et al., 1998). IL-4 is another eosinophil-active Th2-type cytokine that has been examined in the same acute model of *A. fumigatus* allergy. Anti-IL-4 antibodies had only a moderate effect to reduce eosinophilia but abolished airways hyperreactivity, further separating the appearance of eosinophilia from the cause of AHR (Corry et al., 1998). However it remains possible that the role of eosinophils was masked in these relatively short-term experiments.

It is now appreciated that T cells, alveolar macrophages and the cytokines and chemokines produced by them orchestrate the inflammatory response. IL-13 and IL-4 have also been established as two of the key cytokine players in fungal asthma. Further research has focused upon separating the necessary components and mechanisms required for the airways hyperreactivity (AHR), peribronchial inflammation and airways remodeling characteristic of this allergic inflammatory condition.

2.3.1. Th2 cells

Atopic asthma is associated with a predominant $CD4^+$ Th2-type T lymphocyte population and Th2-type cytokine profile in the lung compartment (Robinson et al., 1992). The rapid and substantial production of cytokines and chemokines is a major feature of allergic responses. The Th2 cytokines, IL-4, IL-5, IL-9 and IL-13, are specifically involved in the recruitment and activation of Th2 lymphocytes, eosinophils and basophils and have been implicated in the development of allergic disease (Romagnani, 2002). IL-10, IL-12 and IFN-γ signaling are known to be important determinants of Th1/Th2 balance in immune responses (Chung, 2001). Asthmatic patients are known to have persistently elevated levels of Th2-type cytokines (Robinson et al., 1992). Atopy also correlates with IL-4-, IL-5-, IL-9- and IL-13-dominated *in vitro* responses of antigen-stimulated Th2 cells while IL-10, IFN-g and TNF-a responses were not above that of *in vitro* responses of non-atopics (Heaton et al., 2005). Stimulation with *A. fumigatus* antigens induces proliferation of $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ T cells, which correspond with the Th2 cell phenotype. Th2 cells are also an important source of IL-4, a critical cytokine in the development of allergic airways inflammation, which also has an autocrine proliferative effect upon Th2 cells (Knutsen et al., 1994). $CD4^+$ T cells are essential for the asthma phenotype in mice sensitized and challenged with *A. fumigatus* antigens. This was elegantly demonstrated with adoptive transfer of $CD4^+$ T cells into T and B cell deficient

RAG-knock-out mice (Corry et al., 1998). RAG-deficient mice were protected from development of airways hyperreactivity and associated lung inflammation. However, the protection was reversed with reconstitution of just $CD4^{+}$ T cells. Sensitized RAG-deficient mice that received $CD4^{+}$ T cells by adoptive transfer exhibited pronounced eosinophilic lung inflammation with all the hallmarks of ABPA following allergen challenge.

2.3.2. Th2 cytokines in fungal allergy

IL-3, IL-4, IL-5, IL-13 and GM-CSF are produced by cells at the site of inflammation but also have important functions in haematopoiesis. These cytokines circulate in the blood to the bone marrow where they co-ordinate, individually or in concert with chemokines such as CCL11, the maturation and mobilization of leukocyte and mast cell progenitors, ensuring the continued supply of leukocytes to the inflammatory site (Palframan et al., 1998; Romagnani, 2002). Release of IL-10 and IL-18 may have evolved to readjust the balance of pro-inflammatory cytokines and often act to attenuate inflammatory processes (Lynch et al., 2003). The vast quantity of research in the larger field of allergy has provided insight into the mechanisms of all allergic diseases. The cytokines IL-4, IL-5, IL-13, and chemokines, such as CCL2 and CCL3, have been identified as major players in fungal allergy but were first highlighted as prominent players in the pathophysiology of allergic responses to other allergens.

The primary cytokine requirements for asthma pathophysiology in fungal allergy are IL-4 and IL-13 (Blease et al., 2001a; Grunig et al., 1998; Kurup et al., 1999). Elevated pulmonary levels of IL-4 and IL-13 is a feature of clinical and experimental asthma (Hogaboam et al., 2000; Huang et al., 1995; Kotsimbos et al., 1996). However, IL-4 and IL-13 share a common receptor, which may explain the apparent redundancy of IL-4 in the development of allergic responses to *A. fumigatus* exposed by experiments with IL-4 deficient mice (Kurup et al., 1997).

IL-13 is a product of Th2 cells and alveolar macrophages and is known to induce immunoglobulin production, B cell proliferation and monocyte differentiation. IL-13 has been identified as a major mediator of airway hyperreactivity, and targeted expression of this cytokine in the lung induced substantial eosinophil and mononuclear cell recruitment, mucous overproduction and subepithelial fibrosis (Grunig et al., 1998; Zhu et al., 1999). IL-13 is critical for the induction of goblet cell hyperplasia in mice challenged with *A. fumigatus* conidia (Blease et al., 2001a; Blease et al., 2001b) or antigens (Grunig et al., 1998). Immunoneutralization of IL-13 in *A. fumigatus*-challenged mice reduced goblet cell hyperplasia as well as AHR and collagen deposition but was only effective when treatment occurred at 14–30 or 30–38 days after the intra-tracheal allergen challenge with live *A. fumigatus* conidia emphasizing the precise temporal role of this cytokine in the development of pathology (Blease et al., 2001b). Chronic over-expression of IL-13 in the mouse lung recapitulated many of the features of asthma, including mononuclear and eosinophil infiltration, fibrosis, goblet cell hyperplasia and thickening of the lamina propria, and airways hyperreactivity (Zhu et al., 1999). As eosinophils are largely cleared

from the lungs by day 14 the mechanism of IL-13-induced remodeling responses are unlikely to be mediated via eosinophils. The cytokine TGF-β was significantly reduced by anti-IL-13 treatment while whole lung and BAL levels of IFN-γ were elevated in mice treated with IL-13R targeted cytotoxin (IL-13PE) providing a plausible mechanism for the reduction in clinical symptoms seen with this treatment (Blease et al., 2001a; Blease et al., 2001b). Murine macrophage Dectin-1 expression is upregulated by IL-4, IL-13 and GM-CSF but can be downregulated by IL-10 (Willment et al., 2003). This profile of cytokine upregulation matches the conditions known to induce alternatively activated macrophages.

A model of fungal allergy which enables the study of immune responses following a live conidial challenge in sensitized mice has been used to elucidate the normal pattern of immune responses in the lung following challenge with a substantial tracheal challenge of conidia spores. Following exposure to Aspergillus fumigatus in sensitized mice there is an initial recruitment of neutrophils, eosinophils and lymphocytes to the interstitial space. Neutrophil numbers spike sharply with a peak recruitment at day 3 post-intracheal (i.t.) challenge. Eosinophil numbers are also greatest at day 3 but their presence gradually fades over the following 7–10 days. In contrast lymphocyte recruitment is delayed, becoming notable at around seven days post-i.t. but persisting for several weeks following allergen challenge (see Figure 2). Studies using this chronic fungal asthma model revealed distinct kinetics of the recruitment of leukocytes and ensuing inflammatory processes are achieved via precise temporal expression of cytokines and in particular chemokines (see Figure 3 and Table 2).

2.3.3. CC Chemokines in ABPA

Intra-pulmonary levels of chemokines CCL3 and CCL5 are both elevated following challenge of *A. fumigatus*-sensitized mice and are detected at high levels in the BAL fluid from asthmatics (Alam et al., 1996; Blease et al., 2000b). Both chemokines have been shown to exert major effects on the recruitment of eosinophils and their blockade *in vivo* reduces eosinophilia and moderately reduces AHR in acute models of allergic airways disease (Gonzalo et al., 1998; Lukacs et al., 1996;

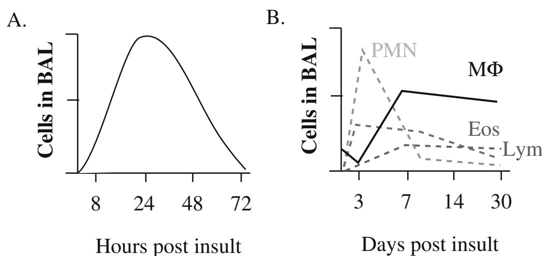

Figure 2. (A) The time course of total cell infiltration in the acute response to Aspergillus fumigatus occurring in the allergic airway. (B) Each leukocyte and macrophage population typically follows a distinct time course of recruitment during the chronic allergic response (See Color Section.)

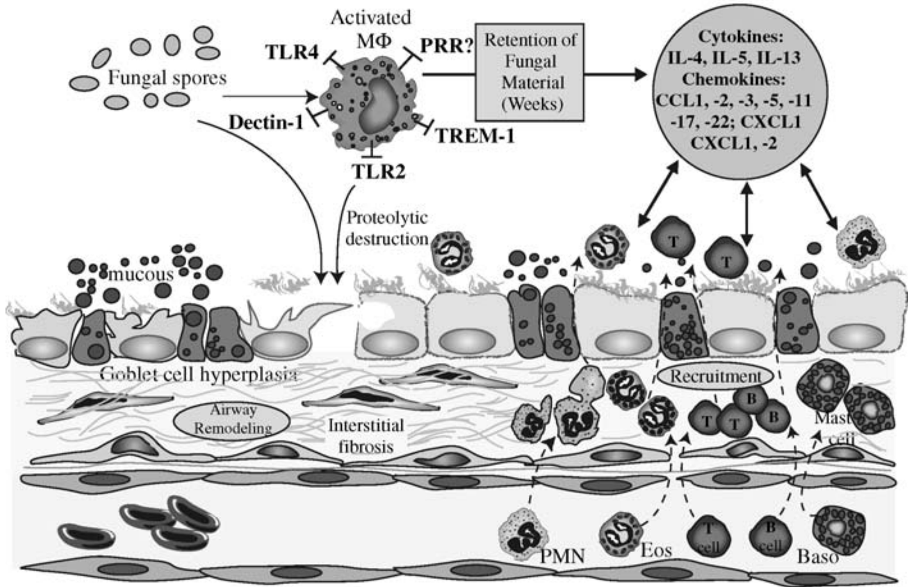

Figure 3. An outline of the chronic responses occurring in the allergic airway following challenge with *Aspergillus fumigatus*, featuring a central role of macrophage activation and cytokine and chemokine production (See Color Section.)

Table 2. Manipulation of Cytokine & Chemokine Targets and their Effect Upon the Major Parameters of Fungal Asthma and ABPA Assessed in Mice

Mouse Genotype	Antibody/ Treatment	Prominent Cytokine Response	Serum IgE	Cellular Recruitment	AHR	Remodeling
Wild Type	0	IL-4, IL-5, IL-13	+++	+++	+++	√
″ ″	Anti-IL-5	ND	+++	+++	+++	√
″ ″	Anti-IL-4	IFN-γ	0	++	0	√
″ ″	Anti-IL-13					↓
″ ″	IL-13-PE (targets IL-13R)	IFN-γ, IL-13	+++	++	+	↓
$RAG^{-/-}$ (B & T cell deficient)	0	0	0	+	0	0
″ ″	Adoptive transfer of WT $CD4^{+}$ T cells	IL-4, IL-5, IFN-γ	0	++++	++	√

IFN-γ$^{-/-}$	0	IL-4, IL-5	+++	+++	+++	
IL-4$^{-/-}$	0	IFN-γ			+++	√
IL-5$^{-/-}$	0	IFN-γ	++	+++	0	√
IL-13$^{-/-}$	0	IL-4, IL-5, IFN-γ	++	+++	+++	↓
″ ″	Anti-IL-5	IFN-γ	++	++	++	↓
″ ″	Anti-IL-4	IFN-γ	0	++	+	↓
IL-13$^{+/+}$	Not sensitized	IL-13, IL-4, IL-5	+++	+++	+++	√
IL-10$^{-/-}$	0	IL-4, IL-5, IFN-γ	+++	++++	+++	
B cell$^{-/-}$	0	IL-4, IL-5	0	+++	+++	√
″ ″	Anti-IL-5	ND	0	+++	+++	
″ ″	Anti-IL-4	ND	0	++	0	
IgE$^{-/-}$	0	IL-4, IL-5,	0	+++	+++	√
IgG$_1$$^{-/-}$	0	IL-4, IL-5, IFN-γ	++	+++	+++	√
Wild type	Anti-IL-18	ND	ND	++++	++++	
Eosinophil$^{-/-}$	0	IL-13, CCL6	ND	++++	+++	√
Wild type (CCL6$^{+/+}$)	*0*	*IL-4, IL-5, IL-13*	+++	+++	+++	
″ ″	Anti-CCL6	↓IL-13, ↓IL-10	+++	+	0	
Wild type (CCR1$^{+/+}$)	*0*	*IL-4, IL-5, IL-13*	++	+++	+++	√
″ ″	BX-471	CXCL2, CXCL15	++	+	+	↓
CCR1$^{-/-}$	0	IFN-γ	++	+	0	0
CCR2$^{-/-}$	0	IL-4, IL-5, IL-13	+++	++++	++++	↑
CCL2$^{-/-}$	0	IL-4, IL-5, IL-13, IFN-γ	+++	+++	+++	√
CCR4$^{-/-}$	0	Th2, CCL17, CCL22	+	++	++	√
CCR5$^{-/-}$	0	ND	+++	+		↓
Wild type	Anti-CCL5	ND	+++	+	+	↓
CXCR2$^{-/-}$	0	CXC9, CXC10	++++	+++	↑↓	0
CCL11$^{-/-}$	0		+++	++	+	√
STAT-6$^{-/-}$	0	↓ Th2 cytokines	0	++	+++	↓
″ ″	IL-13-PE		0	+	+	√

AHR = airways hyperreactivity; 0 = none; + = slight; ++ = mild; +++ = moderate; ++++ high; ND = not determined. $^{-/-}$ = gene knockout; $^{+/+}$ = wild-type; IL-13-PE = IL-13 & pseudomonas exotoxin, IL-13-PE38QQR; BX-471 = CCR1 antagonist; √ = extent of remodeling similar to wild-type

Lukacs et al., 1997). However genetic deletion of CCR1, a receptor shared by these two ligands, had little affect on AHR or eosinophil and neutrophil infiltration in the chronic fungal asthma model. In contrast, CCR1 deletion was found to substantially reduce evidence of remodeling at later time points in this model, which corresponded with reduced IL-4, CCL6, CCL11 and CCL22, and increased IFN-γ. Thus CCR1 was proposed as another promoter of Th2 cytokine responses. CCR1 likely mediates increased Th2-type cytokine production via recruitment of leukocytes including monocytes, lymphocytes, neutrophils and eosinophils, with recruitment of the latter being particularly auspicious in the instigation of pulmonary damage and repair mechanisms. Blockade of CCR1 with a receptor antagonist, BX-471, reduced airway inflammation, evidence of remodeling and AHR in the

fungal asthma model revealing a major role for CCR1 in the development of AHR in genetically intact mice (Carpenter, 2005).

Alveolar macrophages are the primary source of CCL6, yet another CCR1 ligand. Levels of this chemokine in BAL were found to be five-fold greater than other CC chemokines, including CCL3, following challenge of sensitized mice with soluble *A. fumigatus* antigen (Hogaboam et al., 1999). Immunoneutralization of CCL6 significantly reduced AHR, eosinophil and lymphocyte infiltration and levels of CCL11, CCL2, IL-10 and IL-13 in the BAL, while the number of macrophages in the BAL was increased. Therefore CCL6 appears to have a prominent role in recruitment of inflammatory cells and development of AHR, via the manipulation of the cytokine response. However CCL6 is probably not required for the initial sensitization to *A. fumigatus* antigens as neutralization of CCL6 did not affect IgE levels (Hogaboam et al., 1999). CCL6 production can be induced by IL-4 and IL-1 β but the mechanism downstream of CCL6, by which this chemokine modulates macrophage numbers and cytokine production, warrants further investigation. The contrasting effects of deletion of CCR1 on remodeling responses compared to the reduction of AHR following ligand neutralization or pharmacological antagonism demonstrates the lack of redundancy amongst the family of chemokines that use this receptor *in vivo*.

CCR2 knock-out mice exhibited an exaggerated asthma phenotype, with increases in AHR, perivascular and peribronchial inflammation, mucous overproduction and fibrosis (Blease et al., 2000a). This phenotype was attributed to enhanced levels of IL-5, IL-13, CCL11 and CCL5, while decreases in CXCL1 and the lack of receptor for CCL2 would explain the reduction in neutrophil and monocyte infiltration of the lung. The lack of neutrophils and monocytes allows the persistence of fungal conidia within the lung consequently prolonging the allergic response. CCR2 is activated by several ligands, including CCL2, CCL7, CCL8, CCL12 and CCL13 all of which are found in clinical lung samples and may be responsible for CCR2 mediated modulation of allergic responses (Taub, 1996). Further experiments uncovered a dichotomous role of the CCR2 ligand, CCL2. Immunoneutralization during the first two weeks following conidial challenge in *A. fumigatus*-sensitized mice, enhanced AHR, eosinophilia, peribronchial fibrosis and goblet cell hyperplasia. In contrast, treatment with anti-CCL2 antibody during days 14–30 post-allergen challenge attenuated allergic inflammatory responses (Blease et al., 2001c). The reduced fungal clearance observed in the earlier treatment group was again attributed to a reduction in phagocytes, as CCL2 is a potent chemoattractant. In a more recent study, CCR2 and CCL2 deletion each had little effect upon the magnitude of allergic responses seen at day 4 post-challenge with soluble *A. fumigatus* antigens further underlining the complexity of the contribution of CCR2 and CCL2 (Koth et al., 2004). Interesingly CCR2 is also expressed on fibrocytes and can mediate their recruitment to the lung. Thus CCR2 may have a crucial role in the fibrotic response to fungal allergens (Moore et al., 2005).

Genetic deletion of CCL11, a major eosinophil chemokine, reduced AHR and eosinophil numbers in the acute model but without affecting T cell recruitment

or lung inflammation (Schuh et al., 2002b). CCL11 deletion also failed to affect disease parameters in the chronic allergic airways disease model suggesting that CCL11 has a role in the initiation of allergic airway disease but little input in maintenance of established allergic inflammation (Schuh et al., 2002b).

2.3.4. *CXC Chemokines and ABPA*

IL-8/CXCL8 protein and mRNA are elevated in induced sputum samples from ABPA patients compared to other asthmatics and normal controls. IL-8/CXCL8 levels in sputum also correlated with the degree of sputum neutrophilia and airway obstruction as measured by FEV_1 (Gibson et al., 2003). CXCR2 is a receptor for neutrophil chemoattractants including IL-8/CXCL8 and CXCL5. CXCR2-deficient ($CXCR2^{-/-}$) mice failed to develop histological features of *A. fumigatus*-induced allergic airways disease and resulting airways remodeling in the chronic model of fungal asthma (Schuh et al., 2002a). Wild-type mice failed to clear fungal material from the lung compartment allowing inflammatory responses to persist. In $CXCR2^{-/-}$ mice fungal material was eliminated from the lungs and pulmonary infiltration of eosinophils and lymphocytes was absent. However, neutrophil recruitment remained intact in the sensitized $CXCR2^{-/-}$ mice and was found to be dependent on CXCL10 and CXCL9 in the absence of CXCR2. Monocytic inflammation although present was diffuse compared to the peribronchial localization of leukocytes in the lungs of wild-type controls. Significantly lower levels of IL-4 and IL-5 and elevated IFN-γ, CXCL9 and CXCL10 in the lungs of $CXCR2^{-/-}$ mice suggested that a shift towards a Th1-type cytokine environment, which facilitated the clearance of fungal material by neutrophils and alveolar macrophages in the absence of CXCR2 (Schuh et al., 2002a). Although there was an initial AHR at days 3 and 7 post-allergen challenge, and pulmonary inflammation evident at all time-points in $CXCR2^{-/-}$ mice, the late phase response with eosinophil and lymphocyte infiltration was substantially reduced compared to wild-type controls. The deletion of CXCR2 conferred protection against the damaging remodeling of the airways in response to *A. fumigatus* challenge. At day 37 post-allergen challenge, there was still no evidence of peribronchial inflammation, fungal growth or remodeling of the airways in the $CXCR2^{-/-}$ mice (Schuh et al., 2002a). Together these results suggested that CXCR2 is required for the development and maintenance of the characteristic pulmonary features of chronic fungal asthma.

Lung concentrations of CC chemokines CCL3, CCL2, CCL5, CCL6, CCL11, CCL17, and CXC chemokine CXCL1, are all increased during experimental fungal asthma (Hogaboam et al., 2000). The role of individual cytokines and chemokines in fungal allergic inflammation has been studied by a great number of laboratories. The results and implications of these experiments are summarized in Table 2.

2.3.5. *Cytokine production by stromal cells*

The cellular localization of *A. fumigatus* is probably also incumbent on its modulation of allergic responses toward a Th2-type response. *A. fumigatus* is known to produce a number of proteases which incite production of cytokines

and chemokines including IL-8/CXCL8, IL-6 and CCL2 by epithelial cells (Borger et al., 1999; Tomee et al., 1997). Both IL-8/CXCL8 and CCL2 are found at an elevated level in BAL and lung tissue samples from *A. fumigatus*-allergic mice. When co-cultured *in vitro* spores of *A. fumigatus* attach to epithelial cells (Paris et al., 1997). These spores are found on and between epithelial cells in the respiratory epithelium of ABPA patients (Slavin et al., 1988). In fact, lung epithelial cells are capable of internalizing Aspergillus conidia (Wasylnka et al., 2002) but unlike their phagocytic lung counterparts epithelial cells lack the ability to kill internalized conidia, which then persist and, worse still, may germinate within alveolar epithelial cells (Wasylnka et al., 2003). In the lung, epithelial cells are an important source of chemokines including CCL2, CCL5, CCL7, CCL11 and IL-8/CXCL8 (Kwon et al., 1995; Lilly et al., 1997; Sousa et al., 1994; Stafford et al., 1997; Standiford et al., 1990). In ovalbumin allergic asthma models, epithelial cells have been shown to be the predominant source of cytokines and chemokines including IL-13 and CCL11 in the allergic lung (Humbles et al., 1997). Thus, epithelial cytokine production is likely to be equally important as macrophage and T cell cytokine production in the pathogenesis of fungal allergic asthma, particularly considering the physical presence of *A. fumigatus* on, in and between epithelial cells. Although there is no specific evidence of epithelial cell cytokine/chemokine production in IPA, presumably the same mechanisms are available to conidia for invasion of epithelial cells and epithelial cells respond to fungal presence with chemokine production. Aspergillus proteases are also more potent inducers of epithelial cell detachment and cytokine production than Alternaria and Cladosporium species, this may explain the rapid destruction of lung architecture and extreme morbidity in aspergillus infection (Kauffman et al., 2000). Damage and repair mechanisms of the airway mucosa may further facilitate the binding of *A. fumigatus* spores and mycelium, which are able to interact with molecules of the extracellular matrix at further detriment to the IPA patient. In the case of ABPA, the continuous presence of *A. fumigatus* in close consort to the epithelial surface will ensure a continuous pro-inflammatory response by the epithelial cells themselves, resident mast cells and other immune cells, resulting in severe and prolonged periods of inflammation of the airways.

4. CONCLUDING REMARKS AND INDICATIONS FROM ANTI-CYTOKINE THERAPY

The cells that participate in the asthmatic response including macrophages, lymphocytes, eosinophils and fibroblasts produce a great number of chemokines during the inflammatory response. Th2 cytokines IL-4 and IL-13 are potent inducers of chemokine production by these cells. Several chemokines have been found to have essential roles in both the development and maintenance of fungal allergy. Considerable clinical and experimental research has demonstrated that chemokines mediate the recruitment and activation of a variety of cells to the airways in allergic airways disease. Much research has been directed toward the blockade of particular

chemokines or chemokine receptors in order to attenuate the accumulation and/or activation of leukocyte populations that drive asthmatic responses. Conversely, in treatment of IPA new therapeutics are sought to stimulate leukocyte generation, recruitment and activation all of which could be achieved by selective upregulation of cytokine-cytokine receptor signaling.

Corticosteroid treatment in mice prior to infection with *A. fumigatus* has been shown to prevent production of the pro-inflammatory cytokine TNF-α and neutrophil influx (Balloy et al., 2005a). As TNF-α and neutrophils have been shown to be essential for defence against *A. fumigatus*, this demonstrates one mechanism by which transplant-recipients are rendered susceptible to invasive fungal infections. The progression of the fungal infection in immunosuppressed mice differed between the treatments administered to cause immunosuppression: a corticosteroid and a chemotherapeutic agent. Thus the requirement for distinct anti-fungal therapy regimens in management of cancer versus post-transplant patients should be considered. The use of corticosteroids in exacerbations of COPD and in septic shock appears to be precipitating a rising incidence of IPA in non-immunocompromised ICU patients (Denning, 2004). However, despite substantial immuno-suppressive corticosteroid therapy, CF patients rarely develop IPA. These individuals are repeatedly exposed to A. fumigatus conidia as proven by the high incidence of ABPA as one of three major diseases that greatly complicate Cystic Fibrosis (CF). Yet their resistance to IPA shows that these individuals still possess a high ability to kill fungal conidia unless they receive a lung transplant, after which they may succumb to IPA.

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CHAPTER 10

ANTIBODIES

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Abstract: Generation of antibodies against fungal antigens is one of the hallmarks of the host/fungus interaction, and antibodies are important contributors to host immunity during these fastidious infections. Many studies have examined the role of antibody production as a prerequisite for protective immunity to different pathogenic fungi, both *in vivo* and *in vitro*. Surprisingly, this has proved a problematic issue to definitively determine, mainly because fungal infections have been traditionally associated with defects in cell mediated immunity and because of the heterogeneity of antibodies in polyclonal sera. However, in the recent years there has been a growing body of evidence for the protective role of antibodies and a revival on the interest and potential use of antibodies in novel, immune-based approaches for the management (diagnosis, prevention and treatment) of fungal infections

1. INTRODUCTION AND GENERAL CONSIDERATIONS

One of the major functions of the immune system is the production antibodies, which are soluble proteins that circulate freely and contribute to immunity against foreign aggressors, including pathogenic fungi. Antibodies belong to the class of molecules collectively known as immunoglobulins. Immunoglobulins consist of two domains, an antigen binding region composed of variable elements and a constant region. The constant region includes an Fc fragment, that determines the isotypic (class) structure and biological functions common to many antibodies, whereas the variable region determines binding to an unique epitope in an antigen, conferring specificity. The classical functions of antibodies include direct activities, such as neutralization of toxins and microorganisms, and indirect activities requiring participation of other components of the immune system, such

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as opsonization, complement activation, antibody-dependent cellular cytotoxicity, immunomodulation and the generation of protective memory antifungal immunity.

Generation of antibody responses is virtually universal during the interactions between the host and fungi. This occurs in response to normal exposure to environmental fungi, as result of commensalism, and during active infection caused by opportunistic and true pathogenic fungi. Antibody production influences immunity in a manner that can be either beneficial, neutral or detrimental for the host, and contradictory reports that either support or deny the importance of antibody immunity during fungal infections abound in the literature. Traditionally, the role of antibodies during fungal infections was mostly ignored, since cell-mediated immunity was believed to play a leading role in protection (Casadevall, 1995). In the few instances that antibodies were investigated results were often inconclusive, which may be explained by the complex nature of the polyclonal humoral response, with the presence in immune sera of protective, non-protective, and even deleterious (infection-enhancing) antibodies (Casadevall, 1995). Also, the efficacy of antibodies is complex and variable, and depends on antibody specificity, isotype, idiotype, dose (with normally a lack of efficacy at both extremes of the antibody concentration – *i.e.* prozone effect), timing of administration, the microbial strain and initial infecting innoculum, as well as host properties (*i.e.* mouse strain used in experimental infections) (Casadevall, 2005). In some instances the efficacy of antibodies is dependent on the presence of intact cellular immunity. Because of all these reasons, antibody protection experiments need to be interpreted with caution and negative results do not exclude the existence of protective antibodies (Casadevall, 2003).

In the last few years the advent of new technologies, such as hybridoma technology for the generation of monoclonal antibodies (mAbs), techniques for antibody engineering and proteomic techniques that facilitate identification of antigenic components present in complex antigenic mixtures, together with the need for alternative approaches for the management of recalcitrant fungal infections have led to a renewed interest in the study of antibody responses (Casadevall et al., 2004; Casadevall and Scharff, 1994). Experiments using mAbs, of a single specificity an isotype, have permitted a more mechanistic and more rigorously controlled assessment of antibody function. Conversely, protective mAbs can be used to identify fungal antigens that elicit useful humoral responses, that by extension represent candidates for vaccine development (Casadevall et al., 2002). Newer human and humanized antibodies, together with genetically engineered antibody fragments offer new perspectives for their clinical use as prophylactic and therapeutic reagents (Burnie and Matthews, 2004). Also, a better understanding of the antigenic composition and antibody response during fungal infections, facilitated by the implementation of powerful proteomic technologies, may lead to novel vaccines and serodiagnostic techniques that are urgently needed (Pitarch et al., 2003).

2. ANTIBODY RESPONSES AGAINST DIFFERENT PATHOGENIC FUNGI

2.1. *Cryptococcus neoformans*

C. neoformans is a ubiquitous environmental yeast that is unique among the pathogenic fungi because it has a polysaccharide capsule. Serological studies suggest that *C. neoformans* infections are very common among immunocompetent individuals, including children, but disease is rare (Goldman et al., 2001). However, as an opportunistic pathogen *C. neoformans* is able to cause life-threatening infections, mainly in patients with cellular immune deficiencies, most notably AIDS (Perfect and Casadevall, 2002). Current therapy for cryptococcosis is sub-optimal and human infection is often incurable (Perfect and Casadevall, 2002). Since the majority of patients with cryptococcal infection are immunosuppressed a logical approach to the improvement of treatment outcomes is through enhancing host immune responses. Host defense against *C. neoformans* is believed to depend primarily on the cellular response (Levitz, 1992). Early experiments with polyclonal sera produced conflicting evidence for and against the importance of antibody immunity in host defense during cryptococcosis. However, numerous studies have now convincingly demonstrated that antibody-mediated immunity may play a role in resistance against cryptococcal infection (Casadevall, 1995).

C. neoformans cells are surrounded by a polysaccharide capsule that is antiphagocytic and represents a major virulence factor (McFadden and Casadevall, 2001; Perfect et al., 1998). During human infection, the capsular polysaccharide, glucuronoxylomannan (GXM), accumulates in tissue, which negatively impacts host immunity (Murphy, 1999; Vecchiarelli, 2000). The majority of patients with *C. neoformans* infections have a high concentration of serum polysaccharide antigen but low titers of serum antibodies to the capsular polysaccharide. Experiments with monoclonal antibodies to GXM have demonstrated the existence of protective, non-protective and even deleterious antibodies, thus providing an explanation for the earlier divergent results using polyclonal sera (Casadevall, 1995). Several groups have now convincingly demonstrated that administration of antibodies to GXM can modify the course of infection to the benefit of the host (Dromer et al., 1987; Fleuridor et al., 1998; Mukherjee et al., 1992; Sanford et al., 1990). Antibodies have been shown to enhance opsonization, activate the complement system, increase phagocytosis (thereby increasing the number of organisms killed) and also promote the clearance of polysaccharide antigen from the serum. Overall, in animal models administration of anti-GXM antibodies prolonged survival, reduced tissue fungal burden, promoted granuloma formation, and enhanced the killing of *C. neoformans* by antifungal agents. Efficacy of mAb-treatment against *C. neoformans* in mice is dependent on isotype, specificity (which in turn is affected by immunoglobulin variable region usage, somatic mutation and constant region usage), binding characteristics, dose and infecting inoculum, timing of administration, fungal strain, mouse strain and availability of T cells and other mediators of cellular immunity (reviewed in (Casadevall and Pirofski, 2005)). Altogether, the data generated indicates that

anti- GXM antibodies mediate protection by immunomodulatory effects, thereby linking antibody efficacy to the overall host immune response (Casadevall et al., 2002; Feldmesser et al., 2002). Based on these observations, MAb 18B7, a murine IgG1 developed by the Casadevall group, is currently in clinical trials which represents the first application of mAb therapy for the treatment of a fungal infections in humans (Casadevall et al., 1998; Larsen et al., 2005).

In addition, the protective efficacy of anti-GXM provides a rational basis for the development of vaccines against *C. neoformans* infections if such a vaccine could induce similar antibodies (Casadevall et al., 2002). A GXM–tetanus toxoid (GXM–TT) conjugate vaccine protected mice against infection (Devi , 1996). Also, peptide mimetics of GXM oligosaccharide epitopes have been identified using random peptide phage display libraries (Fleuridor et al., 2001; Valadon et al., 1998). A peptide mimetic of a human IgM proved to be a GXM mimotope capable of eliciting an antibody response to GXM (Fleuridor et al., 2001). Importantly, vaccination with this mimotope prolonged survival of mice infected with *C. neoformans*.

Melanin synthesis has also been associated with virulence for this fungus (Casadevall et al., 2000). *C. neoformans* cells synthesize melanin during infection and this pigment protects the fungus against immune defense mechanisms. Passive immunization with mAbs to melanin prolonged the survival of and reduced the fungal burden in infected mice (Rosas et al., 2001). The anti-melanin antibodies reduced the growth rate of in vitro-melanized *C. neoformans* cells, suggesting a new mechanism of antibody-mediated protection. Similarly, antibodies to cell wall glucosylceramide exhibit fungistatic effects (Rodrigues et al., 2000). Antibody responses to several cytosolic and membrane proteins have been associated with increased survival in murine models (Neuville et al., 2000). Interestingly, the antibody response to protein antigens was bimodal, with nonsurvivors mounting a strong antibody response early during the acute phase, but a majority of survivors producing antibodies later, during the chronic phase of the infection. These observations highlight the existence of additional targets for the humoral response during cryptococcosis.

2.2. *Candida albicans*

Candida albicans is the most common fungal pathogen of humans. Although in normal individuals this microorganism is a commensal of mucosal surfaces, in patients with predisposing conditions it is able to cause a variety of infections that range from superficial (oral and vaginal) candidiasis to life threatening disseminated candidiasis. Although *C. albicans* remains the most frequent causative agent of candidiasis, other species have been increasingly associated with infections in an expanding population of immunocompromised patients. Morbidity and mortality rates associated with candidiasis remain unacceptably high, the main reasons being the difficulties encountered in the diagnosis and treatment of this type of infections (Banerjee et al., 1991; Pfaller et al., 1999; Pfaller et al., 1998; Viudes et al., 2002).

As a result of its commensal status anti-*Candida* antibodies have been shown to be ubiquitous in human sera, presumably because the immune system can be stimulated as a result of colonization by *C. albicans* in the absence of disease (Domer, 1989; Lopez-Ribot et al., 2004; Martinez et al., 1998; Reiss and Morrison, 1993), starting early during infancy. During the host-fungus relationship, both as a commensal and during infection, *C. albicans* antigens elicit strong immune responses, including production of antibodies. Cytosolic components, cell wall and other secreted moieties, both protein and carbohydrate in nature, represent the major immunogenic components during candidiasis as manifested by proteomic analyses of antibodies present in sera from patients and animal models (Lopez-Ribot et al., 2004; Martinez et al., 1998; Pitarch et al., 2004; Pitarch et al., 2001; Pitarch et al., 1999). Despite the complex antigenic make-up and considerable heterogeneity of the antibody responses to candidal antigens in humans (Chaffin et al., 1998; Lopez-Ribot et al., 2004; Martinez et al., 1998), several immunodominant antigens have been identified, including glycolytic enzymes (enolase), heat shock proteins, mannans and mannoproteins. The role of antibody immunity in protection against candidiasis has been controversial, the antibody response against *Candida* is complex, with the presence in immune sera of protective, non-protective, and deleterious (infection-enhancing) antibodies (Casadevall, 1995; Fernandez-Arenas et al., 2004a; Fernandez-Arenas et al., 2004b; Lopez-Ribot et al., 2004; Martinez et al., 1998). Fernandez-Arenas et al described two categories of serum, protective and non protective, and the antibody profiles were analyzed by a proteomic approach (Fernandez-Arenas et al., 2004a). Immunoglobulin levels (particularly IgG2a) in the protective sera were significantly higher when compared with the non protective sera. The pattern of a "non protective" profile was composed of enolase (Eno1p), transketolase (Tkl1p), heat shock protein (Hs78p) and methionine synthase (Met6p), only antibodies against enolase were IgG2a isotype. The pattern of a "protective" sera, on the other hand, was composed of antibodies against the following antigens: several isoforms of enolase (Eno1p), pyruvate decarboxylase (Pdc11p), pyruvate kynase (Cdc19p), a protein of the 40S ribosomal subunit (Bel1p), triosephosphate isomerase (Tpi1p), DL-glycerol phosphatase (Rhr2p) and fructose-bisphosphate aldolase (Fba1p), and all these antibodies are IgG2a isotype.

As a result, early immunoprotection experiments using polyclonal antibodies often lead to inconclusive observations (Casadevall, 1995). However, recent evidence clearly demonstrates that antibodies with defined specificities show different degrees of protection against systemic and mucosal candidiasis (De Bernardis et al., 1997; Han and Cutler, 1995; Matthews and Burnie, 2001), favoring the host during the course of infection (Casadevall, 1995; Lopez-Ribot et al., 2004; Martinez et al., 1998). The exact mechanisms by which these antibodies protect against *Candida* infection are unknown but are likely to include the inhibition of adhesion or germ tube formation, opsonization, neutralization of virulence-related enzymes, and direct candidacidal activity (Casadevall, 1995; Martinez et al., 1998). Further data supporting the protective role of antibodies during candidiasis are: i) the fact that functional B cells are required to protect mice from a primary

intravenous challenge with *C. albicans* (Wagner et al., 1996) and ii) the fact that opsonizing antibodies may condition the nature of the dendritic cell interaction with fungi and the generation of memory antifungal immunity (Montagnoli et al., 2003). A recent work has addressed the importance of the collaboration between cellular and humoral responses, in the eliciting a long lasting and effective protection (Fernandez-Arenas et al., 2004b). The authors vaccinated mice with a series of "avirulent" mutants and using a proteomic approach were able to define an antibody pattern in the sera from effectively vaccinated animals surviving the infection that differed from the antibody pattern in non-protected mice. The use of this proteomic approach led to the identification of antigens eliciting protective IgG2a antibodies in vaccinated animals, which may represent excellent candidates for a future fungal vaccine (Fernandez-Arenas et al., 2004b).

Mannan is found in the cell wall as large N-linked and shorter O-linked mannooligosaccharides associated with mannoproteins. Anti-mannan antibodies are prevalent in human sera, including patients and normal population (Domer, 1989; Lopez-Ribot et al., 2004; Martinez et al., 1998; Reiss and Morrison, 1993). The antigenic specificity of serotypes A and B of *C. albicans* is determined by structural peculiarities of the carbohydrate moiety of mannans (Hasenclever and Mitchell, 1964). The mannan component is also involved in adhesive interactions (Chaffin et al., 1998). Han and Cutler immunized mice with a mannan fraction (previously encapsulated into liposomes) to induce protective antibody responses. In the same study, these authors tested two monoclonal antibodies specific for different mannan epitopes in the adhesin fraction. Both antibodies agglutinated *Candida* cells but only one of them protected mice against disseminated candidiasis. The protective antibody recognized the acid-labile adhesin while the non-protective antibody recognized a different epitope in the fraction (Han and Cutler, 1995; Han et al., 2000). It seems that the ability of the antibody to rapidly deposit high amounts of complement factor C3 onto the yeast cell wall is needed for protection against disseminated candidiasis (Han et al., 2001). Anti-mannan antibodies can also mediate protection in animal models of *Candida* vaginitis (De Bernardis et al., 2002; De Bernardis et al., 1997; Han et al., 1998). Combined detection of mannanemia and anti-mannan antibodies may have diagnostic value and also help monitor patients with candidiasis (Sendid et al., 2002).

Although β-glucans are present in greater abundance than mannan in the wall of *C. albicans*, they are immunologically less active (Chaffin et al., 1998; Martinez et al., 1998). However, it has been recently reported that anti-glucan antibodies contributed to the passive protection against *Candida* infection in an animal model (Bromuro et al., 2002). Also, β-glucan has recently been identified as a the receptor for the killer toxin produced by the yeast *P. anomala* (Guyard et al., 2002; Magliani et al., 2005). Interestingly protective yeast killer toxin-like antibodies ("antibiobodies", KT-Abs), are able to exert a direct microbicidal activity by mimicking a killer toxin (*Pa*KT) and its interaction with cell wall receptors on susceptible *C. albicans* cells. These antibodies are produced during the course of experimental and natural infections and can also be produced by idiotypic vaccination with

a KT-neutralizing mAb, and they exert strong therapeutic activity against both mucosal and systemic candidiasis (Beninati et al., 2000; Magliani et al., 2005; Magliani et al., 2002; Magliani et al., 2004; Polonelli et al., 2000; Polonelli et al., 1996; Polonelli et al., 1994; Polonelli et al., 2003; Polonelli et al., 1997), and also different fungal infections, including aspergillosis (Cenci et al., 2002) and experimental models of *Pneumocystis carinii* pneumonia (Magliani et al., 2001).

Immunoblotting experiments with sera from patients suffering from systemic candidiasis showed the presence of a 47-kDa immunodominant antigen present in whole cell extracts of the fungus later identified as a heat-stable breakdown product of hsp90 with a cell wall location (Matthews and Burnie, 1989; Matthews et al., 1988b; Matthews et al., 1991b; Matthews et al., 1984). Antibodies to the 47-kDa antigen are present in serum samples from a high proportion of patients with chronic mucocutaneous candidiasis and AIDS (Matthews and Burnie, 1988; Matthews et al., 1988a; Matthews et al., 1991b). Patients who recover from invasive candidiasis generate a major antibody response to the 47-kDa component, whereas fatal cases seem to have little antibody or declining titers (Matthews and Burnie, 1988; Matthews et al., 1988a; Matthews et al., 1991b). In a mouse model of disseminated candidiasis, passive administration of sera from two infected patients containing anti-hsp90 antibodies led to decreased mortality. Epitope mapping of *C. albicans* hsp90 with patients' sera revealed that patients recovering from systemic candidiasis produce antibodies against both fungal-specific and conserved epitopes of hsp90 (Matthews et al., 1991a). A highly conserved epitope LKVIRK was recognized by sera from all patients with antibody to the 47-kDa antigen. When administered prophylactically, a monoclonal antibody raised against this epitope reduced mortality in a mouse model of hematogenously disseminated candidiasis (Matthews et al., 1995). Mycograb (*Neu*Tec Pharma plc.) is a human genetically recombinant antibody against fungal hsp 90. Mycograb acts synergistically with amphotericin B and caspofungin both *in vitro* and *in vivo*. Based on these properties, Mycograb is now being assessed in preclinical trials in patients with invasive candidiasis (Matthews et al., 2003). In addition to hsp90, several members of the hsp70 family of proteins have been reported in the cell wall of *C. albicans*. It was reported that serum samples from both healthy people and patients suffering from candidiasis contained antibodies against the C-terminal portion of a member of the hsp70 family of proteins (Lopez-Ribot et al., 1996a). A more recent report demonstrated the elevated immunogenicity of hsp70, however, no protection against but rather some enhancement of *Candida* infection seemed to occur in a murine model of candidiasis after vaccination with recombinant protein (Bromuro et al., 1998). Immunization with a stress mannoprotein of >200 kDa from the cell wall of *C. albicans*, a major target of secretory IgA, led to the production of mAbC7. This mAb displays three different biological activities (inhibition of adherence, inhibition of germination and direct candidacidal activity) (Moragues et al., 2003), and also has tumoricidal activity (Omaetxebarria et al., 2005).

Perhaps the glycolytic enzyme enolase is the most immunodominant component in *C. albicans* and antibodies against enolase and other cytosolic

and cell wall-associated glycolytic enzymes, including phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, triose phosphate isomerase, phosphoglycerate mutase, pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase, seem to be prevalent in sera from patients and animal models (Alloush et al., 1997; Chaffin et al., 1998; Lopez-Ribot et al., 2004; Martinez et al., 1998; Pitarch et al., 2004; Pitarch et al., 2001; Pitarch et al., 1999). Strockbine et al. (Strockbine et al., 1984) reported that sera from patients with disseminated candidiasis had circulating antibodies directed against a 48-kDa protein antigen, which was subsequently identified as enolase (Franklyn et al., 1990; Mason et al., 1989). A protective role for anti-enolase antibodies has been suggested since repeated administration of immune anti-enolase serum conferred partial protection against murine candidiasis (van Deventer et al., 1996). In a recent report mice immunized with enolase plus IL-12 showed increased antibody titres against enolase, as well as increased median survival time and decreased fungal burden in kidneys, although antibodies did not seem to play a role in protection (Montagnoli et al., 2004).

C. albicans secreted aspartyl proteinases (Saps) represent key virulence determinants during candidiasis. Saps are immunogenic and elicit mucosal and systemic antibody responses (Naglik et al., 2003). Perhaps the most promising results were reported by De Bernardis et al. (De Bernardis et al., 2002; De Bernardis et al., 1997), who showed that immunization with Sap2 antigen, or administration of an anti-Sap2 monoclonal antibody or anti-Sap2 antibody-containing vaginal fluids, partially protected rats against candidal vaginitis in an experimental model. One study analyzed the B-cell epitopes of *Candida* Sap proteins, particularly Sap2, and provided some information regarding the Sap2 epitopes recognized by serum IgG and IgM antibodies (Ghadjari et al., 1997).

The 58-kilodalton fibrinogen binding cell wall mannoprotein (mp58/Pra1p) belongs to a family of immunodominant fungal antigens and is expressed by fungal cells during infection (Casanova et al., 1992; Lopez-Ribot et al., 1996b). Immunoblotting analyses demonstrated the presence of antibodies against the mp58 in sera from patients with different types of candidiasis (Navarro et al., 1993). Continuous B-cell epitopes on the protein moiety of *C. albicans* mp58 species, have been recently identified in epitope mapping experiments using a complete set of overlapping dodecapeptides synthesized from amino acid sequence of the protein portion of mp58 as deduced from the DNA sequence of its encoding gene (*FBP1/PRA1*) (Viudes et al., 2004; Viudes et al., 2001). These experiments used sera from patients with candidiasis and from animal models and revealed the presence of multiple IgG-reactive continuous epitopes on the protein, expanding both the amino- and carboxy-terminal domains and several internal regions. A synthetic peptide corresponding to the last 10 amino acid residues at the C terminus of the protein elicited a strong antibody response in mice (Viudes et al., 2001). Patients who survived the infection displayed increased antibody reactivity towards the C-terminal epitope as compared to those succumbing to candidiasis (Viudes et al., 2004). Moreover, a monoclonal antibody directed towards this epitopic region

conferred protection in serum therapy experiments in a murine model of hematogenously disseminated candidiasis (Viudes et al., 2004).

Other interesting use of antibodies as tools in the study of pathogenesis of candidiasis (and other fungal infections) is their use to screen expression libraries leading to the identification of genes encoding immunoreactive proteins and also those expressed *in vivo* during infection (Alloush et al., 1996; Nguyen et al., 2004). Also, the fact that a mAb recognizing a pH sensitive carbohydrate epitope on the surface of *C. albicans* cells selectively identifies invasive forms of candidiasis offers new hope for the development of effective diagnostic techniques that could discriminate between commensal status and active infection (Monteagudo et al., 2004).

2.3. *Pneumocytis carinii*

P. carinni is an opportunistic fungus that casues a potentially severe and fatal pneumonia (PCP) in a variety of patients with compromised immune status due to AIDS, chemotherapeutic regimes for cancer, immunosuppressive therapy for organ transplant and congenital immune diseases (Wazir and Ansari, 2004).

Roth and Janitschke demonstrated the formation of antibodies in mice, rats and rabbits immunized with different *P. carinii* antigens (Roth and Janitschke, 1991). Hyperimmune sera was highly effective at reducing the number of fungal organisms in early, intermediate, and advanced stages of PCP and was capable of increasing the mean life expectancy of infected mice (Roths and Sidman, 1993) and passive immunization with polyclonal antibodies in sera from animals that were allowed to recover from severe *P. carinii* pneumonia conferred protection to naïve mice (Bartlett et al., 1998).

Gigliotti et al., developed a battery of monoclonal antibodies against *P. carinii* surface components (Gigliotti et al., 1986). Only one of these antibodies, an IgM, recognized *P. carinii* obtained from rabbits, ferrets, and human. Interestingly, passive immunoprophylaxis with this specific mAb conferred partial protection against PCP in animal models (Gigliotti and Hughes, 1988). This mAb recognizes a conserved epitope on a major mannosylated surface glycoprotein (gpA) of *P. carinii* (Haidaris et al., 1992). However, immunization with gpA produced an specific antibody response but did not protect mice from the development of PCP (Gigliotti et al., 1998). Subsequently this same group generated a panel of mAbs against *P. carinii* antigens other than gpA. Some of these mAbs were protective when administered intranasally, with two IgM antibodies recognizing and epitope on the kexin-like molecule (KEX1) and also the A12 antigen shared by mouse and human organisms leading to a reduction in fungal burden of more than 99% (Gigliotti et al., 2002). Epitope mapping with one of these mAbs indicated recognition of multiple proline-rich epitopes which may explain its degenerate recognition of multiple antigens on the surface of the fungus, including KEX1 and also the A12 antigen (Wells et al., 2004).

2.4. *Aspergillus fumigatus*

Over the past two decades *Aspergillus fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunosupressed patients (Denning , 1998; Marr et al., 2002; Patel and Paya, 1997). Invasive aspergillosis is now a major cause of death at leukemia treatment centers, and bone marrow and solid organ transplantation units (Denning , 1998; Marr et al., 2002; Patel and Paya, 1997). Other spp. of *Aspergillus*, such as *A. terreus*, *A. flavus* and *A. niger* can also cause invasive aspergillosis (Marr et al., 2002). This severe opportunistic fungal infection is characterized by a high mortality rate in these at-risk patients (Lin et al., 2001) (the crude mortality rate of invasive aspergillosis approaches 100%). Although rarely, in immunocompetent patients this fungus can also cause aspergilloma (an overgrowth of the fungus on the surface of preexisting cavities in the lungs of patients treated for tuberculosis) and allergic brochopulmonary aspergillosis (ABPA, a clinical condition observed among individuals exposed repeatedly to conidia and those suffering from atopic asthma or cystic fibrosis).

Most studies to date have examined antibodies against *Aspergillus* as contributing to allergic disease or because of their use in serodiagnostic techniques. Sera from most healthy individuals contain anti-*Aspergillus* antibodies due to continuous environmental exposure. In contrast to immunocompetent hosts, growth of *A. fumigatus* in the tissues of an immunocompromised host, who either lack a sufficient antibody response or who mount variable antibody response, is not correlated with an increase in anti-*Aspergillus* antibody titers. Indeed, presence of antibodies against *Aspergillus* in immunosuppressed individuals is more likely to represent circulating antibodies prior to the onset of immunosuppressive therapy rather than antibodies formed during invasive infection (Latge, 1999). Increasing antibody titers at the end of immunosuppression are normally indicative of recovery from invasive aspergillosis, whereas declining antibody levels are normally associated with poor prognosis. On the other hand, high antibody titers (precipitins) are normally detected in patients with aspergillomas, and those with ABPA show elevated levels of serum IgE (Jaques et al., 1995). Immunoblot analyses have been used to examine the antibody response during infection and to identify antigenic components of *Aspergillus* spp. These have also served as the basis for the development of serodiagnostic assays for the diagnosis of aspergillosis in immunocompetent hosts, although studies were complicated by the variability of antigenic extracts used. Important antigenic components identified include 58-kDa, 88-kDa and 18-kDa proteins, catalase, alkaline protease, a serine protease, a superoxide dismutase, elastase and catalase, among others (reviewed in (Latge, 1999)). More recently Denikus et al used antibodies in sera from infected rabbits to screen an *A. fumigatus* expression library, and identified thirty six antigens, most of which were associated with the cell surface, and included Asp f 16, hsp90 and enolase (Denikus et al., 2005). Importantly, the authors suggested that antibody production may be associated with the development of an early immune response and primary protection.

But without any question, a polysaccharide antigen, galactomannan (GM), is the most important antigen in *Aspergillus*. GM is a component of the *Aspergillus* cell wall, was the first antigen detected in experimentally infected animals and in patients with invasive aspergillosis (Latge, 1999). Recently, Stynen et al. have introduced a sandwich enzyme-linked immunosorbent assay (ELISA) that is based on the use of the rat monoclonal antibody EB-A2, which recognizes the $(1 \rightarrow 5)$-β-D-galactofuranoside side chain of the GM molecule. Since each GM molecule harbors several epitopes, the same monoclonal antibody can function as capture and detector antibody (Stynen et al., 1995). The test is commercialized as Platelia *Aspergillus* EIA (Bio Rad), for the early diagnosis of invasive aspergillosis, which is of paramount importance since antifungal therapy must be begun promptly in these highly immunosuppressed patients to maximize success. Of note, this test could also be important for monitoring therapeutic responses (Bennett et al., 2003).

Presently very little is known about the potential of anti-*Aspergillus* antibodies to protect during infection. Although many mAbs against different *Aspergillus* antigens have been generated, the majority have not been tested for protective efficacy (Casadevall et al., 2002). Most attempts at passive transfer of protection with immune sera have been unsuccessful, although most studies did not examine different parameters known to affect outcome in other experimental systems. For example, one study used mAbs to elastase, which did not confer protection to immunosuppressed mice (Frosco et al., 1994). However, the possibility exists that antibodies to *Aspergillus* may mediate protection, for example, by modulating cellular immune responses, interfering with spore germination, increasing phagocytosis or neutralizing hydrolytic enzymes (Casadevall et al., 2002). For example, recent results suggest that the availability of opsonizing antibodies may condition the nature of the dendritic cell interaction with fungi, and play an important role in the generation of memory antifungal immunity against aspergillosis (Montagnoli et al., 2003).

2.5. *Histoplasma capsulatum*

H. capsulatum is a dimorphic fungus and is the most prevalent cause of fungal respiratory disease, infecting approximately 500,000 individuals in the US each year (Cano and Hajjeh, 2001). Infection usually results in a mild, often asymptomatic respiratory illness, but may progress to life-threatening systemic disease, especially in individuals with AIDS (Graybill, 1988). The clinical manifestations are principally caused by intracellular yeast forms that parasitize mammalian phagocytes.

The antibody response to natural infection by *H. capsulatum* in humans has been investigated (for a review see (Nosanchuk, 2005)), and is characterized by induction and increase in IgM in the first two weeks, followed by increasing titers of IgG and IgA antibodies (Chandler et al., 1969). The IgG fraction contains both complement-fixing and precipitating antibodies (Chandler et al., 1969). Experiments in animal models (mouse) indicate that antibody levels peak by day 21 (Fojtasek et al., 1993). Antibody detection may give a clue to the diagnosis of this infection,

even in immunosuppressed hosts. Antibodies to the H antigen of histoplasmin develop during active histoplasmosis while antibodies to M antigen are indicative of prior infection and is the first to rise with seroconversion (Wheat et al., 1983). Nevertheless, limitations of serological detection of histoplasmosis include lack of humoral response in those patients with more severe immunosuppression and due to the lack of sensitivity or specificity of that response (Wheat et al., 1983). More useful, particularly in immunosuppressed hosts, is the use of antibodies for the detection of *Histoplasma* polysaccharide, which is detectable during disseminated infection and correlates with response to therapy (Wheat, 2003). This assay system developed by Wheat and colleagues, utilizes a polyclonal antibody for both the capture and detection steps in an ELISA sandwich method to detect circulating antigen that provides a rapid and reliable means of diagnosing the more severe forms of histoplasmosis (Wheat, 2003).

The role of humoral immunity in protection against *H. capsulatum* is uncertain. Early experiments indicated that passive immunization with immune serum did not mediate protection (Tewari et al., 1977). Also, B cell–deficient mice are not particularly susceptible to infection and high antibody titers do not correlate with resistance to infection (Allendorfer et al., 1999; Chandler et al., 1969). Consequently, the consensus in the field has traditionally been that humoral immunity has little or no role in host defense. Although murine mAbs to specific components of *H. capsulatum* have been identified (Hamilton et al., 1990; Jeavons et al., 1994; Reiss et al., 1986), for most of them there are no published reports of studies evaluating their protective efficacy in mouse models of histoplasmosis (Nosanchuk, 2005). However, more recently Nosanchuk et al. described the generation of protective antibodies to *H. capsulatum* that bind to a histone H2B–like protein on the surface of the fungus (Nosanchuk et al., 2003). Administration of mAbs before infection reduced fungal burden, decreased pulmonary inflammation, and prolonged survival in a murine infection model. Protection was associated with enhanced levels of cytokines in the lungs of infected mice and the antibodies increased phagocytosis of yeast by macrophages through a CR3-dependent process leading to yeast cell growth inhibition and killing. The authors also suggested that that the histone H2B–like protein in *H. capsulatum* could be a potential candidate for vaccine development (Nosanchuk et al., 2003).

2.6. *Blastomyces dermatitidis*

Blastomycosis is an endemic mycoses in the central United States caused by a dimorphic fungus, *Blastomyces dermatitidis*. In nature this fungus exists in the mycelial phase but upon inhalation of spores into the lung, at body temperature, it converts to yeast phase. It has a wide spectrum of clinical manifestations, including asymptomatic self-limiting pulmonary illness, focal pulmonary or cutaneous illness, and disseminated disease (Bradsher et al., 2003).

Antibodies in human serum can mediate activation of complement that is required for killing of *B. dermatitidis* cells by human neutrophils (Ponton et al., 2001). Also,

serological approaches can occasionally be helpful in establishing the diagnosis as antibodies to *B. dermatiditis* are produced in response to the infection. Early tests detected antibodies to *Blastomyces* A antigen, a yeast antigen (Klein et al., 1986), but recent efforts by Klein and others have focused on the BAD-1 antigen (previously designated WI-1), an important adhesin and virulence factor in *B. dermatitidis*, that is also the main antigenic target of humoral responses during human and experimental infections. (Klein and Jones, 1994; Rooney et al., 2001). Antibodies to this 120-kDa cell surface protein are detected earlier than A antigen and decline by 6 months after illness in patients who respond to therapy (Klein and Jones, 1994). Although vaccination with BAD-1 elicits strong antibody responses, protection was associated mostly with cell-mediated mechanisms (Wuthrich et al., 1998). Also, passive immunization with mAbs to BAD-1 did not protect animals against experimental infection (Wuthrich and Klein, 2000).

2.7. *Coccidioides immitis and C. posadasii*

Coccidioidomycosis is caused by the dimorphic fungi in the genus *Coccidioides*. These fungi live as mycelia in the soil of desert areas of the American Southwest. Humans acquire the infection by inhalation of the arthroconidia, which subsequently convert into the parasitic spherule/endospore phase. Most infections are mild, but these organisms are formidable pathogens capable of causing progressive pulmonary and/or disseminated disease in fully immunocompetent individuals (Cox and Magee, 2004).

Chronic or progressive coccidioidomycosis is associated with the generation of a strong polyclonal antibody response, with elevated levels of IgG, IgA, and IgE in serum of patients (Calhoun et al., 1986; Cox and Arnold, 1979; Cox et al., 1982; Pappagianis et al., 1965). Serum IgG levels directly correlate with disease involvement, being highest in patients with multifocal involvement, whereas serum IgA levels are elevated in approximately 20% of patients, mostly in those with chronic pulmonary disease (Pappagianis, 2001; Pappagianis and Zimmer, 1990). High titers of IgE consistent with a Th2 deleterious response have been demonstrated in approximately 23% of patients with active disease, with the highest incidence occurring in patients with disseminated disease. Serologic analyses using crude antigens prepared from filtrates or lysates of mycelial or spherule-endospore phases are useful in establishing a diagnosis of coccidioidomycosis and in determining prognosis (Pappagianis, 2001; Pappagianis and Zimmer, 1990). IgM antibodies may be detected within the first few weeks of infection, while IgGs are detected after a few weeks of infection and usually disappear in several months if the infection resolves (Pappagianis, 2001; Pappagianis and Zimmer, 1990). Serial antibody titers can be used to assess efficacy of therapy: rising titers are normally a poor prognostic sign whereas falling titers are associated with a disease resolution (Stevens, 1995).

Little is known about the potential role of antibody in the protection against coccidioidomycosis. Kong et al. (Kong et al., 1965) reported that passive transfer of serum from vaccinated mice (with formalin killed spherules, FKS) did not protect recipients. Beaman et al. (Beaman et al., 1977; Beaman et al., 1979) demonstrated

that neither serum nor B cells from immune mice transferred protection in a murine model of coccidioidomycosis, and that preincubation of arthroconidia with serum from immune mice did not neutralize the infectivity of the arthrospores. Also, susceptible C57BL/6 mice produced higher titers of antibodies than resistant A/J did. Although several vaccine formulations containing *Coccidioides* killed cells (i.e. FKS), antigenic extracts or purified antigenic components (i.e. Ag2/PRA, SOW) have been tested in murine models, protection was generally associated with cell mediated immune mechanisms and not antibody responses (Cox and Magee, 2004). However, the possibility exists that there are protective antibodies that could be identified by using monoclonal antibody technology, as has been described for other fungal organisms.

2.8. *Paraccoccidioides brasiliensis*

P. brasiliensis is the etiologic agent of paracoccidioidomycosis, a prevalent systemic mycosis in South America where the lung is the primary target for infection. It is generally believed that antibodies are not effective in the control of chronic infection, but rather are abundant in anergic cases (de Camargo and de Franco, 2000).

The gp43 antigen, first described by Pucia et al. (Puccia et al., 1991), represents a major antigen of *P. brasiliensis* and forms the basis of a variety of serological tests, and antibody titers against gp43 can also be used to monitor therapeutic responses (Bueno et al., 1997). MAbs against gp43 modulate laminin-mediated fungal adhesion to epithelial cells and ameliorate pathogenicity *in vivo* either by inhibiting or enhancing granuloma formation and tissue destruction (Gesztesi et al., 1996). The gp70 is another immunodominant antigen in *P. brasiliensis* that can be used to monitor therapy in human infections (de Mattos Grosso et al., 2003). MAbs against gp70 were generated and exerted a protective effect against intratracheal infection, most likely through the inhibition of granuloma formation in the lungs (de Mattos Grosso et al., 2003). Also, de Fonseca et al. used immunoproteomics to identify six new antigens of *P. brasiliensis* that reacted with IgG antibodies present in sera from patients with paracoccidioidomycosis (da Fonseca et al., 2001).

3. CONCLUSIONS AND OUTLOOK

Antibody production in response to fungal infection is virtually universal. Seminal observations by the Casadevall group with *C. neoformans* have inspired a reappraisal of the role of antibodies in the field of Medical Mycology. It is now clear that discussing about the relative importance of antibody- and cell-mediated immune responses represents an unnecessary and futile dispute, and that the most pressing issue is to understand the mechanisms by which these two arms of the immune system function coordinately to achieve their ultimate goal of clearing an infection. New and powerful technologies in this genomic era offer unprecedented opportunities for the examination of antibody responses during fungal infections. Without

any questions, a better understanding of the antibody response may lead to novel strategies for the diagnosis, prophylaxis, treatment and monitoring of patients that are urgently needed.

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SECTION 3

NON-OPSONIC FUNGAL RECEPTORS

CHAPTER 11

RECOGNITION OF FUNGAL PATHOGENS BY TOLL-LIKE RECEPTORS

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Abstract: Toll-like receptors (TLRs) have been identified as a major class of pattern-recognition receptors. Recognition of pathogen-associated molecular patterns (PAMPs) by TLRs, either alone or in heterodimerization with other TLR or non-TLR receptors, induces signals responsible for the activation of innate immune response. Recent studies have demonstrated a crucial involvement of TLRs in the recognition of fungal pathogens such as *Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans*. By studying fungal infections in knock-out mice deficient in either TLRs or TLR-associated adaptor molecules, it appeared that specific TLRs such as TLR2, TLR4, TLR6 and TLR9 play differential roles in the activation of the various arms of the innate immune response. In addition, stimulation of TLR2 can also induce immunological tolerance and in certain conditions offer escape mechanisms, especially through induction of antiinflamatory cytokines. These developments provide crucial information for understanding the mechanisms of fungal recognition by cells of the immune system, and provide hope for designing new therapeutical approaches to fungal infections

1. TOLL-LIKE RECEPTORS AS PATTERN RECOGNITION RECEPTORS

At the end of the 90's, the the discovery of a novel class of receptors, the Toll-like receptors (TLR), has challenged the dogma of the non-specific nature of innate immunity. The Toll protein has been initially described in *Drosophila melanogaster* as a type I transmembrane receptor, with an important role in the dorso-ventral development of the *Drosophila* embryo. Later on, it has been observed

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that the absence of Toll in genetically-deficient *Drosophila* also results in a severely impaired defense against fungi (initial experiments performed with *Aspergillus fumigatus* infection) and Gram-positive bacteria (Lemaitre et al. 1996). It has been shown that these effects are mediated by inducing expression of a potent antifungal defensin called drosomycin. Soon after the initial description of Toll, it became apparent that while the extracellular domain of Toll contains leucine-rich repeats (LRR), the intracellular tail of the receptor has a striking homology with the intracellular domain of the interleukin-1 receptor type I (IL-1RI), being designated as Toll/IL-1R (TIR) domain (Rock et al. 1998). Moreover, the whole intracellular signaling pathway induced by *Drosophila* Toll is highly similar to the intracellular pathways activated by IL-1RI (Beutler 2004). Based on these theoretical arguments, it has been predicted that Toll homologues may be present in higher organisms and that mammalian homologues might have similar functions. Indeed, 11 different Toll-like receptors (TLR) have been identified in mammals, and they have been demonstrated to be crucial for recognition of pathogenic microorganisms and activation of the innate immune response (Takeda et al. 2003).

During the last few years, extensive research in this field has identified TLRs as a major class of signaling receptors, recognizing conserved bacterial structures called pathogen-associated molecular patterns (PAMPs) (Underhill et al. 2002; Kopp et al. 2003). The specificity of TLR recognition for several important PAMPs has been identified, including recognition of lipoteichoic acid, bacterial lipoproteins and zymosan by TLR2, double-stranded RNA by TLR3, lipopolysaccharide by TLR4, flagellin by TLR5, single-stranded RNA by TLR7, and CpG motifs of unmethylated bacterial DNA by TLR9. It became gradually apparent that by using differential recognition of the various microbial PAMPs by specific combinations of TLRs (and non-TLR pathogen recognition receptors), cells of the innate immune system are able to discern between various classes of pathogens and react with a tailored response (Underhill et al. 2002). The focus of this review is to provide an overview of the role of TLRs as recognition receptors for the medically-important fungal pathogens, and to demonstrate the crucial role of this class of receptors for the activation of antifungal innate immunity.

2. RECOGNITION OF *CANDIDA ALBICANS* BY TLRs

The first suggestion for a role of TLRs for the antifungal host defense was provided by the very high susceptibility of Toll-deficient *Drosophila* to aspergillosis (Lemaitre et al. 1996). Shortly after the discovery of the human homologues of Toll it became apparent that TLR2/TLR6 heterodimers recognize zymosan, a structure derived from the fungus *Saccharomyces cerevisiae* (Underhill et al. 1999; Ozinsky et al. 2000). The cell-wall structure of *Saccharomyces* greatly resembles that of the fungal pathogen *Candida albicans* (Klis et al. 2001), which prompted the investigation of the role of TLRs in the host defense against disseminated candidiasis. In an experimental infection model, it has been demonstrated that the absence of TLR4-mediated signals results in an increased susceptibility to disseminated candidiasis in TLR4-defective mice (Netea et al. 2002). This effect was mediated through

decreased release of the chemokines keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2, and impaired recruitment of neutrophils at the site of infection. In contrast, production of the proinflammatory cytokines tumor necrosis factor (TNF) and IL-1β was only marginally influenced, whereas the candidal killing capacity of TLR-4 -defective phagocytes was normal. In addition, by using a TLR2-specific antibody it has been show that production of TNF and IL-1β was dependent of TLR2 (Netea et al. 2002). These results lead to the conclusion that one fungal species, *C. albicans*, recruits different TLRs to activate specific arms of innate immunity.

Since this first observation, additional studies have confirmed the important role played by TLRs in recognition of *C. albicans* and anticandidal host defense. A global role for the TLRs in the host defense against disseminated candidiasis was demonstrated by the increased susceptibility of $MyD88^{-/-}$ mice, an intracellular adaptor molecule of the TIR domain, to *C. albicans* infection (Bellocchio et al. 2004; Villamon et al. 2004a). MyD88 was also involved in mediation of cytokine synthesis by *C. albicans*, as well as phagocytosis and killing of the yeast (Marr et al. 2003; Bellocchio et al. 2004).

The role of TLR2 in recognition of *Candida* and *Saccharomyces* was later demonstrated by two independent studies showing collaborative recognition of fungal β-glucan by TLR2 and dectin-1, with subsequent induction of proinflammatory cytokines (Brown et al. 2003; Gantner et al. 2003). In addition, TLR2 mediates prostaglandin E2 production in response to *C. albicans* (Villamon et al. 2005). Mannoproteins from *C. albicans* also influence the function of dendritic cells through TLR2 and TLR4 (Pietrella et al. 2006), while TLR2, in collaboration with dectin-1, is involved in the induction of regulatory antigen-presenting cells and immunological tolerance (Dillon et al. 2006). Experimental infection models of disseminated candidiasis in $TLR2^{-/-}$ mice have also shown modulatory effects of TLR2 on host defense. An initial recent study reported an increased susceptibility of $TLR2^{-/-}$ mice to disseminated candidiasis, and this effect was attributed to decreased production of TNF and MIP-2, as well as decreased neutrophil recruitment (Villamon et al. 2004c). In contrast, TLR2 proved dispensable for the acquired immunity in a model of *Candida*-reinfection (Villamon et al. 2004b). However, the increased susceptibility of $TLR2^{-/-}$ mice to the infection with *C. albicans* was not found by two subsequent studies, who found decreased a fungal burden in $TLR2^{-/-}$ mice, accompanied by decreased production of IL-10, and increased IL-12 and IFNγ production (Bellocchio et al. 2004; Netea et al. 2004). An additional in-vitro study has also shown an increased ability of macrophages from $TLR2^{-/-}$ mice to contain *C. albicans* (Blasi et al. 2005). The source of this discrepancies is unclear, one possible explanation being the use of different *Candida* strains.

In addition to TLR2 and TLR4, Bellocchio and colleagues have also investigated the role of TLR9 for the host defense against *Candida albicans* (Bellocchio et al. 2004). While no increased susceptibility of $TLR9^{-/-}$ mice to disseminated candidiasis has been observed, the fungal burden in the organs of deficient animals tended to be lower than in control mice. However, in contrast to $TLR2^{-/-}$ mice, $TLR9^{-/-}$ animals produced less IL-12 and more IL-10 and IL-4 than control mice

(Bellocchio et al. 2004). It is unclear why this shift towards an antiinflammatory cytokine profile, known to be deleterious for the anticandidal host defense, did not result in a deleterious effect on the outcome of the infection.

The nature of the fungal PAMPs recognized by TLRs during the recognition of *C. albicans* is still under intensive investigation. Whereas collaboration between TLR2 and the β-glucan receptor dectin-1 in the recognition of zymosan and *Candida* microorganisms has been demonstrated, it is likely that β-glucan interacts solely with dectin-1 and induces an amplification loop on cytokine release induced by the interaction of a different candidal PAMP with TLR2 (Brown et al. 2003; Gantner et al. 2003). Alternatively, it has been recently suggested that dectin-1 alone can stimulate IL-2 and IL-10 synthesis through direct interaction with the intracellular adaptor protein Syk (Rogers et al. 2005). As a putative ligand of TLR2 on the surface of *C.albicans*, Joualt and colleagues have proposed the phospholipomannan component (Jouault et al. 2003), although it is yet unclear whether the signals resulting from the phospholipomannan/TLR2 interaction are amplified by dectin-1 mediated signals. Mannans isolated from *C. albicans* and *S. cerevisiae* have been proposed as the fungal TLR4 agonist by Tada and colleagues (Tada et al. 2002), but which of the various mannan structures of *Candida* is responsible for this effect has not been presented. In a recent study, by using *C. albicans* mutant strains defective in either N-linked or O-linked mannans, we have demonstrated that stimulation of cytokine production by *C. albicans* is mediated by three receptors, each of them recognizing a specific fungal PAMP: TLR4 recognizes O-linked mannans, mannose receptor (MR) recognizes N-linked mannans, and dectin-1 recognizes β–glucans (Netea et al. 2006).

In conclusion, TLRs play an important role in the recognition of *C. albicans*. Both TLR2 and TLR4 seem to play an important role in the recognition of the fungus, alone or in cooperation with lectin receptors such as dectin-1 or MR. No complete picture is yet available regarding the candidal PAMPs which activate the various receptors. However, the studies performed to date point out that different TLRs activate specific arms of the antifungal defense, with TLR4 activating especially chemokine production and neutrophil recruitment, while TLR2 induces a Th2 bias (Figure 1). In addition, the recognition of *Candida* by TLRs takes place in the context of multiple recognition pathways involving additional classes of receptors. Thus, lectin-like receptors are very likely to be of great importance for recognition of *Candida spp*. Dectin-1 is involved in recognition of the fungus by interacting with TLR2 (as detailed above), but other lectin receptors such as mannose receptor and DC-SIGN are also involved in recognition of *C. albicans* (Yamamoto et al. 1997; Cambi et al. 2003; Porcaro et al. 2003). In addition, complement receptors are crucial for phagocytosis of opsonized yeasts, an important antifungal host defense mechanism (Morrison et al. 1981a; Alaei et al. 1993).

An important aspect is represented by the consequences of these experimental findings on the role of TLRs for the human disease. In study of women with urogenital *C. albicans* infection, the Asp299Gly TLR4 polymorphism was not associated with either the susceptibility or the severity of urogenital candidiasis (Morre et al. 2003). Moreover, no association between TLR2 and TLR4

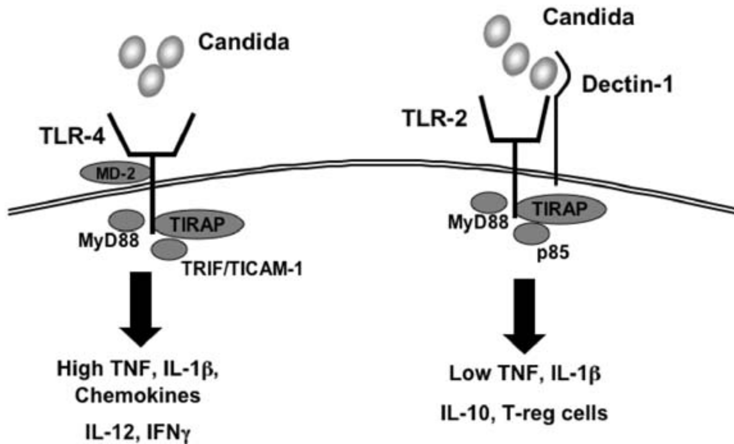

Figure 1. Diffrerential activation of TLR2 and TLR4 by C. albicans. While interaction of *Candida* mannan with TLR4 induces chemokine release, leukocyte recruitment and protection, interaction of *Candida* glucans and phospholipomannan with TLR2 primarily mediates release of IL-10 and T-regulatory cell proliferation, resulting in anti-inflammatory effects

polymorphisms and mucocutanous candidiasis has been found (van der Graaf et al. 2003). In contrast, when we investigated the prevalence of the Asp299Gly TLR4 mutation in patients with disseminated candidiasis, it appeared that this mutation increases significantly the susceptibility of patients to the systemic *Candida* infections (van der Graaf et al, personal communication).

3. RECOGNITION OF *ASPERGILLUS FUMIGATUS* BY TLRs

An important role of TLRs has been also demonstrated for the recognition of *Aspergillus fumigatus*. Invasive aspergillosis is a life-threatening infection which occurs predominantly in immunocompromised patients. As the number of immunocompromised patients has increased, *A. fumigatus* has become the second most common opportunistic fungal infection. A role for TLRs in the recognition of *Aspergillus* was suggested for the first time by Wang et al. who have proposed TLR4, but not TLR2, as a receptor for *Aspergillus* hyphae (Wang et al. 2001). Subsequent studies have supported the hypothesis of TLR involvement in recognition of *Aspergillus* by showing an important role of MyD88 in the *Aspergillus*-induced cytokine production (Mambula et al. 2002; Bellocchio et al. 2004). However, the exclusive role of TLR4 in the recognition of *Aspergillus* hyphae was not confirmed. In contrast, both TLR2 and TLR4 have been demonstrated to be important for recognition of *A. fumigatus* (Mambula et al. 2002; Meier et al. 2003; Netea et al. 2005) and *A. niger* (Meier et al. 2003). Several recent studies have shown that in addition to TLRs, recognition of β–glucans by dectin-1 is also an important feature of host defense (Hohl et al. 2005; Steele et al. 2005; Gersuk et al. 2006).

In addition, two studies have suggested that TLR4 is able to recognize either conidia or swollen conidia, but not hyphae (Mambula et al. 2002; Netea et al. 2005). It has been therefore proposed that the loss of TLR4-mediated proinflammatory signals during germination from conidia to hyphae represents an escape mechanism of *Aspergillus* from the host defense (Netea et al. 2005) (Figure 2). The concept that TLR4-mediated proinflammatory effects are protective against invasive aspergillosis is supported by the recent data showing increased susceptibility of TLR4$^{-/-}$ mice to *A. fumigatus* infection (Bellocchio et al. 2004). In contrast, TLR2$^{-/-}$ and TLR9$^{-/-}$ mice did not have an increased susceptibility to aspergillosis (Bellocchio et al. 2004).

The nature of the cell-wall components of *A. fumigatus* which interact with TLRs has yet to be identified. However, a rational presumption could be that the polysaccharide chains of the *Aspergillus* cells wall, containing glucans, chitin and galactomannan, would be the most logical structures to be recognized by pattern-recognition receptors such as TLRs.

4. RECOGNITION OF CRYPTOCOCCUS NEOFORMANS BY TLRs

The first study on the role of TLRs for the recognition of *C. neoformans* has shown that cryptococcal glucuronoxylomannan binds to TLR2 and TLR4, which is followed by NF-kB translocation in the case of interaction with TLR4, but not TLR2 (Shoham et al. 2001). However, despite induction of NF-kB activation,

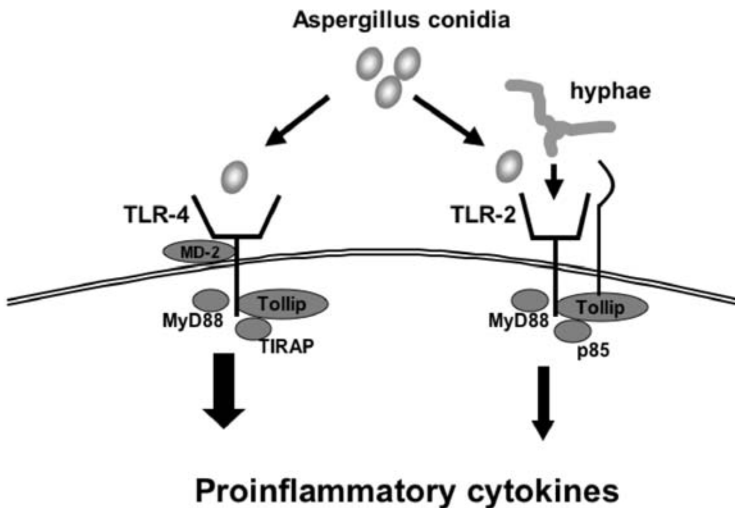

Figure 2. Loss of TLR4-mediated signals during germination of A. fumigatus. The recognition of *A. fumigatus* conidia by monocytes involves both TLR2 and TLR4. Germination from conidia to hyphae results in the loss of proinflammatory TLR4-mediated signals and a TLR2-mediated antriinflammatory cytokine profile, used by *A. fumigatus* as an escape mechanism from the host defense

glucuronoxylomannan interaction with TLR4 failed to result in release of proinflammatory cytokines (Shoham et al. 2001). Later, it has demonstrated that the TIR-associated adapter molecule MyD88 is involved in the induction of cytokines by *C. neoformans*, and the MyD88$^{-/-}$ mice displayed increased susceptibility of to *Cryptococcus* infection (Yauch et al. 2004; Biondo et al. 2005). These data strongly suggest that TLRs are important for the recognition of *C. neoformans* and for the resistance against cryptococcosis.

The role of the specific TLRs for the recognition of *C. neoformans* is still a matter of debate. In a recent study, the defective cytokine production induced by *C. neoformans* in TLR4-defective, but not TLR2, TLR1 or TLR6-deficient mice, suggests that *C. neoformans* induces cytokine release through TLR4/MyD88-mediated signals (Netea et al, personal communication). These findings are supported by Shoham and colleagues who reported the involvement of TLR4 in the recognition of cryptococcal glucuronoxylomannan (Shoham et al. 2001), and by the study of Yauch et al. which reported the lack of involvement of TLR2 in the cryptococcal-induced cytokine production. Although in the latter study the TLR2$^{-/-}$ mice displayed a slightly diminished survival compared to controls, this was not accompanied by a higher outgrowth of *C. neoformans* in the organs, and the mechanism behind the increased mortality is still unknown (Yauch et al. 2004). However, another study has found that it is TLR2, and not TLR4, which mediates cytokine release induced by *C. neoformans* (Biondo et al. 2005). Finally, one recent study failed to find a significant role of either TLR2 or TLR4 for the host defense against cryptococcosis (Nakamura et al. 2006). The source of these discrepancies is yet unknown, although differences such as strain of *Cryptococcus*, or type of deficient mice (missense TLR4 mutation in C3H/HeJ mice vs. stop TLR4 mutation in ScCr mice) might be partly responsible.

The interaction of *C. neoformans* with TLR4 involves glucuronoxylomannan (Shoham et al. 2001), but this does not result in cytokine release. While it is yet unknown which specific cryptococcal component interacts with TLR4 to induce cytokine release, it has been previously demonstrated that mannoprotein constituents of *Cryptococcus* stimulate production of TNF (Chaka et al. 1997). Because *Candida* mannan components stimulate TNF production through interaction with TLR4 (Tada et al. 2002), it is possible that a similar mannan/mannoprotein constituent of *Cryptococcus* is responsible for the TLR4-mediated TNF release. Moreover, it is tempting to speculate that this putative mannoprotein/TLR4 interaction is masked by the GXM component of the cryptococcal capsule, as GXM binds to TLR4 and induces NF-kB translocation, but is unable to stimulate TNF release (Shoham et al. 2001). In this way, by masking stimulatory *Cryptococcus*/TLR4 interactions, the polysaccharide cryptococcal capsule represents an effective virulence factor of *C. neoformans*.

In conclusion, TLRs are important receptors for the recognition of *C. neoformans*. TLR2 and TLR4 seem to be two most important TLRs for the recognition of *C. neoformans*, but a clear picture of the role of these two receptors, as well as of the cryptococcal PAMPs which are being recognized, still needs further research.

5. RECOGNITION OF OTHER FUNGI BY TLRs

In addition to the three major medically important fungi described above, several studies described a role for TLRs in the recognition of other fungal microorganisms. Susceptibility to experimental infection with *Pneumocystis carinii* has been shown to be dependent on effective recognition through TLR4: mice deficient in TLR4 showed increased susceptibility to *Pneumocystis* pneumonia, and this involved impaired production of cytokines by alveolary macrophages (Ding et al. 2005). Another fungal pathogen, *Coccidioides posadasii*, is also recognized by TLRs. However, induction of cytokines by *C. posadasii* is mediated by a mechanism involving MyD88, TLR2 and dectin-1, while TLR4 does not seem to be involved (Viriyakosol et al. 2005). No information is available on other fungal pathogens such as *Histoplasma, Fusarium* or *Scedosporium*.

6. FUNGAL PATHOGENS CAN USE PATHOGEN RECOGNITION SYSTEMS AS ESCAPE MECHANISMS

Recent aspects of PRR biology show that while innate recognition is crucial for an efficient immune response, certain fungal pathogens use PRR-based strategies to evade host defense. Several studies have demonstrated that whereas TLR2 ligation can also induce proinflammatory cytokines, this effect is weaker than that mediated by TLR4 (Hirschfeld et al. 2001). In contrast, TLR2 signals are strong mediators of antiinflammatory effects. The TLR2-induced immunosuppression is either an exaggeration or a premature activation of the normal antiinflammatory effects of TLR stimulation, needed during the recovery phase of the infection for reversing the inflammation. The first study investigating the differential effects of TLR2 and TLR4 stimulation on dendritic cells reported the failure of TLR2 ligands to induce release of IL-12 and IFNγ, thus promoting conditions favorable for Th2-type responses (Re et al. 2001). These initial findings were supported by additional studies (Agrawal et al. 2003; Dillon et al. 2004; Redecke et al. 2004). Studies to elucidate the molecular mechanism behind this phenomenon showed that engagement of TLR2/TLR1 heterodimers by the bacterial lipopeptide Pam3Cys results in stabilization of the transcription factor c-Fos, a suppressor of IL-12, and this leads to the Th2 bias (Agrawal et al. 2003).

These in-vitro data have been accompanied by in-vivo studies demonstrating immunosuppressive effects of TLR2 in fungal infections. The fungal cell wall component zymosan stimulates TLR2 and dectin-1, inducing immunological tolerance (Dillon et al. 2006). A study of anticandidal properties of macrophages has demonstrated that the macrophages defective in TLR2 exhibit increased ability to contain *C. albicans* infection (Blasi et al. 2005). In line with this, it has been demonstrated that TLR2$^{-/-}$ mice are more resistant to disseminated candidiasis, and this is accompanied by a Th1-bias in these mice (Bellocchio et al. 2004; Netea et al. 2004). *Candida albicans* induce immunosuppression

through TLR2-mediated IL-10 release, and this leads to generation of CD4+ CD25+ T-regulatory cells with immunosuppressive potential (Netea et al. 2004; Sutmuller et al. 2006). Similar data have been reported in case of schistosomal lyso-phosphatidylserine–induced TLR2 stimulation leading to generation of IL-10-producing T-regulatory cells (Van der Kleij et al. 2002). The decreased survival to *Candida* infection in $TLR2^{-/-}$ mice in another study is very likely due to the different experimental design (Villamon et al. 2004c). Similarly to *Candida*, tolerance induction by *Borrelia burgdorferi* is conferred through TLR2-mediated release of IL-10, and this has been proposed to explain the immunosuppresion of chronic Lyme borreliosis with persistence of the microorganisms in immunocompetent hosts (Diterich et al. 2003). These effects of TLR2 are reminiscent of those of other pathogen-recognition receptors such as DC-SIGN or mannose receptors, which also mediate microbial evasion through their interaction with mannose-capped lipoarabinomannan from mycobacteria and induction of a Th2 bias (Nigou et al. 2001; Geijtenbeek et al. 2003).

In addition to the induction of antiinflammatory signals through TLRs, certain fungi have developed strategies to either block or avoid their recognition by TLRs and subsequent activation of the innate defence. Thus, *Aspergillus fumigatus* evades immune recognition by germination into hyphae with subsequent loss of TLR4-recognition, whereas the TLR2-mediated IL-10 pathways remain intact, thus shifting the balance towards a permissive Th2-type profile (Netea et al. 2003). In recent experiments, we were able to document a similar evasion of TLR4 recognition by *Candida* hyphae, which stimulate mainly anti-inflammatory cytokines through TLR2, while being unable to be recognized by TLR4 and stimulate IL-12 or IFNγ synthesis (d'Ostiani et al. 2000; van der Graaf et al. 2005). TLR4 is not the only pattern-recognition receptor targeted during fungal germination. In an elegant recent study, Gantner et al. have shown that dectin-1 recognizes the β–glucans at the level of budding scars in the yeast, but it cannot recognize the β–glucans in the hyphae, where they are shielded by a layer of mannans (Gantner et al. 2005). Similar data have been recently reported for *Saccharomyces cerevisiae* (Wheeler et al. 2006). In this way, two major recognition systems (TLR4 and dectin-1) are not able to recognize candidal hyphae, shifting the balance towards an anti-inflammatory response (Figure 3).

All these data suggest that fungal pathogens can use specific signals induced by pattern recognition receptors to either down-modulate the microbicidal functions of leukocytes, or escape immune recognition.

7. CONCLUSIONS AND FUTURE DIRECTIONS

TLRs are a major class of pathogen recognition receptors and the recent studies reviewed here demonstrate that they recognize PAMPs from fungal pathogens, leading to production of cytokines, activation of the microbicidal mechanisms of leukocytes, and resistance to infection. However, in certain circumstances such as interaction of *C. albicans* with TLR2, they induce immunotolerance and an

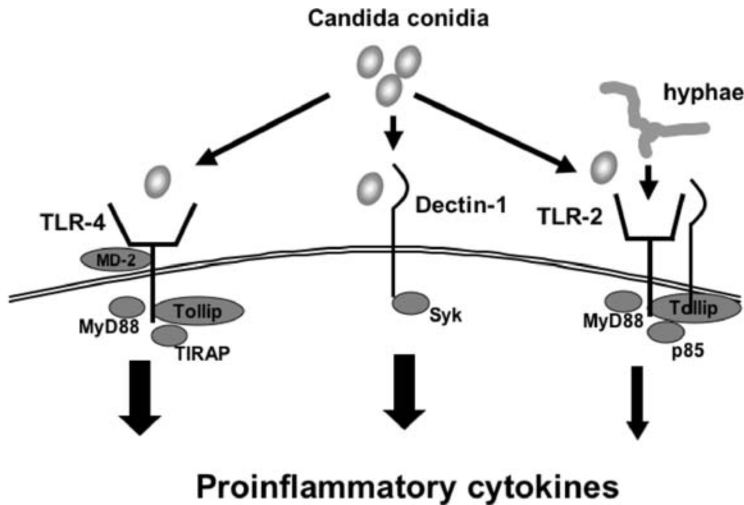

Figure 3. Evasion from pattern recognition during germination by C. albicans. C. albicans escapes immune recognition during germination from yeasts to hyphae by avoiding recognition by at least two pattern-recognition receptors: whereas yeasts are recognized by TLR4, dectin-1 and TLR2 (in addition to mannose receptor and complement receptors, which are not depicted in the figure), the hyphae are recognized only by TLR2

antiinflammatory cytokine profile, which can lead to an evasion from the host defense.

Much has still to be done to fully understand the recognition of fungal pathogens by TLRs:

- are there other TLRs involved in the recognition of fungi: e.g. TLR6 or TLR1, which are known to form heterodimers with TLR2?
- which are the fungal PAMPs interacting with each of the TLRs?
- is there an interaction of other important fungal pathogens (e.g. *Histoplasma capsulatum, Coccidioides imitis, Fusarium* and *Scedosporium*) with TLRs?
- what is the nature of interaction between TLR and non-TLR pattern-recognition receptors in the recognition of fungal pathogens?
- how can these in-vitro and experimental studies on TLR-fungal interaction translate into treatment strategies for the various patients groups with fungal infections?

All these and other potential questions assure us that the field of TLRs research in fungal infections will be a very active one in the years to come. Further understanding of this interaction between TLRs and fungi could potentially provide a basis for new therapeutic strategies, which will contribute to avert the threat of severe fungal infections.

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CHAPTER 12

FUNGAL β-GLUCANS AND THEIR RECEPTORS

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Abstract: β-Glucans are predominant carbohydrates found in the cell wall of many fungi which possess immune-modulating activities. Recognition of these carbohydrates is thought to occur via multiple receptors and at least four different receptors have been identified, including Dectin-1, complement receptor 3, lactosylceramide, and scavenger receptors. There is growing evidence that β-glucan recognition is an important component of anti-fungal immunity, but may also contribute to development of certain diseases. In this chapter we will review each of these aspects, highlighting the roles of β-glucans and their receptors in fungal recognition and immunity

1. INTRODUCTION

The innate immune system is the first line of host defence and must immediately recognise and counter fungal invasion. This arm of the immune system is comprised principally of phagocytic cells, such as macrophages and neutrophils, which ingest and kill these microbial invaders. Information pertaining to the nature of the fungus is then transmitted to T and B lymphocytes, through cytokines, chemokines and the presentation of microbial antigens. This leads to the development of a highly specific, or adaptive, immune response which targets the individual fungal pathogen. For protection against fungal infection in healthy individuals, a T_H1-type immune response is generally required which activates of the fungicidal mechanisms of the phagocytic cells (Romani, 2004).

The ability of the innate immune system to immediately recognise and respond to fungi relies on evolutionarily ancient germ-line-encoded receptors, the pattern-recognition receptors (PRRs) (Janeway, 1992). These receptors recognise highly conserved fungal structures, the pathogen-associated molecular patterns (PAMPs) (Janeway, 1992), such as β-glucan or mannan-based cell wall structures. This enables the host to recognise a broad range of organisms without the need for somatic recombination, characteristically required for the development of the

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adaptive response. PRR recognition occurs either directly (non-opsonic recognition), or indirectly (opsonic recognition) whereby distinct opsonic receptors, such as complement receptors, recognise serum PRR-coated (opsonised) pathogens. PRRs also promote fungal uptake, through the actin-dependent process of phagocytosis, and can induce inflammatory responses, such as cytokine and chemokine production, which activates and recruits other cells to the site of infection, leading ultimately to the generation of the adaptive response. The Toll-like receptors (TLRs; discussed in an earlier chapter) have been of particular interest, as their discovery has revealed the mechanisms of intracellular signalling following pathogen recognition. However, there are many other non-TLR PRRs and although their role is less well defined, these receptors play an equally important role in initiating innate immune responses.

The innate recognition and response to fungi has been attributed to several PRRs which recognise a range of fungal PAMPs, including β-glucan (Table 1 and described in detail in other chapters). Here we will focus on the innate mammalian β-glucan receptors which, as we shall see, play a central role in anti-fungal immunity. We will discuss how the study of these receptors, particularly Dectin-1, has highlighted the importance of non-TLR PRRs in the generation of immune responses. We will also cover β-glucans more generally; touching on their structure, immune modulating activities, potential role in disease, and the possibility that fungal pathogens mask their β-glucan to avoid recognition by the immune system.

2. STRUCTURE AND FUNCTION OF FUNGAL β-GLUCANS

The cell wall of fungi is rigid giving the organism its shape and providing physical protection from the environment. Despite its potential as an anti-fungal drug target, the biosynthesis and structure of the cell wall has only been extensively studied in a few species of fungi, including *Aspergillus* (Fontaine et al., 2000), *Candida* (Klis et al., 2001) and *Saccharomyces* (Klis et al., 2002). In these organisms, the cell wall consists primarily of carbohydrates, including β-glucan, chitin and mannosylated cell wall proteins, the mannoproteins.

The β-glucans, in particular, are the most predominant carbohydrates, making upto more than 50% of the dry weight of the cell wall. These polysaccharides, which are also found in plants and some bacteria, are thought to form hollow helices, like springs, providing elasticity and mechanical strength. β-Glucans consist primarily of long linear $\beta 1 \rightarrow 3$ linked glucose polymers, with varying degrees of branching, which form extensive interchain hydrogen bonds, creating a strong meshwork within the cell wall. Some species also possess $\beta 1 \rightarrow 6$ linked polymers, that can be highly branched, and which are thought to be involved in anchoring the cell wall mannoproteins to the $\beta 1 \rightarrow 3$ glucan layer. A graphical representation of a β-glucan structure and the proposed *Saccharomyces cerevisiae* cell wall is shown in Figure 1.

The classical cell wall structure, as shown in Figure 1, depicts β-glucans buried within the fungal cell wall. However, the recent demonstration that monoclonal

Table 1. PRRs involved in fungal recognition

PRR	Fungal PAMPs	Effect of Receptor Deletion on Fungal Infection in mice
?Chitinase	chitin	ND
?Chitotriosidase	chitin	no effect (*Candida albicans*)[#]
CD14	glucuronoxylomannan	↑ susceptibility (*Cryptococcus neoformans*)
C3	fungal surfaces	ND
CR3	mannose, β-glucan, N-acetylglucosamine, methylmannoside, methylglucoside, complement opsonised pathogens	ND
DC-SIGN	internal mannose, terminal di-mannose	ND
Dectin-1	β-glucan	↑ susceptibility (*Candida albicans*)*
Lactosylceramide	β-glucan	ND
mannose receptor	terminal mannose	no effect (*Pneumocystis carinii, Candida albicans)*
mannose binding lectin	selected monosaccharides (such as mannose, fucose, glucose)	no effect (*Candida albicans*, *Aspergillus fumigatus*)
Pentraxin 3	galactomannan, zymosan	↑ susceptibility (*Aspergillus fumigatus*)
SP-A	selected monosaccharides (such as mannose, fucose, glucose)	↑ susceptibility (*Pneumocystis carini*)
SP-D	selected monosaccharides (such as mannose, glucose), β-glucan	delayed clearance (*Pneumocystis carinii)*
TLR2	phospholipomannan, zymosan, lipoproteins, lipopetides, glycolipids	↑ susceptibility (*Cryptococcus neoformans*) ↑ resistance (*Candida albicans*) no effect (*Aspergillus fumigatus*)
TLR4	mannan, glucuronoxylomannan	no effect (*Cryptococcus neoformans*) ↑ susceptibility (*Candida albicans, Aspergillus fumigatus*
TLR9	CpG DNA	no effect (*Aspergillus fumigatus*) no effect on susceptibility but altered cytokine profile and fungal burden (*Candida albicans*)

?, role as PRR not confirmed; [#]Human studies; ND, not determined; * (P. Taylor, M. Botto, K. Haynes, S. Gordon and G.D.B. unpublished results); CR3, complement receptor 3; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; SP-A, surfactant protein A; SP-D, surfactant protein D; TLR, Toll-like receptor). reprinted in part from (Brown, 2006)

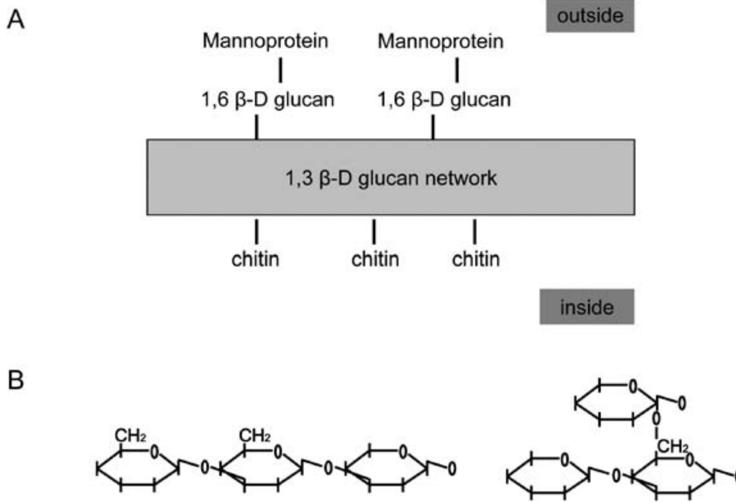

Figure 1. Cartoon representation of the (A) *Saccharomyces cerevisiae* cell wall and (B) the basic linear and branched β-glucan structure

antibodies can detect β-glucans on the cell surface (Torosantucci et al., 2005) and the ability of receptors, such as Dectin-1, to recognise fungi through their cell wall β-glucans (see below), suggests that the traditional view of the fungal cell wall architecture is incorrect. It therefore appears that these carbohydrates are in fact exposed on the fungal cell surface, although they may be restricted to specific areas, such as bud scars, in certain species (Brown, 2006; Gantner et al., 2005).

3. EFFECT OF PURIFIED β-GLUCANS ON THE IMMUNE SYSTEM

Mushrooms and their components have long been known to possess healing qualities, but scientific interest over the last century has largely concentrated on their β-glucans. This focus stemmed from early work in the 1900's, where yeast particles were shown to be capable of inactivating serum complement (von Dungren, 1900), leading to the description of the alternative pathway of complement activation and the development of zymosan, an insoluble particulate *Saccharomyces cerevisiae* cell wall extract, which is composed primarily of β-glucans, but also mannans, chitin, protein and lipids (Di Carlo and Fiore, 1958). Zymosan was subsequently shown to possess a variety of immune stimulating properties, which were attributed to its β-glucan content (Riggi and Di Luzio, 1961). Since then, zymosan has been widely used to study *in vitro* and *in vivo* immune functions, including inflammation, arachidonate release, cell migration and phagocytosis (Brown and Gordon, 2003). Although not truly reflecting the complexity of intact fungal cells, zymosan is often used as a representative fungal particle.

Table 2. Examples of biologically active soluble β-glucans used *in vivo* and *in vitro*

β-Glucan	Source
Glucan Phosphate	*Saccharomyces cerevisiae*
PGG-Glucan	*Saccharomyces cerevisiae*
SSG-Glucan	*Sclerotinia sclerotium*
Scleroglucan	*Sclerotium glucanicum*
Lentinan	*Lentinus edodes*

Administration of purified β-glucans (Table 2) also has a number of beneficial effects on the host, including protection against tumour development and infections with fungal, bacterial, viral and protozoal pathogens (Ross et al., 1999; Tzianabos, 2000). These abilities are thought to stem from the ability of β-glucans to activate leukocytes, although this is influenced by their degree of branching, polymer length, and tertiary structure (Bohn and BeMiller, 1995). β-Glucans have been shown to stimulate the phagocytic, cytotoxic and anti-microbial activities of leukocytes, including the production of reactive oxygen and nitrogen intermediates, as well as inducing a variety of pro-inflammatory mediators, cytokines, chemokines and nuclear transcription factors (Adams et al., 1997; Battle et al., 1998; Czop, 1986; Williams et al., 1996). β-Glucans are also long-lived in mammalian systems, which lack the appropriate glucanases, and, depending on their molecular weight, are excreted by glomerular filtration or degraded slowly through oxidation (Nono et al., 1991; Suda et al., 1996).

β-Glucans can have negative effects on the host. Systemic administration of particulate β-glucan, for example, can induce granuloma formation, hepatosplenomegaly, microembolisation and endotoxin sensitivity, although this has largely been overcome with the development of soluble versions of these carbohydrates (Williams et al., 1996). β-Glucans can also induce lethal toxicity when used in combination with certain non-steroidal anti-inflammatory drugs (Takahashi et al., 2001) and may contribute to respiratory diseases, such as asthma (Rylander and Lin, 2000)(see below).

4. β-GLUCANS AND ANTI-FUNGAL IMMUNITY

As discussed above, fungal β-glucans have long been known to stimulate immune-responses, but the role of β-glucans in the immune response to fungal infection has only been fully appreciated since the identification of the PRRs involved in the recognition of these carbohydrates. Much of the earlier work had focussed on the role of mannose-based cell wall structures, and many of these studies were performed using extracts, such as mannan, which were often contaminated with β-glucans (Goldman, 1988). Indeed, evidence from knockout mice suggests that the mannose receptor, for example, which was long thought to play a role in fungal recognition, was not required for the control of infections with these organisms

(Lee et al., 2003; Swain et al., 2003). However, mannose based recognition systems can play a role in the fungal recognition in selected leukocyte populations, in collaboration with other molecules, such as the β-glucan receptors (Taylor et al., 2004)).

The study of the role of β-glucans in anti-fungal immunity has also been hampered by the inability to generate fungal mutants with complete defects in synthesis of these carbohydrates. Even partial β-glucan mutants have growth defects *in vitro*, highlighting the essential role of these carbohydrates in maintaining the cell wall architecture. Not surprisingly, nearly all these mutants have attenuated virulence in mouse models (see (Umeyama et al., 2006) and (Mouyna et al., 2005) for recent examples), but given their inherent growth defects, it is difficult to infer a role for β-glucans from these studies. Interestingly, one *S. cerevisiae* mutant with changes in its cell wall architecture, including alterations in the composition of its β-glucans, was more virulent in mice, possibly due to the induction of enhanced inflammatory responses, but the mechanisms behind this are unclear (Wheeler et al., 2003).

It is worth noting that β-glucans are released into the circulation during systemic fungal infections, including those caused by *Candida, Aspergillus* and *Cryptococcus* (Obayashi et al., 1995). These carbohydrates could potentially modify immune responses during infection, but their characteristics and effects are unknown. These circulating β-glucans have, however, been proposed to be a diagnostic indicator for the detection of invasive fungal infections and a number of assays have been developed, including one licensed by the FDA which is based on factor G, an invertebrate β-glucan PRR from the horseshoe crab (Miyazaki et al., 1995).

5. β-GLUCAN RECEPTORS

β-Glucan receptors were first identified on monocytes over 20 years ago as phagocytic receptors for zymosan (Czop, 1986). β-glucan receptor activity was subsequently described on a variety of other leukocytes, including macrophages, neutrophils, eosinophils and NK cells, as well as on non-immune cells including endothelial cells, alveolar epithelial cells and fibroblasts. Recognition of β-glucans on these cells is thought to occur via multiple receptors (Kougias et al., 2001; Mueller et al., 2000) and at least four receptors have been identified to date (Figure 2), including complement receptor 3 (CR3), lactosylceramide, scavenger receptors and Dectin-1, which has been identified as the primary leukocyte receptor for these carbohydrates.

5.1. Dectin-1

Dectin-1, or '**De**ntritic cell-associated **C**-type lec**tin-1**', was originally identified by subtractive cloning from an epidermal dendritic cell (DC) line as a receptor that recognised an unidentified T cell ligand (Ariizumi et al., 2000). The receptor was

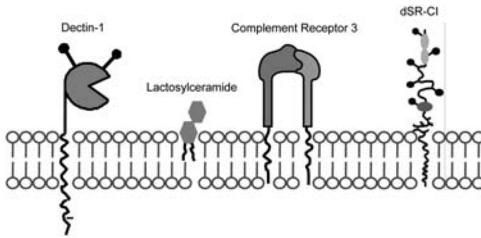

Figure 2. Pattern recognition receptors for β-glucans, including Dectin-1, CR3 and lactosylceramide. Also shown is SR-CI, a Drosophila scavenger receptor shown to recognise β-glucans, as the mammalian scavenger receptor(s) which recognise these carbohydrates has not been identified (See Color Section.)

subsequently re-identified as a β-glucan receptor from a screen of a macrophage-derived cDNA expression library (Brown and Gordon, 2001). Structurally, Dectin-1 is a glycosylated type-II trans-membrane glycoprotein that possesses a single non-classical C-type lectin-like domain, a stalk region and a cytoplasmic tail that contains an immunoreceptor tyrosine-based activation-like motif (ITAM). Dectin-1 is most similar to NK C-type lectins, such as NKG2D and Ly49, which typically recognize endogenous proteins, including MHC class I molecules (Yokoyama and Plougastel, 2003), and the encoding gene is located within the NK receptor gene complex found on human chromosome 12 and murine chromosome 6. Dectin-1 is alternatively spliced into two major isoforms, differing by the presence or absence of the stalk region, which have different functionalities (Heinsbroek et al., 2006; Willment et al., 2001).

Although originally considered to be restricted to dendritic cells (DCs) (Ariizumi et al., 2000), Dectin-1 has been shown to be expressed by a number of other cell types. In mouse, the receptor appears to be largely myeloid restricted and is expressed on macrophages, monocytes, dendritic cells and neutrophils, although a subpopulation of Dectin-1 expressing T cells is also identified (Reid et al., 2004; Taylor et al., 2002). Dectin-1 is highly expressed on alveolar macrophages and cell populations present in inflammatory infiltrates which, along with its distribution in tissues, is consistent with a role in pathogen surveillance (Reid et al., 2004; Taylor et al., 2002). Interestingly, Dectin-1 expression is absent on marginal zone macrophages, suggesting that this receptor does not have an antigen trapping and clearing role similar to that of SIGNR1 (Geijtenbeek et al., 2002), and is also not detected in immune-privileged tissues, such as the eye and the brain. Expression of Dectin-1 can be significantly modulated by cytokines and other biological response modifiers, much of which correlates with the known effects of these agents on the immune response to fungal pathogens (Willment et al., 2003). The human receptor is similarly distributed but is also expressed on B-cells and eosinophils and is not induced in inflammatory infiltrates (Willment et al., 2005).

Functionally, Dectin-1 acts as a typical pattern recognition receptor, recognising both soluble and particulate fungal β-glucans, such as zymosan, but also β-glucans from other organisms including plants and bacteria (Brown and Gordon, 2001).

The recognition of these carbohydrates is specific and metal-ion independent and this receptor does not recognise monomers or polymers with other linkages. Using neoglycolipid microarray technology, Dectin-1 was shown to specifically recognise $\beta 1 \rightarrow 3$ and not $\beta 1 \rightarrow 6$ linkages, and its minimum unit ligand was determined to be a decamer (Palma et al., 2006). Dectin-1 lacks the residues involved in calcium coordination and carbohydrate recognition found in typical C-type lectins and although two residues in the carbohydrate recognition domain, Trp^{221} and His^{223}, were shown to be required for β-glucan binding (Adachi et al., 2004), the mechanism by which this receptor recognises carbohydrates is unknown. Prior to the identification of Dectin-1, CR3 had been proposed to be the principal β-glucan receptor on leukocytes (Thornton et al., 1996). The use of specific carbohydrate inhibitors, blocking monoclonal antibodies, and leukocytes from CR3 deficient mice suggested that Dectin-1 was the primary β-glucan receptor (Brown et al., 2002), and this has now been formally demonstrated with the Dectin-1 deficient mouse (Brown, 2006).

Upon β-glucan binding, Dectin-1 can mediate a variety of cellular responses, including the respiratory burst (Gantner et al., 2003; Underhill et al., 2005), PLA_2 and COX activation (Suram et al., 2006) and ligand uptake through phagocytosis and endocytosis (Figure 3; (Herre et al., 2004; Underhill et al., 2005). Dectin-1 can also induce the production of a variety of cytokines and chemokines, including TNF-α, MIP-2, IL-12, IL-2 and IL-10 and probably others (Brown et al., 2003; Gantner et al., 2003; Rogers et al., 2005; Steele et al., 2003). These responses are mediated by the cytoplasmic ITAM-like motif of Dectin-1, which becomes tyrosine phosphorylated upon ligand binding (Gantner et al., 2003). Although the ITAM-like motif resembles the tandem repeat sequences found in other activation receptors, such as the Fc receptors (Van den Herik-Oudijk et al., 1995)) and DAP12 (Lanier et al., 1998), only the membrane-proximal repeat of Dectin-1 seems to be required for signalling (Brown et al., 2003; Gantner et al., 2003; Herre et al., 2004; Rogers et al., 2005; Underhill et al., 2005).

Unusually, this sequence is able to mediate an interaction with Syk kinase (Rogers et al., 2005; Underhill et al., 2005), which was previously thought to recognise only tandem repeats. Although Syk initiates all downstream signalling events in traditional ITAM containing receptors, such as cellular activation (Turner et al., 1995) and phagocytosis (Crowley et al., 1997), this kinase appears to required for only some Dectin-1 functions and in cell-type specific manner. For example, Syk is required for the respiratory burst (Underhill et al., 2005), IL-2 and IL-10 production, but not TNF-α and IL-12 (Rogers et al., 2005) or phagocytosis in macrophages (Herre et al., 2004; Underhill et al., 2005). These Syk-independent signalling pathways are unknown and are likely to be novel.

The induction of certain cytokines also requires collaborative signalling from the Toll-like receptor (TLR) pathway. In addition to Dectin-1, signals from TLR2 (Underhill et al., 1999) and TLR6 (Ozinsky et al., 2000) were shown to be required for the induction of TNF-α, IL-12 (Brown et al., 2003; Gantner et al., 2003) and IL-2 (Rogers et al., 2005) in response to zymosan. How signalling from Dectin-1

integrates with the TLR pathway is unclear, but as TLRs do not recognise β-glucans it must be triggered through recognition of different ligands in zymosan (Gantner et al., 2003). Dectin-1 and TLR2 colocalise upon binding of zymosan (Brown et al., 2003), and they may form a signalling complex, similar to that required for LPS signalling (Shimazu et al., 1999). It is worth noting that these activities of Dectin-1 were the first example a non-TLR PRR directly contributing to the generation of immune responses.

5.1.1. Role of Dectin-1 in anti-fungal immunity

Although the definitive role of Dectin-1 is yet to be formally demonstrated, there is much evidence to suggest that this receptor has an important function in the immune response to fungi. Dectin-1 can recognise a number of fungal species, including *Saccharomyces* (Brown et al., 2003), *Candida* (Brown et al., 2003), *Coccidoides* (Viriyakosol et al., 2005), *Pneumocystis* (Steele et al., 2003), and *Aspergillus* (Gersuk et al., 2006; Hohl et al., 2005; Steele et al., 2005). This receptor mediates fungal uptake and killing, in part via induction of the respiratory burst (Gantner et al., 2005; Steele et al., 2003), and the production of cytokines and chemokines, including TNFα, MIP-2, MIP-1 α, GM-CSF, G-CSF, IL-1β, IL-1α and IL-6 (Brown et al., 2003; Steele et al., 2003; Steele et al., 2005; Viriyakosol et al., 2005), many of which are known to be protective (Romani, 2004).

Although only demonstrated for zymosan (Rogers et al., 2005), Dectin-1 may induce the production of IL-12, IL-10 and IL-2 in response to whole fungi. While IL-12 induces T_H1-cell polarisation, and would be considered to be protective, the role of IL-10 and IL-2 production is unclear. Indeed, IL-10 has inhibitory effects on anti-fungal immune responses (Vazquez-Torres et al., 1999). However, these cytokines contribute to the development of regulatory T cells (Belkaid and Rouse,

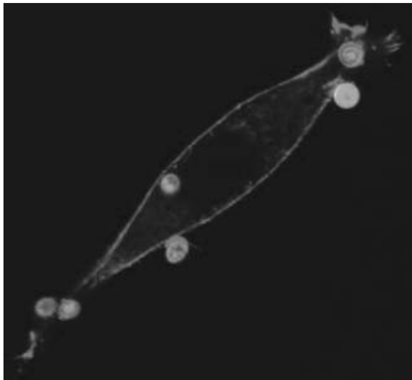

Figure 3. Dectin-1 can mediate the phagocytosis of zymosan and the binding and phagocytosis of yeast particles in transfected cells. Shown are Dectin-1 transfected NIH3T3 fibroblasts binding and internalising fluorescently labelled zymosan (green) via actin (red)-based phagocytic cups. Reproduced with permission from (Brown and Gordon, 2001) (See Color Section.)

2005) and they may be involved in limiting inflammatory pathology and promoting fungal persistence and long-term immunity (Montagnoli et al., 2002; Netea et al., 2004).

The role of Dectin-1 as a key innate receptor in anti-fungal immunity is further supported by emerging *in vivo* evidence. By far the most compelling data is from the Dectin-1 deficient mice which show a greatly increased susceptibility to systemic infection with *Candida albicans*, but are otherwise apparently normal (Brown, 2006). In addition, blockage of Dectin-1 inhibited inflammatory cytokine production and cellular recruitment in the lungs of wild-type mice infected with *Aspergillus fumigatus*, highlighting the central role of Dectin-1 in the recognition of this pathogen (Steele et al., 2005). Unexpectedly, this response was largely TLR2 independent in alveolar macrophages (Steele et al., 2005), implying that Dectin-1 may be capable of directly inducing inflammatory cytokine production in these cells.

5.2. Complement Receptor 3 (CR3)

Complement receptor 3 (CR3, Mac-1, $\alpha_m\beta_2$) is a non-covalently linked heterodimeric glycoprotein consisting of two chains; CD11b, which is encoded on human chromosome 16 and unique to CR3, and CD18, which is encoded on human chromosome 21 and common to all β_2 integrins. Integrins, in general, play important roles in cell – extracellular matrix interactions, in terms of intracellular signalling and physical attachment, and in immunity. The latter is particularly highlighted by diseases such as leukocyte adhesion deficiency, where the lack of β_2 integrins (due to loss of CD18) results in recurrent life-threatening infections (Ehlers, 2000). Many integrins recognise several matrix proteins, often through the recognition of a common tripeptide (Arg-Gly-Asp or RGD), and can induce transmembrane signalling in either direction; ie: “inside-out” signalling, which controls the ligand binding affinity of the receptor, and “outside-in” signalling, whereby ligand binding to the integrin induces intracellular signals (Coppolino and Dedhar, 2000; Giancotti and Ruoslahti, 1999). The signalling mechanisms involved in these processes are not fully understood, but is an area of active interest.

In addition to several matrix proteins, CR3 recognises a large variety of other endogenous ligands and is considered to be one of the most promiscuous integrins (Plow and Zhang, 1997). CR3 acts as a receptor for the complement component, iC3b, opsonised on the surface of pathogens, and as a non-opsonic PRR, recognising a number of exogenous ligands, including microbial products. CR3 also acts as a lectin, recognising a variety of carbohydrates, including β-glucan (Thornton et al., 1996). While most ligands are thought to bind to the I domain, the lectin domain maps to a different, more C-terminal, site of CR3 (Diamond et al., 1993; Thornton et al., 1996).

CR3 is widely expressed on leukocytes and can mediate many functions on these cells. The receptor has been described on monocytes, macrophages, dendritic cells, neutrophils, eosinophils, natural killer cells and on some T and B cells, and can be upregulated following cellular activation (Ross, 2000). Upon ligand recognition,

CR3 can induce cellular responses, including cytotoxicity, phagocytosis, adhesion and migration. However, the generation of some of these responses, such as phagocytosis (Wright and Silverstein, 1982) and cytotoxicity (Xia et al., 1999), require additional stimuli. Furthermore, CR-mediated recognition does not induce protective responses, such as the respiratory burst (Wright and Silverstein, 1983), and can repress proinflammatory signals (Marth and Kelsall, 1997). As a result, CR3 is thought to be targeted by many intracellular pathogens for "stealthy" cellular entry.

The ability of CR3 to recognise β-glucans was first described over twenty years ago (Ross et al., 1985) and this receptor has been shown to be involved in a number of responses to these carbohydrates, perhaps the most notable of which, is its involvement in the anti-tumourigenic properties of β-glucans. Binding these carbohydrates to the lectin domain has been shown to prime leukocytes for CR3-dependent cytotoxicity of iC3b coated tumour cells, and the requirement for CR3 in this process has been demonstrated in $CR3^{-/-}$ knockout mice (Vetvicka et al., 1996; Xia et al., 1999). Similarly, β-glucans can enhance complement-mediated haematopoietic recovery (Cramer et al., 2006), and in both these processes, the initial complement deposition appears to be antibody mediated (Cramer et al., 2006; Yan et al., 1999). Interaction of CR3 with β-glucan has also been shown to affect neutrophil chemotaxis, adhesion and transendothelial migration (Harler et al., 1999; Leblanc et al., 2006; Tsikitis et al., 2004; Xia et al., 2002).

It should be mentioned that the presence of these activities led to the proposal that CR3 was the principal β-glucan receptor on leukocytes (Ross, 2000). However, the demonstration that leukocytes lacking CR3 could still recognise and respond to β-glucans (Brown et al., 2002; Gantner et al., 2005; Muller et al., 1996) and the subsequent discovery of Dectin-1 (see above), has cast some uncertainty on exact role of this receptor.

5.2.1. Role of CR3 in anti-fungal immunity

Of its ligands, the ability of CR3 to act as an opsonic receptor for iC3b coated pathogens is perhaps one of its most relevant functions in terms of fungal recognition (this is discussed in detail in an earlier chapter). CR3 can directly (non-opsonically) recognize a number of fungi, including *Candida, Blastomyces, Histoplasma* and *Cryptococcus*, although the mechanisms of recognition by this receptor appear to be different for each species and may not involve β-glucan (Brandhorst et al., 2004; Bullock and Wright, 1987; Forsyth and Mathews, 2002; Forsyth et al., 1998; Taborda and Casadevall, 2002). Fungal pathogens may actually target this receptor to avoid inducing protective host responses, as evidenced by the increased resistance of $CR3^{-/-}$ mice to infection with *Blastomyces dermatitidis* (Brandhorst et al., 2004), for example.

5.3. Lactosylceramide (LacCer)

Lactosylceramide (LacCer; Galβ4Glcβ1Cer; CDw17) is a glycosphingolipid consisting of a hydrophobic ceramide lipid and a hydrophilic sugar moiety, which is found in microdomains in the plasma membranes of many cell types. LacCer was

identified as a β-glucan receptor through a biochemical screen with a radiolabelled high molecular weight β-glucan (PGG-glucan) (Zimmerman et al., 1998). The interaction of LacCer with β-glucan has been shown to induce a number of cellular responses *in vitro*, including cytokine production (MIP-2 and TNF-α (Evans et al., 2005; Hahn et al., 2003)), NF-κB activation (Evans et al., 2005; Wakshull et al., 1999) and enhancing the respiratory burst and microbicidal activity of leukocytes (Wakshull et al., 1999). How LacCer mediates these activities is unclear, but it may involve clustering and subsequent activation of Lyn kinase (Iwabuchi and Nagaoka, 2002).

5.3.1. Role of LacCer in anti-fungal immunity

Lactosylceramide has been to shown to mediate the attachment of a number of microbes and may play a role as an adhesion receptor for pathogens (Karlsson, 1989). Indeed a number of fungi, including *Candida albicans, Cryptococcus neoformans, Saccharomyces cerevisiase* and *Sporotrichum schenckii*, can bind to this receptor (Jimenez-Lucho et al., 1990). More recently, *in vitro* evidence using isolated β-glucan extracts from *Pneumocystis carinii*, suggests that LacCer may play an important role in triggering innate responses to these organisms, especially in non-immune cells (Evans et al., 2005; Hahn et al., 2003).

5.4. Scavenger Receptor (SR)

Scavenger receptors are a large heterogeneous family of cell-surface glycoprotein PRRs that differ greatly in their structure but which all recognise modified low-density lipoproteins, selected polyanionic ligands and a variety of microbes (Peiser et al., 2002). These receptors are variously expressed on myeloid and some endothelial cells and are involved in both immunity and homeostasis (Taylor et al., 2005). Scavenger receptors have been implicated in β-glucan recognition in several studies (Dushkin et al., 1996; Rice et al., 2002; Vereschagin et al., 1998), but only a Drosophila receptor, SR-CI, has been shown to bind these carbohydrates (Pearson et al., 1995). Although thought to recognise the basic β-glucan structure, the affinity of scavenger receptor(s) for these carbohydrates may be influenced by the charge of the polymer (Rice et al., 2002).

5.4.1. Role of scavenger receptors in anti-fungal immunity

Little is known of the role of scavenger receptors in fungal recognition, and of the receptors described to date, only SRCL-P1 has been shown to recognise yeast (Ohtani et al., 2001). Others, including MARCO, appear unable to recognise these organisms (Elomaa et al., 1995). Given that scavenger receptors have important functions in host defence against other pathogens, particularly bacteria (Mukhopadhyay and Gordon, 2004), it is likely that they will be shown to have some role in anti-fungal immunity.

6. FUNGAL EVASION OF β-GLUCAN RECEPTORS

As the recognition of β-glucan appears to play an important role in anti-fungal immunity is it not surprising that fungal pathogens have evolved mechanisms to avoid recognition through these PRRs (Brown, 2006). The ability of *Candida albicans* to undergo morphogenic changes between yeast and hyphal forms, for example, is thought to contribute to the virulence of this organism (Gale et al., 1998; Lo et al., 1997), and although this is still controversial (Gow et al., 2002), yeast forms induce host-protective responses whereas hyphae do not (d'Ostiani et al., 2000). Indeed, hyphal filaments lack surface-exposed β-glucan and do not induce β-glucan-mediated responses *in vitro* (Gantner et al., 2005), indicating that hyphae may evade immune recognition by masking these carbohydrates. This may also contribute to the ability of the organism to exist as a commensal.

Inflammatory responses to *Aspergillus fumigatus* are similarly β-glucan dependent during conidial swelling and germination, when these carbohydrates are exposed, prior to filamentation and β-glucan masking (Steele et al., 2005; Gersuk et al., 2006; Hohl et al., 2005). Furthermore, the masking of β-glucan by encapsulation in *Cryptococcus neoformans* (Cross and Bancroft, 1995) and the change in cell-wall β-glucan content to α-glucan in *Paracoccidioides brasiliensis*, upon infection of the lung (Borges-Walmsley et al., 2002), indicates that evasion of β-glucan receptors may be a common strategy for fungal pathogens and that it can be achieved through a variety of mechanisms.

7. β-GLUCANS AND DISEASE

Rheumatoid arthritis is a chronic inflammatory autoimmune disease of the joints, characterized by changes in the synovial membranes, and is thought to be mediated by self-reactive T cells (Firestein, 2003). Although intra-articular injection of particulate β-glucans has long been used to model non-autoimmune arthritis in animals (Keystone et al., 1977), these carbohydrates were recently shown also to induce autoimmune forms of this disease in genetically susceptible SKG mice, which harbour a mutation in ZAP70 (Sakaguchi et al., 2003; Yoshitomi et al., 2005). These mice continuously produce arthritogenic T cells, but only develop disease under specific-pathogen free conditions after immune activation by fungal infection, purified β-glucans or other microbial stimuli (Yoshitomi et al., 2005). Inhibition of Dectin-1 function prevented the β-glucan-mediated induction of this disease, indicating that in certain genetic backgrounds, the immune response triggered by this receptor can result in autoimmune disease. β-Glucan-induced rheumatoid arthritis has been proposed to stem from the activation of Dectin-1-expressing synovial macrophages and/or DCs, although there is no evidence that synovial phagocytes are exposed to β-glucan and the mechanism(s) leading to the activation of arthritogenic T cells are unknown (Brown, 2006).

Fungi and their spores can also cause many respiratory disorders in humans, and although the contribution of β-glucans to these is not firmly established (Douwes,

2005), there is much evidence to indicate that they play a role (Rylander and Lin, 2000). *Aspergillus fumigatus*, for example, can induce hypersensitivity disorders in non-immunocompromised patients, including allergic asthma, hypersensitivity pneumonitis and allergic bronchopulmonary aspergillosis (ABPA). Although the mechanisms leading to these disorders are mostly unknown, the high levels of Dectin-1 expressed by alveolar macrophages (Taylor et al., 2002) and the ability of Dectin-1 to initiate β-glucan-dependent inflammatory responses to *A. fumigatus* in the lungs of infected mice (Steele et al., 2005) indicates that Dectin-1, and probably the other β-glucan receptors (Evans et al., 2005; Hahn et al., 2003), are involved in these diseases (Brown, 2006).

8. CONCLUSIONS

The identification of PRRs has provided us with a molecular understanding of pathogen recognition. In fungal infections, the recognition of β-glucans appears to be a central component and the study of the receptors involved have provided us with some unique insights into the mechanics of innate immunity. In addition to infection, understanding the immune recognition of β-glucans also holds promise for the development of therapeutic agents and a better understanding of the role of these carbohydrates in disease. Future studies in knockout mice are now required to provide a more definitive description of the role of these receptors.

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CHAPTER 13

DETECTION OF FUNGI BY MANNOSE-BASED RECOGNITION RECEPTORS

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Abstract: Fungi characteristically display mannose groups on the surface of their cell wall. These mannose residues are crucial for cell wall integrity and fungal viability. They constitute Pathogen-Associated-Molecular-Patterns (PAMPs) that can be detected by specific recognition receptors expressed by mammalian antigen-presenting cells. Fungal mannosylation, the mammalian membrane receptors that have evolved to detect fungal mannose groups and the immune responses resulting from fungal recognition by these receptors are the topics of this chapter. It consists of four parts. First, we give an introduction into fungal mannosylation to lay the ground for understanding mannose-based fungal recognition receptors. In the second part of the chapter we describe common features of mannose receptors. We then focus on two membranous mannose-receptors for which fungal binding has been demonstrated, the dendritic cell specific ICAM-3 grabbing non-integrin receptor (DC-SIGN) and the classic Mannose Receptor (MR). Finally, we give an outlook into potential therapeutic applications by targeting DC-SIGN and MR to prevent and treat fungal infections

1. FUNGAL MANNOSE-GROUPS: PATHOGEN-ASSOCIATED-MOLECULAR-PATTERNS?

The outer shape and stability of fungi is largely determined by the cell wall, whose primary component is a matrix of small polysaccharides, proteins, lipids, and inorganic salts that contains chitin microfibrils. In the yeast *Candida albicans* the cell wall consists of 20 to 50% mannan (mannose polymers), 30 to 60% glucan (D-glucose polymers with glycosidic bonds), 1 to 2% chitin, 2 to 14% lipid, and 5 to 15% protein (McGinnis and Tyring 1996). Additionally, yeasts contain soluble peptidomannans embedded in α- and β-glucans in their outer cell wall (McGinnis and Tyring 1996). Mannose polymers thus form an integral part of the cell wall of most fungi (Masuoka 2004).

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Mechanisms underlying fungal and mammalian protein glycosylation are only partially understood. They differ greatly between different cell types and species (Spiro 2002). Protein mannosylation has been most extensively studied in yeast (Herscovics and Orlean, 1993), where mannose residues are linked to serine or threonine groups of polypeptide chains by O-glycosidic bonds in the endoplasmatic reticulum. In a second step they are elongated and modified in the Golgi apparatus (Figure 1). Patterns of glycosylated proteins exposed at the fungal surface may change depending on the stage of fungal development. In the fungus *Saccharomyces cerevisiae* O-mannosylation is essential for cell wall integrity and cell viability (Strahl-Bolsinger et al. 1999). Reduced protein O-mannosylation in *Candida albicans* leads to defective morphogenesis, attenuated virulence in mouse model systems and reduced adherence to host cells (Ernst and Prill 2001). Since protein mannosylation is indispensable for fungal viability, it is evolutionarily conserved. Mannosylated proteins are a hallmark of fungal cell walls. They form molecular patterns, the so-called pathogen associated molecular patterns (PAMPs) that can be recognised by mammalian pattern recognition receptors (PRRs) expressed on cells of the immune system (Medzhitov and Janeway, 2000). Among them, Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) play the most prominent role. It is important to keep in mind that mammalian cells also display endogenous mannose residues on their surface. The ability of host immune cells to distinguish between endogenous and exogenous mannose residues prevents autoimmune reactions and is a prerequisite for an effective immune response against invading

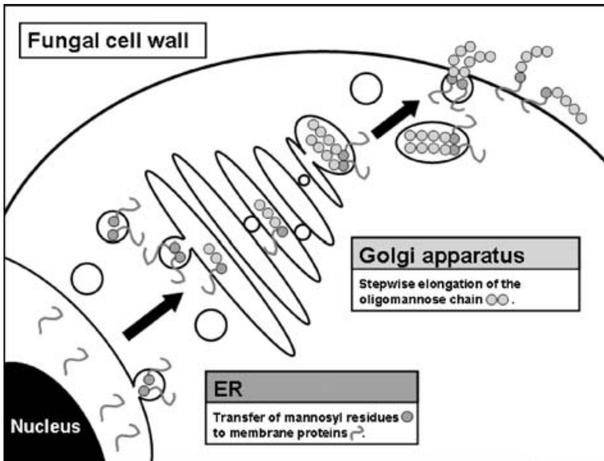

Figure 1. O-mannosylation of fungal cell wall proteins. Proteins are synthesized in the endoplasmatic reticulum (ER) where mannosyl residues are transferred from dolichyl-phosphate-activated mannose to O-groups of serine or threonine residues. Consequently, an α-D-mannosyl linkage is formed. Proteins then move on to the Golgi apparatus. In the Golgi apparatus the oligomannose chains are elongated step by step by α-1,2- and α-1,3-mannosyltransferases. Finally, mannosylated proteins are integrated into the fungal cell wall

fungi. Since CLRs bind to common self and non-self mannose-containing structures, achieving at least partial specificity of the receptor-ligand interaction is a pivotal issue. Characteristic features of the mammalian and fungal cell both contribute to this specificity. On the one hand, CLR specificity is mediated by branching and spacing of fungal mannose residues creating unique carbohydrate recognition profiles for each receptor. Fungi characteristically display densely packed repetitive patterns of carbohydrate residues on their surface, while mammalian cells contain mannose residues at a much lower density. On the other hand, multimerization of CLRs on the surface of APCs increases avidity for specific carbohydrate ligands and contributes to receptor specificity.

Recently, it was shown that *C. albicans* yeast budding and cell separation create permanent scars exposing sufficient beta-glucan to trigger antimicrobial responses through the C-type lectin receptor dectin-1 (Gantner et al. 2005). During filamentous growth, no cell separation or subsequent beta-glucan exposure occurs, and the pathogen fails to activate dectin-1. Therefore, *C. albicans* itself controls whether it is recognized by dectin-1. Most intriguingly, pathogenicity of *C. albicans* is largely determined by its dimorphic growth with the yeast form being required for systemic spreading and the filamentous form being required for pathogenicity (Saville 2003). Similarly, the C-type lectin receptor DC-SIGN binds preferentially to yeast conidia and not to hyphae (Cambi 2005, unpublished results). Fungal and immune cell factors thus control the specificity of the fungus/mannose-receptor interaction.

2. CHARACTERISTICS OF MANNOSE-BINDING RECEPTORS

Mammalian receptors recognising carbohydrate structures are called lectins. The lectins binding to mannose residues belong to the family of C-type lectin receptors (CLRs), the "C" indicating Calcium-dependency of sugar binding (Cambi and Figdor, 2003). This chapter will focus on transmembrane CLRs. Based on the orientation of their amino (N)-terminus, they can be grouped in type I (intracellular N-terminus) and type II receptors (extracellular N-terminus). CLRs are pattern recognition receptors whose primary function is to mediate antigen uptake (Figdor et al. 2002). This is in contrast to the other important family of PRRs, the Toll-like receptors (TLRs), that mainly induce cytokine signalling and immune activation after binding to PAMPs (Takeda et al. 2003). The key functional domain of CLRs mediating carbohydrate binding is the carbohydrate recognition domain (CRD). CLRs either contain one or many CRDs. The capacity of C-type lectins to sense microorganisms is highly dependent on the density of the PAMP present on the microbial surface as well as on the degree of multimerization of the lectin receptor. In fact, the arrangement of several CRDs in multimers projects the binding sites in a common direction, in order to allow interactions with the arrays of carbohydrates on microbial surfaces.

The soluble Mannose-Binding Lectin receptor (MBL) present in the plasma is a classic example of how fungal and host cell characteristics contribute to substrate specificity in the CRD/mannose interaction. The binding affinity of MBL

for its target monosaccharide is rather weak (1 mM for a mannoside), and is most likely biologically insignificant. Biologically relevant interactions (dissociation constant $< \mu$M) are generated by concerted multivalent binding of two or more monosaccharide residues (Lee et al. 1991). Multivalency is achieved by two separate mechanisms. First of all, MBL-CRDs only exist as trimers linked via the neck domain of individual monomers. The separate ligand interactions of monomers are consequently combined and result in increased ligand affinity of the trimer (Sheriff et al. 1994). Additionally, MBL-trimers may form dodecamers to further increase avidity (Wallis and Drickamer, 1999). Mutations that compromise assembly of higher order oligomers of human MBL result in reduced capability to activate components of the complement system. This increases both risk and severity of infections and can even lead to autoimmunity (Larsen et al. 2004). In the same line, the soluble lectin receptor, surfactant protein D, secreted at the lung surface forms trimers, whose assembly into dodecamers seems required for the proper regulation of surfactant phospholipid homeostasis and the prevention of emphysema (Zhang et al. 2001). Multimerization is a common mechanism that is also used by membrane-associated mannose receptors to improve ligand binding. The extracellular portion of the Mannose Receptor is composed of eight CRDs, of which at least three are required for high affinity binding and endocytosis of multivalent glycoconjugates. Thus, several CRDs with only weak affinity for single carbohydrates are clustered in one single molecule to achieve higher affinity binding (Taylor et al. 1992).

The membrane bound mannose binding CLR DC-SIGN forms tetramers to increase avidity (Mitchell et al. 2001). Moreover, in the membrane of immature dendritic cells, DC-SIGN resides in clusters that change its ligand binding properties and are even associated with other membrane domains called lipid rafts (Cambi et al. 2004).

The second mechanism to achieve multivalency in the CLR-mannose interaction is the display of multiple mannose residues on the fungal surface. The distance between individual CRDs in the MBL trimers is 45–53 Å. Fungi such as *C. albicans* are coated with tightly packed terminal mannose residues, enabling efficient, multivalent binding of MBL trimers. In vertebrates, high mannose groups are displayed at distances of approximately 20–30 Å. The larger distance between mannose residues in vertebrates can hinder multivalent binding of MBL trimers. Therefore, the differences in mannose residue spacing between yeasts and vertebrates may contribute to MBL substrate specificity and may prevent auto-opsonisation by MBL binding to endogenous proteins.

Among the human CLRs five membranous mannose receptors have been identified (Table 1): the classic macrophage mannose receptor (MR), Endo-180, the dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN), its liver homologue L-SIGN, and Langerin. Until now recognition of fungal mannosylated proteins has been demonstrated only for DC-SIGN and the MR. The latter recognizes end-standing single mannose structures and di-mannose clusters, while DC-SIGN binds to end-standing di-mannoses as well as to internally branched mannose

Table 1. Recognition of fungi by membranous mannose receptors. Only MR and DC-SIGN have been shown to bind to fungi. The immunological consequences of fungal binding to these receptors range from immune activation to immune inhibition and are not fully understood

Receptor	Fungi recognised	Effects of fungal binding
Mannose Receptor (MR)	*P. carinii*,	Immune inhibition
	C. albicans,	Endocytosis, TNFα production
	C. neoformans	T cell activation, DC maturation, IL-12 production
Endo-180	Not known	
DC-SIGN	*C. albicans*,	Endocytosis
	A. fumigatus	DC maturation
L-SIGN	Not known	
Langerin	Not known	

structures (Feinberg et al. 2001). Recognition of fungal mannose groups by CLRs is a complex process involving various receptors and thus providing a high degree of redundancy (Cambi and Figdor 2005).

3. RECOGNITION OF FUNGI BY DC-SIGN AND THE MANNOSE RECEPTOR

3.1. DC-SIGN

DC-SIGN (CD209) is a type II CLR that contains one CRD (Geijtenbeek et al. 2000a). It functions as both cell adhesion and pathogen receptor, is mainly expressed by immature DCs, but can also be detected on tissue resident alveolar and decidual macrophages (Soilleux et al. 2002) and on macrophages in normal lymph nodes (Granelli-Piperno et al. 2005). DC-SIGN mediates contact between T lymphocytes and DCs by binding to ICAM-3 (Geijtenbeek et al. 2000a) and between DCs and neutrophils by interacting with CD11b/CD18 (van Gisbergen et al. 2005). Furthermore, DC-SIGN mediates rolling of DCs on endothelial cells by binding to ICAM-2 (Geijtenbeek et al. 2000b). As pathogen receptor, it recognises various microorganisms such as *HIV, Mycobacterium tuberculosis, Helicobacter pylori, Leishmania mexicana, Schistosoma mansoni, Candida albicans* and *Aspergillus fumigatus* (reviewed in Cambi et al. 2005). DC-SIGN achieves ligand specificity not only by binding primary mannose groups, but also by recognizing specific conformations of mannose residues bound to the primary monosaccharide (Guo et al. 2004). It forms tetramers to increase ligand avidity (Mitchell et al. 2001) and resides in clusters in the cell membrane of immature DCs (Cambi et al. 2004). Bound antigens are taken up and delivered to late endosomes (Engering et al. 2002). We have recently shown that targeting antigens to DCs via DC-SIGN leads to an effective induction of naive and recall T-cell responses (Tacken et al. 2005). On the other hand, *M. tuberculosis* induces IL-10 production by DCs through binding of its cell wall component ManLAM to DC-SIGN and by direct infection (Geijtenbeek

et al. 2003). Surprisingly, binding of antigens to DC-SIGN can thus have opposite effects ranging from immune stimulation to immune inhibition (Figure 3). The mechanisms underlying these contradictory outcomes of mannose/DC-SIGN interactions have not yet been uncovered.

Until now two fungi binding to DC-SIGN have been identified: the yeast *C. albicans* (Cambi et al. 2003) and the fungus *Aspergillus fumigatus* (Serrano-Gomez et al. 2004). DC-SIGN binds to both live and heat-inactivated yeast forms of *C. albicans*. It could even mediate *C. albicans* phagocytosis in the presence of mannose, which inhibits the second important *C. albicans* receptor on DC, MR. Interestingly, on DCs, we observed that *C. albicans* was internalized in vesicles containing both MR and DC-SIGN as well as in mutually exclusive vesicles (Figure 2). It will be interesting to characterize these vesicles in more detail to elucidate whether the destiny of the *C. albicans* containing vesicles enriched in DC-SIGN is different from those enriched in MR.

The mechanisms controlling localization of the receptors in separate phagocytic vesicles are not yet understood. Uptake by MR will target fungi to the late endosomes or lysosomes for degradation, while uptake via DC-SIGN may route fungi to the early endosomes. Interestingly, we found that DC-SIGN can discriminate between different species and morphological forms of *Candida*, predominantly interacting with conidia and not with hyphae of *Candida* species (unpublished results). DC-SIGN contributes to binding and internalization of *Candida* conidia on immature DCs, while it could not mediate binding of DCs to fungal hyphae. Previous reports indicate that internalization of *Candida* hyphae by DCs is mainly mediated by non-lectin receptors such as complement receptor 3, Fc gamma receptor II, and Fc gamma receptor III (Romani et al. 2002). This is in agreement with our recent observation that, unlike neutrophils and monocytes/macrophages, DCs are poor in both intracellular killing and damaging of *C. albicans* hyphae (Netea et al. 2004). Since growth in hyphae is a prerequisite for *Candida* pathogenicity, we

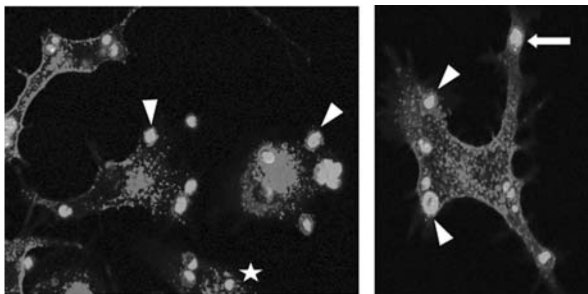

Figure 2. Uptake of zymosan (heat-inactivated yeast cell wall preparation derived from *Saccharomyces cerevisiae*) by immature DCs. FITC-labelled zymosan particles (green) were added to immature DCs and taken up. Cells were fixed and stained with anti-DC-SIGN- (blue) and anti-MR-antibodies (red). Most phagocytic vesicles contain both, MR and DC-SIGN (white arrow heads), while few contain exclusively MR (white arrow) or DC-SIGN (white star) (See Color Section.)

hypothesize that fungal recognition by DC-SIGN might be important at early stages of fungal infection, in the recognition of the conidial form, rather than at later or chronic stages of fungal infection when multicellular filaments of *Candida* have been formed.

We furthermore found that DC-SIGN specifically recognizes *Candida* cell wall mannan and that other mannan preparations do not interfere with the DC-SIGN/*Candida* interaction (Cambi, 2005, unpublished results). Taylor and colleagues showed that in murine resident peritoneal macrophages the DC-SIGN homolog SIGNR1 and the β-glucan receptor dectin-1 co-operate in the nonopsonic recognition of the yeast cell wall component zymosan derived from *Saccharomyces cerevisiae*. Triggering of a SIGNR1- transfected macrophage cell line with zymosan resulted in TNFα production (Taylor et al. 2004). Recently, an association of SIGNR-1 and TLR4 could be detected (Nagaoka et al. 2005). SIGN-R1 enhanced TLR4 oligomerization which was inhibited by treating transfected cells with mannan or with anti-SIGNR1 antibodies. These findings emphasize that fungal recognition by APCs is a complex process involving a network of receptors that can simultaneously interact with each other and with fungal PAMPs (Figure 3).

Next to *C. albicans*, DC-SIGN also binds to *A. fumigatus* which is the second most important fungus causing nosocomial opportunistic infections in immunocompromised patients. DC-SIGN binds to clinical isolates of *A. fumigatus*. Binding and internalization of *A. fumigatus* conidia induce DC maturation and are inhibited by galactomannan derived from *A. fumigatus* cell wall (Serrano-Gómez et al. 2004). DC-SIGN is expressed in lung DC and alveolar macrophages and may therefore play a role in the first line host defence against pulmonary *A. fumigatus* infections. Unravelling the role of DC-SIGN in the immune response against fungi will contribute to our understanding of fungal infections and pathogenicity.

3.2. Mannose Receptor

The mannose receptor (MR, CD206) is the other membrane receptor binding to fungal mannose groups. It was the first receptor identified of a family of four mammalian endocytic receptors with common structural features (reviewed by East, 2002). Next to MR, Endo-180, DEC205, and the phospholipase A2 receptor belong to the family. Recognition of fungal mannose groups has been demonstrated only for MR. In analogy to DC-SIGN, MR binds to endogenous and exogenous ligands. Initially, it was characterized as a receptor responsible for the clearance of endogenous glycoproteins. MR binds to neutrophil-derived myeloperoxidase (Shepherd et al. 1985) and lysosomal hydrolase (Stahl et al. 1978) and protects inflamed tissue from enzymatic destruction by removing these molecules. The anti-inflammatory effects of cytokines, such as IL-4 and IL-10 (Martinez-Pomares et al. 2003) and IL-13 (DeFife et al. 1997, Doyle et al. 1994), and anti-inflammatory drugs such as dexamethasone (Shepherd et al. 1985) are partly mediated by MR upregulation resulting in increased clearance of the above mentioned cytopathic enzymes released in the inflammatory response. Additionally, MR binds to the hormone

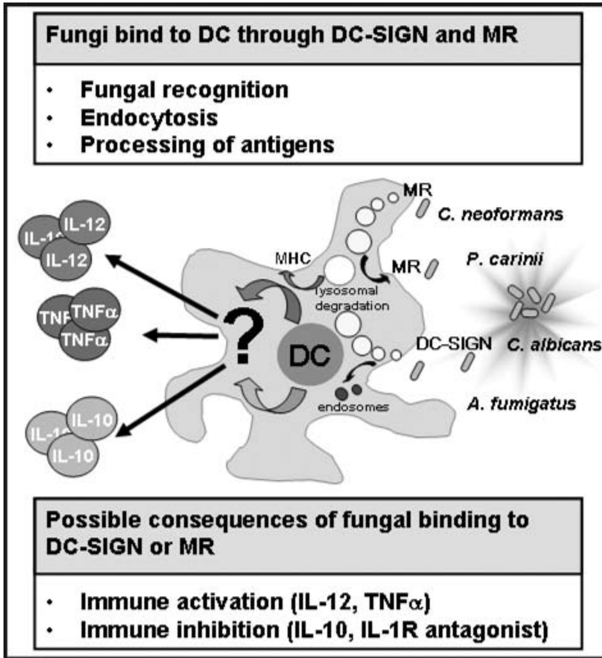

Figure 3. Fungi bind to DCs through DC-SIGN and MR and induce immunity or immune evasion. Binding to fungi induces DC-SIGN- and MR-mediated phagocytosis. After ligand binding MR is routed to the late endosomes while DC-SIGN ends up in early endosomes. It is intriguing that depending on the fungus, its form, and other unknown factors, fungal binding to MR or DC-SIGN may induce immune activation, while under different conditions fungi use the same receptors to evade the host immune system. The underlying molecular mechanisms are not yet known (See Color Section.)

lutropin and participates in controlling its pulsatile release and clearance which are prerequisites for lutropin action (Fiete et al. 1998). MR is a type I C-type lectin expressed on APCs such as immature DCs and macrophages. Murine macrophages and human dendritic cells can shed soluble, functional MR forms (Martinez-Pomares et al. 1998, Jordens et al. 1999). Under steady-state conditions 10–20% of the MR is found at the cell surface while the rest is located intracellulary (Stahl et al. 1980, Wileman et al. 1984). MR contains eight C-type lectin like domains (CTLD), one cysteine-rich domain, and a domain containing fibronectin type II repeats. It is heavily glycosylated which influences its lectin activity (Su et al. 2005). In this respect, terminal sialylation is particularly important. It is required for mannose recognition and influences MR self-aggregation which in turn regulates the avidity of the cysteine-rich domain for sulphated carbohydrate ligands (Su et al. 2005). The CTLDs 4, 5 and 7 are necessary for binding to multivalent ligands (Taylor et al. 1992). Two Ca^{2+} ions are required for mannose binding by domain 4. One is directly involved in binding to carbohydrates while the other interacts with a loop of CTLD4 that may play a role in ph-dependent Ca^{2+} release in the endosome

(Mullin et al. 1997). Research into the function of MR has been hampered by the difficulty of obtaining stable MR cDNA constructs. Just recently, MR was cloned and expressed stably in the non-phagocytic Chinese hamster ovary (CHO) cell line for functional analysis (Le Cabec et al. 2005). Surprisingly, MR expression did not confer the ability to phagocytose particulate MR ligands, such as the *Saccharomyces cerevisiae* cell wall component zymosan, to transfected CHO cells.

Recognition of a plethora of microorganisms such as *HIV, Klebsiella pneumoniae, Mycobacterium tuberculosis, Streptococcus pneumoniae*, and *Leishmania* by MR has been documented (reviewed by Taylor et al. 2005). Furthermore, MR recognizes pathogenic fungi such as *Cryptococcus neoformans, Pneumocystis carinii* and *C. albicans*.

C. neoformans is an encapsulated yeast that causes severe disease in immunocompromised patients (Mitchell and Perfect 1995). Meningoencephalitis is the most common clinical manifestation of cryptococcosis. It usually presents with multiorgan involvement. The involvement of mannose receptor in generating optimal T cell responses against *C. neoformans* mannoprotein was demonstrated by Mansour et al. (2002). Their study showed that *C. neoformans* mannoprotein (MP), consisting 80–90% of mannose, bound to MR-transfected CHO cells and that blocking MR diminished MP-dependent activation of primary T cells significantly. Syme et al. demonstrated that DCs phagocytose *C. neoformans* and that uptake is mostly dependent on MR and CD32 (Syme et al. 2002). After *C. neoformans* uptake DCs induced T cell proliferation. Recently, it was shown that *C. neoformans* mannoproteins activate DCs largely through MR binding (Pietrella et al. 2005). Pietrella et al. demonstrated CD40 and CD86 upregulation on human immature DCs after incubation with two *C. neofomans* MPs (MP1 and MP2). Upregulation could be inhibited by preincubating DCs with anti-MR antibody. Both mannoproteins induced production of the pro-inflammatory cytokines TNFα and IL-12 by DCs, however, only IL-12 production could be inhibited by incubating DCs with anti-MR antibodies. Finally, MP-treated DCs induced proliferative T cell responses of mostly CD4$^+$ T cells and an increase in production of IL-2, IL-10 and IFNγ.

The fungus *P. carinii* also causes life-threatening diseases in immunocompromised patients. *P. carinii* pneumonia is the most prevalent opportunistic infection in patients infected with the human immunodeficiency virus (HIV) (Thomas and Limper 2004). Frequently, it is the AIDS-defining illness and cause of death in afflicted patients. Alveolar macrophages expressing MR are in the first line of innate immune defence against inhaled *P. carinii*. After uptake by alveolar macrophages *P. carinii* is routed to phagolysosomes and degraded (Limper et al. 1997). Binding and uptake of *P. carinii* via MR have been demonstrated (Ezekowitz et al. 1991, O'Riordan et al. 1995). Recently, it was shown that unopsonized *P. carinii* does not induce the production of proinflammatory cytokines such as IL-1β, IL-6 or TNFα and that it inhibits TLR4-mediated TNFα release by alveolar macrophages in response to bacterial lipopolysaccharide (LPS). Furthermore, silencing of MR receptor with siRNA in human alveolar macrophages led to a significant increase in TNFα production in response to *P. carinii* (Zhang et al. 2005). These findings

support an immunosuppressive role of MR in the interaction with *P. carinii* (Figure 3). Similarly, crosslinking MR on human immature monocyte-derived DCs with specific monoclonal antibodies induced production of the anti-inflammatory cytokines IL-10, IL-1R antagonist and the non-signalling IL-1R type II (Chieppa et al. 2003). Additionally, *P. carinii* enhances soluble MR shedding by human macrophages (Fraser et al. 2000). After binding by soluble MR, *Pneumoystis* organisms are less efficiently taken up by membranous MR expressed on alveolar macrophages. Therefore, induction of MR shedding facilitates *P. carinii* immune evasion.

The opportunistic fungus *C. albicans* is not only recognized by DC-SIGN but also by MR. In 1987 Karbassi et al. reported that macrophage-colony stimulating factor (M-CSF) treated peritoneal exudate macrophages showed a significantly enhanced capability to kill *C. albicans* (Karbassi et al. 1987). M-CSF treatment augmented mannose-dependent macrophage binding and ingestion of *C. albicans*. Their data indicated that M-CSF mediated upregulation of MR was at least in part responsible for the observed enhanced killing of *C. albicans* by macrophages. Four years later it was shown that unopsonized *Candida* is only taken up by monocyte-derived macrophages and not by monocytes (Marodi et al. 1991). Uptake was Ca^{2+}-dependent and could be blocked by yeast mannan or mannose-BSA conjugates. Interestingly, the authors did not detect differences in uptake between the pathogenic *C. albicans* and the rarely pathogenic *C. parapsilosis*. These data indicated that mannose-based recognition receptors play an important role in *C. albicans* phagocytosis. In addition, alveolar macrophages were shown to produce TNFα in response to *C. albicans* mannan (Garner et al. 1994). Mannan- but not LPS-mediated cytokine production could be blocked by adding D-mannose or α-methyl-di-mannoside. In human DCs uptake of *C. albicans* is mediated by DC-SIGN (Cambi et al. 2003) and the MR (Newman and Holly 2001). The immunological consequences of *C. albicans* binding to both receptors on the same cells are not yet understood. In analogy to *P. carinii* soluble MR can also bind to *C. albicans* (Martinez-Pomares et al. 1998). Binding to soluble MR could serve as an immune evasion mechanism, inhibiting cellular uptake and efficient killing of *Candida* species.

The functional data of fungi binding to MR obtained so far are inconsistent. While *C. neoformans* and *C. albicans* mannoproteins induced IL-12 and TNFα production respectively through MR triggering, the MR/*P. carinii* interaction seemed to have rather immune-inhibitory effects (Figure 3). In 2002, two groups independently generated $MR^{-/-}$ mice. While $MR^{-/-}$ mice generated by Mi et al. died in utero, $MR^{+/-}$ mice survived and showed increased levels of circulating lutropin due to diminished hormone clearance (Mi et al. 2002). As a result embryonic implantation rates were lower leading to smaller litter sizes. $MR^{-/-}$ mice generated by Lee et al., however, neither showed a lethal phenotype, nor altered lutropin levels. Instead, decreased clearance of glycoproteins with terminal mannose and N-acetylglucosamine residues was demonstrated (Lee et al. 2002). The obvious discrepancies between results obtained by both groups cannot be fully explained.

Surprisingly, $MR^{-/-}$ mice generated by Lee et al. also did not show a higher susceptibility to *C. albicans* and *P. carinii* infections (Lee et al. 2003, Swain et al. 2003). Therefore, the role that MR plays in vivo in host defence against both fungi remains controversial. These findings emphasize the concept of PRR redundancy, with different receptors binding to the same ligand, thus guaranteeing a higher level protection against invading pathogens.

4. TARGETING DC-SIGN AND THE MANNOSE RECEPTOR TO PREVENT AND TREAT FUNGAL INFECTIONS

Detailed knowledge of fungal carbohydrate moieties recognized by individual C-type lectin receptors is a prerequisite for targeting of carbohydrate-protein-interactions for preventive and therapeutic purposes. The enormous complexity of the "glycome" calls for high-throughput analyses of carbohydrate-protein interactions. In the past decade, oligosaccharide microarrays have been developed to screen for protein-carbohydrate interactions. The neoglycolipid technology is one example for generating lipid-linked oligosaccharide arrays from proteoglycans, polysaccharides, glycoproteins and glycolipids and even whole organs or organisms (Feizi and Chai 2004). Soluble C-type lectins are used to probe these microarrays to identify new carbohydrate ligands. Based on the newly identified ligands for known CLRs, it may be possible to develop innovative strategies in treatment and prevention of systemic fungal infections. These systemic infections in particular with *C. albicans* and *A. fumigatus* pose an important clinical problem in the management of critically ill or immunocompromised patients (Sternberg 1994). Possible antifungal strategies include i) treatment with soluble CLRs, ii) induction of antifungal IgG production and iii) alteration of fungal glycosylation processes. Depending on the receptors chosen as targets, patients could be treated with soluble C-type lectin domains. In a scenario where uptake by APC via CLRs is exploited by fungi to escape the immune response, this could make fungi more vulnerable for conventional anti-fungal drugs administered at the same time. Furthermore, anti-mannan antibodies of mostly IgE isotype have been found in some infected patients (Savolainen et al. 1996). Another therapeutic option could be development of agents inducing IgG antibody production to improve fungal opsonisation and uptake. Furthermore, DCs could be targeted via CLRs with carbohydrate structures that mimic fungal sugars to enhance the production of specific antibodies against fungal cell wall components. In the same context hindering fungus uptake via CLRs could lead to improved triggering of other PRRs such as the TLRs improving fungal immunogenicity. Another research approach that may contribute to improved anti-fungal therapies is the analysis of the mechanisms regulating and controlling protein glycosylation. Once these principles are better understood, fungal cells may be targeted by new drugs to specifically alter fungal glycosylation patterns to improve immune responses and to affect fungal viability.

Nevertheless it remains crucial to remember that mannose receptors such as DC-SIGN and the MR also react with endogenous ligands. Treatment with soluble

CRDs or drugs that change protein glycosylation patterns could therefore have unforeseen adverse effects ranging from inhibition of DC invasion into tissues in the case of DC-SIGN, altered lutropin levels in the case of the MR to autoimmunity after treatment with drugs modifying protein glycosylation. In view of the vast progress in understanding protein glycosylation made in recent years, we expect more exciting insights holding therapeutic promises for the future.

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SECTION 4

IMMUNITY TO SPECIFIC PATHOGENS

CHAPTER 14

PNEUMOCYSTIS

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Abstract: *Pneumocystis* is an opportunistic fungal pathogen most prevalent in HIV^+ individuals. Despite a significant reduction in the incidence of *Pneumocystis* pneumonia following the advent of highly active anti-retroviral therapy (HAART), *Pneumocystis* remains the number one AIDS-defining illness. The host response to *Pneumocystis* involves a fine balance between cellular activation leading to organism clearance and lung injury associated with the immune response. This review will focus on the innate and adaptive cellular immune responses to *Pneumocystis* infection, specifically, immune recognition, clearance of infection and resolution of inflammation will be discussed

Keywords: *Pneumocystis*, immunosuppression, pulmonary immunity

1. INTRODUCTION

Pneumocystis carinii organisms were originally described as part of the trypanosomal life cycle and thought to be protozoans for many decades. Sequencing of the small subunit ribosomal RNAs from rat *Pneumocystis* (Edman, Kovacs et al. 1988) and subsequent total RNA sequencing (Stringer, Stringer et al. 1989) determined the organism was a fungal pathogen rather than a protozoan as originally thought. *Pneumocystis* is an atypical fungal pathogen with high levels of cholesterol present in the cell membrane rather than the fungal sterol, ergosterol (Kaneshiro, Ellis et al. 1994). The trophozoite form of the organism, characterized as 3–6 μm in diameter, is the predominant form present in the lung; the cyst form is slightly larger, 4–8 μm in diameter, and contains numerous cysts. *Pneumocystis* organisms isolated from different host species exhibit genetic heterogeneity despite being morphologically similar (Wakefield 1998). As a result of this genetic heterogeneity a nomenclature system has been established such that the host can be identified. Under this system mouse-derived *Pneumocystis* is known as *P. carinii sp. f. muris*

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and human-derived is *P. carinii sp. f. hominis* with each organism designated a special form based on the host it is derived from (Redhead, Cushion et al. 2006). Genetic variation coupled with the inability to grow the organism *in vitro* make studying *Pneumocystis* pneumonia (PCP) especially difficult.

The earliest reported cases of PCP occurred in malnourished children from European orphanages during World War II. Immunocompromised patients were also found to be at risk for developing PCP. A substantial increase in the number of PCP cases was seen following the beginning of the AIDS epidemic of the early 1980s. Peripheral $CD4^+$ T-cell levels below 200 cells/μl were found to be the primary risk factor for HIV^+ patients to develop PCP (Phair, Munoz et al. 1990; Stansell, Osmond et al. 1997). The introduction of anti-Pneumocystis prophylaxis in 1989 and highly active antiretroviral therapy (HAART) in 1996 has led to a significant decrease in the incidence of PCP. Despite the decline in opportunistic infections associated with HIV PCP remains the most common AIDS-defining illness in the United States (Kaplan, Hanson et al. 2000; Morris, Lundgren et al. 2004). These studies found patients not receiving medical care, or non-adherent to treatment, possible drug-resistance and decreased efficacy of treatment when $CD4^+$ T cell counts are low contribute to the inability to eradicate PCP in susceptible populations.

The mode of transmission of *Pneumocystis* has yet to be determined with certainty. Reactivation of latent infection when a patient becomes immunocompromised is the traditional theory of infection supported by studies showing the majority of healthy children test positive for anti-*Pneumocystis* antibodies by the age of 4 years (Pifer, Hughes et al. 1978; Peglow, Smulian et al. 1990; Morris, Beard et al. 2002). Alternatively, clusters of PCP outbreaks suggest airborne person-to-person transmission is likely (Morris, Beard et al. 2002). *Pneumocystis* appears to be ubiquitous in nature and its' DNA sequences have been identified in samples of air spora and soil (Wakefield 1996). One study of HIV^+ patients found gardening or camping to increase the risk of developing PCP (Navin, Rimland et al. 2000). While a definitive answer regarding the transmission of *Pneumocystis* has not been reached it is clear that patients with weakened immune systems are especially at risk for developing pneumonia and investigation into the host defense against *Pneumocystis* is of critical importance.

This chapter will focus on the innate and adaptive immune response against *Pneumocystis* infection. A successful host response against a pathogen involves receptor-mediated recognition, activation of the innate and adaptive immune systems to release inflammatory mediators and oxidants, but also resolution to prevent excessive organ injury. The role of alveolar macrophages, thought to be the key effector cells against *Pneumocystis*, will be reviewed in detail. With regard to adaptive immunity the requirement for $CD4^+$ T cells to prevent infection will be discussed, as will the controversial role of $CD8^+$ T cells. Elucidating the cellular responses involved in PCP-related pathology and clearance of infection remain of central importance as the decline in the rate of infection has leveled off and PCP remains a prevalent opportunistic infection.

2. CELLULAR RESPONSES TO *PNEUMOCYSTIS* INFECTION

2.1. Lymphocytes

As mentioned in the introduction, HIV^+ patients with peripheral blood $CD4^+$ T-lymphocyte counts less than 200 cells/μl are considered at high risk for developing PCP (Phair, Munoz et al. 1990; Stansell, Osmond et al. 1997; Mansharamani, Balachandran et al. 2000). The importance of $CD4^+$ T lymphocytes in host defense against *Pneumocystis* is further supported by CD4 depletion studies in mice. CD4-competent, mice are not naturally susceptible to *Pneumocystis* infection and are able to clear organisms from the lungs within 12 weeks of intratracheal inoculation whereas mice depleted of $CD4^+$ T cells by weekly injections of a monoclonal antibody are unable to clear infection (Harmsen and Stankiewicz 1990; Shellito, Suzara et al. 1990). Discontinuation of antibody depletion permits clearance of the infection in these mice. Due to the clinical relevance and reproducibility of CD4-depletion as a model of *Pneumocystis* infection it has become widely used to study host defense against this pathogen. Another commonly used model to study *Pneumocystis in vivo* exploits mice homozygous for the severe combined immunodeficiency recessive mutation (SCID mice) that lack functional T and B lymphocytes but have functional macrophages, natural killer (NK) cells and myeloid cells (Bosma, Custer et al. 1983; Roths, Marshall et al. 1990). Cohabitation of naïve SCID mice with *Pneumocystis* infected animals leads to spontaneous infection that cannot be cleared and will eventually result in death. The ability to reconstitute these mice with subsets of lymphocytes from wild-type mice makes this an attractive model to study host defense. Immune reconstitution of SCID mice with congenic bone marrow results in clearance of *Pneumocystis* organisms from the lungs but is also associated with a hyperinflammatory reaction that can result in lung injury and death (Roths, Marshall et al. 1990). Treatment of reconstituted SCID mice with CD4 monoclonal antibodies renders them unable to clear *Pneumocystis* infection (Harmsen and Stankiewicz 1990). Furthermore, immune reconstitution of *Pneumocystis* infected SCID mice with purified $CD4^+$ T cells results in clearance of infection similar to animals reconstituted with unsorted lymph node cells (Roths and Sidman 1992). This same study found CD4-reconstituted mice also have lung injury similar to animals reconstituted with unsorted cells.

$CD4^+$ T cells can be divided into Th_1, Th_2, and Th_{IL-17} cells, which are characterized by the secretion of distinct cytokines. Cytokines are small secreted molecules important in mediating the host response to various pathogens. Th_1 cells secrete interferon γ (IFN-γ), Th_2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and the recently described Th_{IL}-17 cells secrete IL-17 (Aggarwal, Ghilardi et al. 2003). Certain cytokines, including TNF-α are secreted by all subsets. Isolation of $CD4^+$ T-lymphocytes from regional lymph nodes and lungs of *Pneumocystis* infected mice found increased precursor frequency of both Th_1 and Th_2 cells in *Pneumocystis* infected animals suggesting both subsets contribute to the host response during *Pneumocystis* infection (Shellito, Tate et al. 2000). Evidence that these subsets have mutually exclusive functions against *Pneumocystis* and individual subsets alone are

sufficient to clear infection can be seen from IL-4 and IFN-γ knockout mice that are both able to resolve *Pneumocystis* infection (Garvy, Wiley et al. 1997). How $CD4^+$ T-cell depletion results in enhanced susceptibility to *Pneumocystis* infection has yet to be determined. It is unlikely that $CD4^+$ T-cells directly mediate *Pneumocystis* killing. Beck et al investigated the possibility that in the absence of $CD4^+$ T cells dampens the recruitment of effector cells to the lung resulting in inability to clear infection (Beck, Warnock et al. 1991). Interestingly, this study found CD4 depleted mice have exacerbated inflammation characterized by increased $CD8^+$ T-lymphocytes and activated AM but are unable to clear infection without CD4 help; in contrast to CD4-competent mice require minimal inflammation to clear infection.

CD4 help may function to promote the production of a *Pneumocystis* -specific antibody response. Helper T-cells mediate the production of antibodies from B lymphocytes. Immunocompetent mice immunized by intratracheal inoculations of *Pneumocystis* prior to depletion of $CD4^+$ T-cells and *Pneumocystis* challenge are able to clear organisms from the lung despite the absence of $CD4^+$ T-cells (Harmsen, Chen et al. 1995). It is believed this clearance may be antibody-dependent as these mice have significant levels of *Pneumocystis* – specific IgG in their sera. In this study the efficacy of the vaccination is dependent on the presence of an intact immune system at the time of vaccination then subsequent immunosuppression and infection. Recently there has been promising data that CD4-independent vaccination against opportunistic pathogens is possible by gene modification. Dendritic cells (DCs) transduced with CD40L and pulsed with *Pneumocystis* are able to induce significant *Pneumocystis*-specific IgG responses in the absence of $CD4^+$ T-cells (Zheng, Shellito et al. 2001). CD4-depleted animals vaccinated with these *Pneumocystis*-pulsed, CD40L-modified DCs are protected from *Pneumocystis* infection.

The significance of the type of antibody response involved in clearance remains under investigation. The type of antibody response initiated is determined by the cytokine environment; cytokine production by Th_1 cells promotes IgG2a production and Th_2 cell products promote IgG1 and IgE production. Both Th_1 and Th_2 antibody responses are associated with clearance of *Pneumocystis* as both IL-4 and IFN-γ knockout mice clear infection though the two mouse strains mount differential antibody responses (Garvy, Wiley et al. 1997). These studies show promising data that resolution of infection involves antibody secretion. However, more recent data provides direct evidence that antibody involvement enhances clearance but is not required for killing (Lund, Schuer et al. 2003). Mice lacking CD40 expression on B-cells, rendering them unable to produce *Pneumocystis* -specific IgG, are still capable of clearing infection though this clearance is delayed (Lund, Schuer et al. 2003). Although antibody production is not required for organism clearance B cell-deficient mice are more susceptible to infection than wild-type animals (Marcotte, Levesque et al. 1996) providing further evidence that while required antibody secretion can enhance clearance and may therefore be therapeutically relevant. Further investigation regarding the mechanism of $CD4^+$ T lymphocyte-mediated

clearance of *Pneumocystis* is essential to understanding the susceptibility to this organism in immunocompromised patients.

$CD8^+$ cytotoxic T cells are increased in the lungs during *Pneumocystis* infection in the absence of CD4 (Beck, Warnock et al. 1991). Depletion of $CD8^+$ T cells alone does not render mice more susceptible to infection nor does it alter their ability to clear organisms from the lungs (Harmsen and Stankiewicz 1990; Beck, Newbury et al. 1996). Additionally, *Pneumocystis* infected SCID mice reconstituted with purified $CD8^+$ T cells are unable to clear infection (Roths and Sidman 1992). Reconstitution with $CD8^+$ T-cells did not lead to a hyperinflammatory reaction as $CD4^+$ T-cell reconstitution of SCID mice does (Roths and Sidman 1992). The role of $CD8^+$ T-cells in the absence of $CD4^+$ T-cells is controversial. One study found depletion of $CD8^+$ T cells in addition to $CD4^+$ T cells results in more intense infection compared to depletion of either alone (Beck, Newbury et al. 1996). *In vitro* $CD8^+$ T-cells proliferate in response to *Pneumocystis* antigen and secrete IFN-γ indicating $CD8^+$ T-cells may have effector functions against *Pneumocystis* (Beck, Newbury et al. 1996). Conflicting with these data Wright et al found depletion of both $CD4^+$ and $CD8^+$ T-cells results in enhanced clearance compared to the depletion of $CD4^+$ T-cells alone (Wright, Gigliotti et al. 1999). This study failed to show data for $CD8^+$ T-cell depletion alone for comparison. Parameters of lung injury, including oxygenation, lung compliance, respiratory rate, albumin leakage and lactate dehydrogenase activity, were exacerbated in $CD4^+$ T-cell depleted mice but were similar to control levels in CD4/CD8 depleted animals. The author's suggest that $CD8^+$ T-cells elicit a hyperinflammatory reaction that is ineffectual at clearing infection. Data from our laboratory suggests that a specific subset of IFN-γ secreting $CD8^+$ T-cells are able to function as effector cells against *Pneumocystis* infection in the absence of $CD4^+$ T-cells (Kolls, Habetz et al. 1999; Mc Allister, Steele et al. 2004). Overexpression of IFN-γ by gene transfer enhanced clearance in the absence of CD4 but was ineffective if both CD4 and CD8 T-cells were depleted (Kolls, Habetz et al. 1999). More direct studies show immune reconstitution of *Pneumocystis* infected SCID mice with $CD8^+$ T-cells from AdIFN treated animals are able to clear infection compared to transfer of $CD8^+$ T-cells from AdEGFP treated mice (Mc Allister, Steele et al. 2004). Interestingly, reconstitution with T-cells from control animals results in increased lung injury compared to mice reconstituted with $CD8^+$ T-cells from AdIFN mice and non-reconstituted mice. Taken together these data indicate $CD8^+$ T-cells with a T-cytotoxic 1 phenotype, i.e. IFN-γ secreting, have the ability to function as effector cells against *Pneumocystis* and modulation of the immune response may be necessary to achieve this effector function and prevent a hyperinflammatory reaction in the absence of $CD4^+$ T-cells.

The hyperinflammatory reaction associated with $CD8^+$ T-cells in the absence of $CD4^+$ T-cells described by Wright et al may also be explained by a loss of regulatory T-cells (T_{Reg}). Thymically derived $CD4^+CD25^+$ T_{Reg} cells expressing the transcriptional repressor forkhead box P3 (FOXP3) are naturally occurring suppressors of immunity important in the prevention of autoimmunity and the

control of immunopathology following resolution of infection (Mills 2004; Belkaid 2005). Reconstitution of *Pneumocystis* infected SCID mice with $CD4^+CD25^-$ effector T-cells (T_{Eff}) leads to clearance of *Pneumocystis* organisms from the lung accompanied by a hyperinflammatory reaction that eventually leads to mortality (Hori, Carvalho et al. 2002). *Pneumocystis* infected SCID mice reconstituted with $CD4^+CD25^+$ T_{Reg} cells are not able to clear infection nor do they develop a hyper-inflammatory reaction. It is possible that enhanced lung injury associated with CD4-depletion is due to loss of regulatory function that results in an uncontrolled $CD8^+$ T-cell inflammatory response. In support of this theory is recent data showing that B-cell deficient mice depleted of $CD8^+$ T-cells have altered levels of T_{Reg} : T_{Eff}, with the level of BAL fluid $CD4^+$ T_{Eff} cells substantially increased in CD8 depleted animals (Swain, Meissner et al. 2006). Further investigation into the role of T_{Reg} cells should clarify the coordination of $CD4^+$ and $CD8^+$ T-cells in immunity against *Pneumocystis*.

$\gamma\delta$-TCR^+ T-cells also exhibit regulatory functions though the role of these cells in *Pneumocystis* infection has not been extensively studied. $\gamma\delta$-TCR^+ T-cells are increased in the BAL fluid of HIV^+ patients with *Pneumocystis* infection, as well as in *Pneumocystis* infected mice (Kagi, Fierz et al. 1993; Agostini, Zambello et al. 1995; Steele, Zheng et al. 2002). $\gamma\delta$-TCR^+ T-cell-deficient mice are more effective at clearing *Pneumocystis* from the lungs than wild-type animals. Augmented clearance was associated with increased $CD8^+$ T-cell recruitment to the lung and higher IFN-γ production (Steele, Zheng et al. 2002). These data indicate $\gamma\delta$-TCR^+ T-cells may function to regulate $CD8^+$ T_{c1} immune response during the course of infection.

2.2. Alveolar Macrophages

While $CD4^+$ T cells have been implicated in susceptibility to *Pneumocystis* infection, alveolar macrophages (AM) are believed to be the primary effector cells responsible for clearance of organisms from the lung. Liposomal administration of bisphosphonates has been used to selectively deplete macrophages in animal models of *Pneumocystis*. Macrophage depletion prior to *Pneumocystis* infection impairs the animals' ability to clear infection with macrophage-depleted animals exhibiting a 2.5 fold higher lung burden of *Pneumocystis* compared to controls (Limper, Hoyte et al. 1997). Recently great strides have been made to better understand the mechanism(s) of *Pneumocystis* recognition, binding, uptake and killing by AM though many more questions remain to be answered. The ability of macrophages to directly recognize pathogens is believed to be mediated through a variety of germline encoded cell surface receptors referred to as pattern recognition receptors (PRR). Recognition of pathogen-associated molecular patterns (PAMPs) by PRR results in macrophage activation, secretion of inflammatory mediators, endocytic and phagocytic activity. Many PRR have been described but perhaps the most extensively studied with regards to *Pneumocystis* is the mannose receptor (MR).

The MR is a 170-kDa type-I transmembrane protein present on macrophages, dendritic cells, and subsets of endothelial cells. It is considered a C-type lectin receptor due to its ability to bind carbohydrates in a Ca^{2+}-dependent manner (McGreal, Miller et al. 2005). The MR has five domains including an amino-terminal cystein-rich region, a fibronectin type II containing domain, a series of carbohydrate-recognition domains (CRDs), a hydrophobic transmembrane domain and a cytoplasmic carboxy-terminal domain. The eight extracellular calcium-dependent CRDs mediate pattern recognition of mannose and fucose on pathogens while the cytoplasmic tail is necessary for phagocytic activity (Ezekowitz, Sastry et al. 1990; Taylor, Conary et al. 1990; Stahl and Ezekowitz 1998). The MR is able to recognize a wide range of pathogens including Gram-negative and Gram-positive bacteria, yeasts, and parasites (Stahl and Ezekowitz 1998). The identification of a 120-kD major surface glycoprotein deemed gp120 or glycoprotein A (gpA) on the surface of *Pneumocystis* with a high-mannose content suggested recognition of *Pneumocystis* by AM may be mediated through the MR (Radding, Armstrong et al. 1989).

Indeed *in vitro* studies have shown AM uptake of *Pneumocystis* is mediated by MR (Ezekowitz, Williams et al. 1991; Zhang, Tachado et al. 2005). Ezekowitz et al incubated fluoresceinated *Pneumocystis* with rat or human AM monolayers and found the macrophages bound and ingested *Pneumocystis* leading to the release of superoxides. Preincubation of the AM monolayers with MR inhibitors led to a dose-dependent inhibition of *Pneumocystis* attachment (Ezekowitz, Williams et al. 1991). Gene silencing of MR also resulted in significant reduction of *Pneumocystis* phagocytosis by human AM (Zhang, Tachado et al. 2005). Further confirmation that MR expression is sufficient for *Pneumocystis* recognition is seen by transfection of COS cells with MR cDNA. Cells expressing MR were not only able to bind organisms but also ingest the attached *Pneumocystis*, whereas untransfected cells fail to interact with *Pneumocystis* (Ezekowitz, Williams et al. 1991). Binding studies demonstrate *Pneumocystis* gpA is the ligand for MR. Addition of soluble gpA, which competes with *Pneumocystis* surface gpA for binding to macrophage receptors, inhibits macrophage interaction with *Pneumocystis* in a concentration-dependent manner (O'Riordan, Standing et al. 1995).

As previously mentioned, HIV^+ individuals with $CD4^+$ T-lymphocyte counts less than 200 cells/μl are susceptible to *Pneumocystis* infection. This increased susceptibility does not appear to be solely a result of diminished $CD4^+$ T-lymphocyte capacity but also altered host AM response. AM from HIV^+ individuals have an impaired ability to bind and phagocytose *Pneumocystis*. This impairment correlated with severity of disease as determined by $CD4^+$ T-lymphocyte count. This same study found MR expression to be significantly reduced in HIV^+ patients; 98% of AM from healthy individuals expressed MR compared to 89% of cells from HIV^+ patients with peripheral $CD4^+$ T lymphocyte counts $> 200\,\text{cells/mm}^3$, 26% of cells from HIV^+ patients with $CD4^+ < 200\,\text{cells/}\mu\text{l}$, and only 19% of cells expressing MR in patients HIV-seropositive with active *Pneumocystis* pneumonia (Koziel, Eichbaum et al. 1998). Expression of MR on AM from healthy individuals

is significantly reduced following *in vitro* infection with HIV-1 compared to uninfected AM (Zhang, Zhu et al. 2004).

The ligation of macrophage MR by *Pneumocystis* leads to the release of superoxides, cytokines and chemokines. The signaling pathways involved in this response have not been extensively studied though a number of recent publications suggest NF-κB activation is involved (Zhang, Zhu et al. 2004; Zhang, Tachado et al. 2005). With a multiplicity of infection (MOI) of 5:1 (*Pneumocystis* to AM) the AM interaction with *Pneumocystis* results in NF-κB nuclear translocation in a MR-dependent manner (Zhang, Zhu et al. 2004). However, increased MOI results in suppression of NF-κB nuclear translocation compared to unstimulated cells. NF-κB nuclear translocation in response to *Pneumocystis* was blunted in AM isolated from HIV^+ individuals, as well as in AM from healthy individuals infected with HIV-1 *in vitro* (Zhang, Zhu et al. 2004). This impaired NF-κB response is not seen following LPS stimulation suggesting there is a specific impairment in recognition of *Pneumocystis* in HIV infected AM. Taken together these studies indicate MR is a prime candidate for host recognition, uptake, and destruction of *Pneumocystis*. Furthermore, susceptibility of HIV^+ individuals for *Pneumocystis* infection may be due to downregulation of macrophage MR on HIV infected cells.

In direct contrast to the aforementioned data, competitively blocking the MR with mannan does not affect AM-mediated killing of *Pneumocystis* (Steele, Marrero et al. 2003) and studies using MR knockout (MR-KO) mice have found MR is not required for *Pneumocystis* phagocytosis and these animals are not more susceptible to *Pneumocystis* infection (Swain, Lee et al. 2003). In this study, both CD4-competent and CD4-depleted MR-KO mice had comparable *Pneumocystis* pulmonary load at various timepoints after infection compared to wt animals. While MR-KO mice were no more susceptible to *Pneumocystis* infection and showed no impairment in clearance they had significant increases in AM and neutrophils compared to wild-type (wt) animals and indicators of lung injury, including BAL albumin, protein and LDH levels, were elevated in MR-KO mice infected with *Pneumocystis* compared to their wt counterparts. This suggests MR-KO mice may be less efficient at clearing infection and thus require greater cellular influx to fight infection though they are clearly capable of *Pneumocystis* clearance. Enhanced cellular influx in the lung may also be due to increased chemokine and inflammatory cytokine production in MR-KO mice, a possibility the author's did not discuss. Zhang et al have shown MR has a negative regulatory role on inflammatory cytokine production in response to *Pneumocystis* in that gene silencing of MR leads to increased production of TNF-α, IL-1β and IL-6 (Zhang, Tachado et al. 2005). Contrasting results regarding the importance of MR in innate immunity to *Pneumocystis* infection may be due to differences in species, cellular and model systems. As a result of difficulties culturing *Pneumocystis* many investigators use *Pneumocystis* isolated from rat lung to expose human macrophages, which may not be the best system to investigate natural course of infection. Swain et al have shown MR-KO mice do not have a lag in *Pneumocystis* clearance compared to

wt mice suggesting other receptors acting alone or in combination are involved in *Pneumocystis* uptake and degradation (Swain, Lee et al. 2003). A number of recent papers suggest another C-type lectin receptor, Dectin-1, plays a significant role in host defense against *Pneumocystis* infection.

Dectin-1 is a small type-II transmembrane receptor containing a lectin-like carbohydrate recognition domain, and an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail (Brown 2001). Dectin-1 is a PRR that recognizes β-1,3-linked and β-1,6-linked glucans from fungi and plants to mediate non-opsonic phagocytosis, as well as a β-glucan-independent endogenous ligand on T cells that promotes proliferation. The receptor is expressed on macrophages, neutrophils, dendritic cells, and a subset of T cells (Taylor, Brown et al. 2002). Dectin-1-dependent phagocytosis requires the ITAM-like motif present in the cytoplasmic tail of the receptor (Herre, Marshall et al. 2004). β-glucans are a major component of the *Pneumocystis* cell wall shown to elicit the release of inflammatory cytokines and chemokines from AM (Lebron, Vassallo et al. 2003). Dectin-1 is the predominant β-glucan receptor present on AM (Brown, Taylor et al. 2002). Blockage of the β-glucan receptors on AM by preincubation with glucan from *Saccharomyces cerevisiae* results in a significant decrease in AM-mediated killing of *Pneumocystis in vitro* (Steele, Marrero et al. 2003). Specifically blocking Dectin-1 on AM using an anti-Dectin-1 monoclonal antibody, 2A11, abrogates AM-mediated killing of *Pneumocystis* suggesting this is the β-glucan receptor that mediates killing of *Pneumocystis* (Steele, Marrero et al. 2003). In this same study, visualization of FITC-labeled *Pneumocystis* interacting with AM shows the *Pneumocystis* cell wall colocalizes with the Dectin-1 β-glucan receptor. The interest in Dectin-1 as a phagocytic receptor involved in clearance of *Pneumocystis* is relatively new and Dectin-1 expression on AM from patients susceptible to *Pneumocystis* infection has yet to be determined.

Perhaps the most well studied PRR to date, the Toll-like receptors are a family of type I transmembrane PRR conserved from *Drosophila* to plants and animals. Nine TLR have been identified with distinct ligand recognition specificities (Reviewed in (Medzhitov 2001; Akira and Takeda 2004). With regards to fungal pathogens TLR2 is the primary TLR involved in recognition. The specific involvement of TLR2 in the recognition, uptake and phagocytosis of *Pneumocystis* has not been investigated. Recently TLR4 has been shown to play a role in the inflammatory response to *Pneumocystis* (Ding, Shibui et al. 2005). TLR4 mutant mice have exacerbated histopathology associated with *Pneumocystis* infection compared to wild-type controls despite no difference in organism burden. The PRR are often discussed as mutually exclusive mediators of host defense. However, there is mounting evidence supporting collaborative mechanisms of pathogen clearance (Mukhopadhyay, Herre et al. 2004). Gantner *et al* show reactive oxygen species elaboration and phagocytosis of the fungal particle zymoxan is dependent on Dectin-1 but not TLR2 (Gantner, Simmons et al. 2003). This same study found Dectin-1 and TLR2 co-expression enhances NF-κB activation and cytokine production (Gantner, Simmons et al. 2003). More intense investigation as to how PRR interact to influence the outcome of infection is integral to understanding host immunity.

2.3. Neutrophils

Neutrophils are increased in the lungs during the course of *Pneumocystis* infection and correlate with pulmonary dysfunction and morbidity (Fleury, Escudier et al. 1985; Smith, el-Sadr et al. 1988; Sadaghdar, Huang et al. 1992). Recruitment of neutrophils to the lung correlates with increased levels of the potent neutrophil chemoattractant IL-8 in the BAL fluid (Benfield, van Steenwijk et al. 1995; Benfield, Vestbo et al. 1995; Benfield, Kharazmi et al. 1997). Experimentally, neutrophils do not contribute to lung injury associated with *Pneumocystis* infection, despite clinical observations (Swain, Wright et al. 2004). In this study antibody depletion of circulating neutrophils had no affect on organism clearance or parameters of lung injury. These data were confirmed in genetically modified animals deficient in chemotactic cytokine receptor CXCR2 (Swain, Wright et al. 2004), which is the primary receptor for chemokine-mediated neutrophil chemotaxis to the lung. Thus neutrophils may be indicators of disease severity but do not have effector functions against *Pneumocystis*.

3. MEDIATORS OF HOST DEFENSE

3.1. Surfactant Components, Adhesive Glycoproteins, and Reactive Oxygen and Nitrogen Intermediates

Pulmonary surfactant is a complex of phospholipids and protein secreted by alveolar type II epithelial cells important in reducing surface tension at the air-liquid interface to maintain stability at low lung volumes (Wright 2005). The primary component of surfactant is phospholipid in nature with only about 10% protein. Four surfactant proteins, SP-A, -B, -C, and –D have been defined. With regards to innate host defense, SP-A and SP-D are considered the primary mediators. SP-A and SP-D are members of the collectin family of proteins characterized by an N-terminal collagen-like domain and C-terminal calcium-dependent carbohydrate recognition domain. It has been suggested that surfactant proteins bind to pathogens and function as opsonins that enhance uptake and phagocytosis (Wright 2005). *Pneumocystis* infection results in increased levels of SP-A and SP-D protein in BAL fluid and alters the distribution of these proteins (Atochina, Beck et al. 2001). Both SP-A and SP-D bind to *Pneumocystis* glycoprotein A leading to enhanced attachment to AM (O'Riordan, Standing et al. 1995; Williams, Wright et al. 1996). Furthermore, neutralization of SP-D significantly inhibits the binding of *Pneumocystis* to AM (O'Riordan, Standing et al. 1995). Genetically modified mice provide further evidence the surfactant proteins are important in *Pneumocystis* clearance. Both SP-A and SP-D deficient mice have increased organism burden compared to wild-type animals (Linke, Harris et al. 2001; Atochina, Beck et al. 2004; Atochina, Gow et al. 2004; Linke, Ashbaugh et al. 2005). These animals display an increased inflammatory response to infection that is ineffective at clearing the organism and results in increased lung injury and altered nitric oxide metabolism. The decreased

ability of AM from SP-$A^{-/-}$ mice to bind *Pneumocystis* (Linke, Harris et al. 2001) supports the hypothesis that surfactant proteins function as opsonins.

Other host proteins have been investigated for their ability to enhance the AM response to *Pneumocystis*. Adhesive glycoproteins found in serum may leak into the lungs as a result of capillary disruption during the course of *Pneumocystis* pneumonia. Vitronectin (VN) and fibronectin (FN) are immunologically distinct adhesive glycoproteins found at increased levels in BAL fluid from PCP patients (Neese, Standing et al., 1994). VN and FN bind β-glucan and glycoprotein A of *Pneumocystis* and are found bound to the surface of freshly isolated *Pneumocystis*. Attachment to host cells is enhanced in the presence of VN and FN (Pottratz and Martin 1990; Limper, Standing et al. 1993). Cytokine release from AM is augmented when *Pneumocystis* is coated with VN or FN (Neese, Standing et al. 1994; Vassallo, Kottom et al. 2001). However, these adhesive glycoproteins do not seem to affect organism phagocytosis at physiological doses (Hoyte, Standing et al. 1997).

In addition to innate responses involved in the binding and uptake of *Pneumocystis* secreted mediators of host inflammation and organism killing have been described with regards to *Pneumocystis*. Production of reactive oxygen and nitrogen intermediates by AM and PMN represents an important effector function of these cells. *Pneumocystis* is susceptible to H_2O_2 and superoxide killing due to its' limited anti-oxidant activity (Pesanti 1984). *Pneumocystis* elicits peroxide and superoxide secretion by AM in a contact-dependent manner that is partially mediated by glycoprotein A (Hidalgo, Helmke et al. 1992; Laursen, Moller et al. 1994; Koziel, Li et al. 2000). AM from HIV^+ patients with CD4 T cell counts less than 200 cells/μl have a significantly attenuated superoxide response following *Pneumocystis* stimulation (Koziel, Li et al. 2000) offering a possible mechanism for the susceptibility of these patients to develop infection. It should be noted that experimental data does not support a major role for reactive oxygen and nitrogen intermediates in organism clearance or lung damage (Swain, Wright et al. 2004). Neither gp91phox knockout mice that have greatly reduced reactive oxygen species production nor double knockout mice lacking gp91phox and inducible nitric oxide synthase that have attenuated nitric oxide and reactive oxygen species production exhibit differences in *Pneumocystis* burden or pulmonary dysfunction compared to wild-type animals (Swain, Wright et al. 2004).

3.2. Cytokines

Stimulation of antigen presenting cells (APC) from naïve mice with *Pneumocystis* major surface glycoprotein induces significant interferon-gamma (IFN-γ) secretion (Theus, Smulian et al. 1997). IL-1β, IL-1α, IL-3, IL-6, TNF-α and TNF-β are increased in the lung following *Pneumocystis* infection in SCID mice reconstituted with unfractionated congenic splenocytes from immunocompetent donor animals (Wright, Johnston et al. 1997). Cytokine levels peak at day 12 post-reconstitution (PR) and return to baseline levels by day 22 PR at which time no *Pneumocystis* organisms are detectable in the lungs. Despite continued organism burden,

unreconstituted SCID mice do not exhibit pulmonary inflammation or upregulation of cytokine message in response to *Pneumocystis* infection with the exception of a moderate increase in IL-1β and TNF-α late in the course of infection at day 35 (Wright, Johnston et al. 1997). This study suggests cytokine production is mediated by lymphocytes as unreconstituted SCID mice lacking lymphocytes of T- and B-cell lineages do not produce cytokines in response to *Pneumocystis* infection. Cytokine production is important in the activation of AM and lymphocytes. Notably, TNF-α and IFN-γ have been shown to be crucial in host defense to various pathogens.

IFN-γ produced by $CD4^+$ lymphocytes enhances AM bactericidal activity, antigen presentation and IL-1 synthesis (Stevens, Exon et al. 1989). The secretion of IFN-γ and other cytokines from mononuclear cells is impaired in HIV^+ patients, which may predispose these patients to opportunistic infection (Murray, Rubin et al. 1984). However, animal studies using IFN-$\gamma^{-/-}$ mice or depleting antibodies do not support this notion. IFN-γ receptor knockout mice are not more susceptible to *Pneumocystis* infection suggesting IFN-γ is not essential to clear infection (Hanano and Kaufmann 1997). Furthermore, immunologically reconstituted SCID mice treated with antibodies against IFN-γ clear *Pneumocystis* from the lungs as effectively as control antibody treated animals (Chen, Havell et al. 1992). In accordance with this study, Garvy et al show SCID mice reconstituted with splenocytes from IFN-$\gamma^{-/-}$ mice or mice treated with an anti-IFN-γ monoclonal antibody do not have altered ability to clear *Pneumocystis* from their lungs (Garvy, Ezekowitz et al. 1997). This study went a step further to investigate the cellular response in the lungs following infection and reconstitution. There is an altered cellular response in the absence of IFN-γ associated with eosinophil and neutrophil infiltration into the lungs and increased activated $CD4^+$ cells compared to control mice. It is unclear from these studies whether there is an increased Th2 cytokine response in the absence of IFN-γ, though the infiltration of eosinophils into the lungs suggest this is a possible mechanism. While IFN-γ does not appear to be required for clearance of *Pneumocystis* it clearly has a major role in directing the inflammatory response elicited by *Pneumocystis*. Selective overexpression of IFN-γ in the lungs of CD4-depleted animals results in clearance of *Pneumocystis* associated with $CD8^+$ T cell and natural killer (NK) cell infiltration into the lung (Kolls, Habetz et al. 1999). CD4-depleted mice infected with *Pneumocystis* have decreased lung burden when treated with aerosolized recombinant IFN-γ compared to control mice (Beck, Liggitt et al. 1991). These data indicate IFN-γ mediated pulmonary inflammation is advantageous in the absence of a $CD4^+$ lymphocyte response, as is seen in HIV^+ patients susceptible to *Pneumocystis* infection.

Proinflammatory cytokines including IL-1, IL-6 and TNF-α are important mediators of host defense. Blocking the type I IL-1 receptor (IL-1R) prevents binding of both IL-1α and IL-1β and results in near complete inhibition of *Pneumocystis* clearance in reconstituted SCID mice (Chen, Havell et al. 1992). This was associated with a substantial decrease in infiltrating $CD4^+$, $CD8^+$ T cells, macrophages and neutrophils into the lung. The role of IL-6 during *Pneumocystis* infection seems to be in the resolution of pulmonary inflammation rather

than organism clearance. Unlike IL-1 and TNF-α that are upregulated only in the lungs following *Pneumocystis* infection, IL-6 is induced both locally and systemically (Chen, Havell et al. 1993). Neutralization of IL-6 in reconstituted SCID mice does not alter *Pneumocystis* clearance from the lungs (Chen, Havell et al. 1993). However, treating mice with anti-IL-6 IgG exacerbates PMN and lymphocyte recruitment to the alveolar space. These data suggest IL-6 has anti-inflammatory properties during the course of *Pneumocystis* infection. In fact IL-6 has been shown to down-regulate the production of other inflammatory cytokines including IL-1β and TNF-α (Akira, Isshiki et al. 1990).

The most widely studied proinflammatory cytokine in host defense is TNF-α. TNF-α is a proinflammatory cytokine that mediates its effects by binding two structurally related receptors, TNFRI and TNFRII. *Pneumocystis* directly stimulates TNF-α release from AM in a manner dependent on β-glucan but not mannose receptors (Hoffman, Standing et al. 1993; Vassallo, Standing et al. 2000). Secretion is primarily by AM independently of $CD4^+$ T cells, as demonstrated by studies showing CD4-competent and CD4-depleted mice have comparable levels of TNF-α in the BAL fluid following *Pneumocystis* infection (Kolls, Beck et al. 1993). TNF-α is directly lethal to *Pneumocystis in vitro* in a manner dependent on the induction of reactive oxidatants (Pesanti 1991). Furthermore, antibody neutralization of endogenous TNF-α in a reconstitiuted SCID model of *Pneumocystis* significantly inhibits the clearance of *Pneumocystis* from the lungs (Chen, Havell et al. 1992). Despite this neither TNF-α nor TNF-α receptor (TNFR) knockout mice are more susceptible to natural *Pneumocystis* infection than wild-type controls and *Pneumocystis* infected knockout mice have similar pulmonary *Pneumocystis* burden when compared to wild-type animals (Chen, Havell et al. 1992; Hanano and Kaufmann 1997; Wright, Pryhuber et al. 2004).

It should also be noted that AM from HIV^+ patients with *Pneumocystis* pneumonia spontaneously release TNF-α (Krishnan, Meager et al. 1990) though higher TNF-α levels were associated with lower *Pneumocystis* organism burden in BAL fluid in this study (Krishnan, Meager et al. 1990). In accordance with the data from humans, CD4-depleted mice develop chronic *Pneumocystis* infection despite TNF-α production (Kolls, Beck et al. 1993). Taken together these data suggest that while TNF-α may be an important regulator of *Pneumocystis* clearance and pulmonary inflammation associated with *Pneumocystis* infection other mediators must be involved to efficiently clear infection from the lungs.

Evidence suggests TNF-α and IFN-γ may work in concert to mediate clearance of *Pneumocystis* (Rudmann, Preston et al. 1998). Simultaneous deletion of TNFR and IFN-γ genes results in severe *Pneumocystis* infection while animals with individual deletions of either TNF-α or IFN-γ completely clear *Pneumocystis* from the lungs (Rudmann, Preston et al. 1998). Though the mechanism of cytokine-mediated clearance of *Pneumocystis* needs to be further investigated it has been shown that priming of AM with IFN-γ is cytotoxic to *Pneumocystis* organisms and this killing is dependent on TNF-α induced reactive nitrogen intermediate secretion (Downing, Kachel et al. 1999). In addition to mediating clearance of *Pneumocystis*

from the lungs this study found TNF-α and IFN-γ may potentiate pulmonary inflammation associated with *Pneumocystis* infection resulting lung injury. Further investigation found TNFR signaling contributes to lung injury during *Pneumocystis* pneumonia in CD4-depleted animals, as seen by attenuation of cellular infiltration into the lung, albumin leakage and pulmonary dysfunction in TNFR-deficient mice (Wright, Pryhuber et al. 2004). Pulmonary inflammation in CD4-depleted mice consists primarily of $CD8^+$ T-lymphocytes and polymorphonuclear (PMN) leukocytes. Chemotactic cytokines, known as chemokines, are integral for the recruitment of leukocytes to sites of infection and injury.

Chemokines are classified as CXC, CC, C, or CX_3C family members based on the relative position of two conserved N-terminal cystein residues. Chemokines' functionality are mediated by seven-transmembrane G-protein-coupled receptors. Receptor ligation results in the activation of phosphatidylinositol 3-kinase (PI(3)K) and the products of this kinase accumulate at the leading edge of the cell (Van Haastert and Devreotes 2004). Ultimately this results in actin polymerization leading to the formation of cytoplasmic extensions, known as lamellipodia, and directional movement towards the source of stimulation. CXC chemokine family members, such as IL-8 and interferon-inducible protein 10 (IP-10), are potent neutrophil chemoattractants, whereas CC chemokines such as macrophage inflammatory proteins 1α and β (MIP-1α, -1β) have receptors present on monocytes and some lymphocytes. In agreement with the aforementioned cytokine expression data, chemokine expression in the lungs of unreconstituted SCID mice infected with *Pneumocystis* is not increased following *Pneumocystis* infection and these animals are unable to clear *Pneumocystis* (Wright, Johnston et al. 1999). Mice that are infected with *Pneumocystis* and immunologically reconstituted with unfractionated congenic splenocytes have elevated chemokine expression peaking at day 12 PR that returns to baseline by day 22 PR when *Pneumocystis* organisms are no longer evident in the lung. Expression of the CC chemokines RANTES, MCP-1, MIP-1α and MIP-1β, as well as the mouse IL-8 homolog MIP-2 were significantly elevated over control mRNA levels (Wright, Johnston et al. 1999). TNFR deficiency downregulates chemokine production in the lung following *Pneumocystis* infection, which may attenuate the pulmonary infiltration of leukocytes (Wright, Pryhuber et al. 2004). TNF-α also mediates the induction of intercellular adhesion molecule-1 (ICAM-1) expression in lung epithelial cells by *Pneumocystis* (Yu and Limper 1997; Qureshi, Cook-Mills et al. 2003). ICAM-1 on vascular endothelium binds $\alpha_L\beta_2$ integrin on leukocytes to cause firm adhesion to the endothelium, which is important in transmigration across vascular endothelium into injured tissues. Thus TNF-α mediates recruitment of inflammatory cells to the lung during *Pneumocystis* infection by activating cells to secrete chemokines and upregulating the expression of adhesion molecules on endothelium.

Resolution of infection involves not only the induction of inflammatory host responses but also downregulation of inflammation to prevent tissue damage. IL-10 is a potent anti-inflammatory cytokine that is increased in the circulation of HIV^+ patients (Stylianou, Aukrust et al. 1999) and released in the lungs during

Pneumocystis infection (Ruan, Tate et al. 2002). Pulmonary delivery of viral IL-10 to mice prior to *Pneumocystis* infection does not alter their ability to clear infection but abrogates pulmonary inflammation and the associated lung injury (Ruan, Tate et al. 2002). CD4-competent mice treated with viral IL-10 or control adenovirus both clear infection completely while CD4-depleted animals develop significant lung burden that is similar between IL-10 treated and control animals. In this study endogenous production of IL-10 in wild-type mice that may mask alterations in clearance of *Pneumocystis* between viral IL-10 treated animals and controls. Indeed, CD4-competent IL-10 knockout mice clear *Pneumocystis* organisms from the lung faster than wild-type animals (Qureshi, Harmsen et al. 2003). Accelerated clearance was associated with enhanced IFN-γ production and chemokine expression in the lungs and increased infiltration of $CD4^+$ and $CD8^+$ T-lymphocytes and neutrophils into the lungs of IL-10 KO animals compared to WT controls. In the absence of CD4 T cells IL-10 KO mice are not able to clear infection and have comparable *Pneumocystis* burden compared to WT CD4-depleted animals. Despite comparable burden IL-10 KO mice depleted of CD4 T cells have significantly greater lung injury than CD4-depleted WT mice (Qureshi, Harmsen et al. 2003). As expected, IL-10 is a key mediator of inflammation in the lungs and may dampen the host's ability to clear infection when CD4 T cells are present.

The data concerning the roles of cytokines and chemokines in host defense against *Pneumocystis* infection are vast and complicated. Taken together these data provide evidence for differential and coordinated roles of cytokines during the course of infection and stress the importance of cytokines not only in the induction of an inflammatory response but the resolution following clearance of infection.

4. CONCLUDING REMARKS

Pneumocystis is an opportunistic pathogen that causes severe morbidity and mortality in immunocompromised patients, particularly HIV^+ patients with decreased $CD4^+$ T-cell counts. The coordination of pro-inflammatory and anti-inflammatory host responses to mediate clearance of infection followed by resolution of inflammation without injury to the host is essential to the successful host response to any pathogen. As investigation into the host defense against *Pneumocystis* continues we may better understand how innate and adaptive immune cells influence each other and work together to fight *Pneumocystis* infection allowing for the development of improved immunotherapy for the treatment of *Pneumocystis* pneumonia. Given the clinical manifestation of this illness, the need for $CD4^+$ T-cell independent immunotherapies are of particular importance.

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CHAPTER 15

INTERACTIONS OF *ASPERGILLUS FUMIGATUS* WITH ITS HOST DURING INVASIVE PULMONARY INFECTIONS

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Abstract: *Aspergillus fumigatus* is a saprophytic fungus that leaves in the soil in decaying organic material. Its thermophilly, the ubiquity of the conidia in the atmosphere, their small size, and their constant inhalation by all individuals, are obvious causes for the induction of pulmonary pathologies in human. The multiplication of immunosuppresive therapies makes aspergillosis now the most life threatening pulmonary mycosis in industrialized countries.

In the immunocompetent host, innate immunity is essential to control *A. fumigatus* conidia. When these conidia enter the pulmonary alveolar cavities, they are phagocytosed by the alveolar macrophages. Killing of the resting conidia is a very slow process. It occurs when conidia swell inside the phagolysosome. Reactive oxidant intermediates (ROIs) are essential for the killing of *A. fumigatus*. Polymorphonuclear neutrophils (PMN) that are recruited following germ tube formation act mainly on hyphae. Hyphal damage by PMN is rapid but the mechanisms responsibles for the killing are poorly understood. Although the immunological perturbations due to chemo- and radio-therapies have been insufficiently analysed, it is obvious that the down regulation of the innate immunity and especially the inhibition of the production of ROIs during immunosuppressive therapies is a major inducer of the development of invasive aspergillosis (IA).

In vivo, A. fumigatus interacts with host secreted glycoproteins and with membrane bound receptors on the surface of the immune cells. Pentraxin 3 secretion is increased during infection, and binds to conidia. C-reactive proteins and fibrinogen have also been correlated with *A. fumigatus* infection. Collectins play a protective role during IA. Involvment of TLRs is limited although it has been shown that TLRs are activated differentialy by conidia or hyphae. Carbohydrate-directed-receptors, such as DC-SIGN and Dectin 1, can bind to *Aspergillus* β1,3 glucan and galactomannan, and induce an inflammatory response. *A. fumigatus* has developed many mechanisms to counteract the phagocyte defense reactions, in particular melanin, present at the surface of the conidia, and anti-oxydant molecules that protect the fungus against ROIs. Regulators of the nitrogen and carbon metabolism, and divalent cations are important fungal virulence factors. Adaptation to environmental pH and thermophilly are also essential determinants of growth *in vivo*.

Adaptative immunity is also important during the anti-*A. fumigatus* host defence reaction. Th1 response plays a protective role whereas Th2 response exacerbates the

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disease. Dendritic cells initiate the T-cell response. It has been known for 30 years that vaccination with *A. fumigatus* conidia confers protection against subsequent infection with *A. fumigatus* conidia in immunosuppressed mice. Immunoprotection assessed in mice was correlated to a significant increase in the level of anti-*A. fumigatus* antibodies directed against 2 major antigens DPPV and catalase. However, the injection of recombinant DPPV protein or DPPV-encoding-DNA vaccination, are not protective in spite of the induction of a strong antibody response. In contrast to B cells, it was shown that *A. fumigatus* activated Tcells that induced a Th1 response protect immununosuppressed mice against *A. fumigatus* infection. This response is initially promoted by dendritic cells that can be activated by conidial molecules. These recent data open the way to the development of "vaccines" for a better management of IA patients

1. INTRODUCTION

In nature, *Aspergillus fumigatus* is a saprophytic fungus that leaves in the soil in decaying organic material and plays a major role in plant degradation (Latgé 1999). A survey of the recently annotated sequence of the genome, showed that this fungus has indeed the full enzymatic armentarium to degrade plant material (Tekaia and Latgé 2005). Accordingly, it is the most common inhabitant of compost (Beffa et al. 1998). *A. fumigatus* sporulates abundantly in its natural habitat. As a consequence, it is present in high concentration in the air of all environments, indoors or outdoors ($1–100\,co/m^3$) (Chazalet et al. 1998). It is also a thermophilic fungus that grows the most extensively at temperatures $> 40\,^{\circ}C$ (Beffa et al. 1998; Bhabhra et al. 2004). The ubiquity of the conidia in the atmosphere, their inhalation by every individuals and the fast growth rate of this species at temperatures above body fever (doubling time $< 2h$ at $37\,^{\circ}C$) are obvious biological causes for the induction of pulmonary pathologies in humans.

Since its first appearance in the mid 1800's (Denning et al. 2002) aspergillosis remained more a curiosity than a serious medical problem. Was not aspergillosis the disease of bird breeders or a secondary burden for ancient miner with pulmonary problems or tuberculosis patients with cavities in their lung (Latgé, 1999)? In recent years, the situation has dramatically changed and aspergillosis is now the most life threatening pulmonary mycosis and the only increasing mycological problem in industrialized countries (Odds et al. 2003; Yamazaki et al. 1999). Several reasons can explain these changes: the number of immunocompromised patients in the hospital has increased dramatically with the development of immunosuppressive therapies and grafts; diagnosis of aspergillosis is difficult and most of the time occurs too late to undertake an efficient therapy; drugs to treat aspergillosis are poorly efficient and even toxic for the patient, and most importantly, the pathobiology of the disease remains poorly understood (Latgé 2001; Latgé 2003).

In the last 20 years, search for specific fungal virulence factors was undertaken due to the amenability of this fungus to molecular biology techniques. To date, avirulent mutants result only from the disruption of genes coding for global regulators of growth (Steinbach et al. 2005). These genes control growth both *in vitro* and *in vivo* and cannot be called virulence factors sensu stricto since *A. fumigatus* virulence does not result of their induced specific expression *in vivo*.

The main reason for *A. fumigatus* to grow in humans seems that it encountered an environment nutritionnaly favorable in an immunodeficient host that is not able to counteract fungal development because of its debilitated immune status. Understanding the immune reactions towards *A. fumigatus* in an immunocompetent or immunocompromised status is today a prerequisite to be able to better manage patients with invasive aspergillosis (IA). This review will summarize our present knowledge in this area.

1.1. Innate Immunity

It has been reported long time ago that fungal conidia and especially *A. fumigatus* conidia can be phagocytosed by blood cells. Early studies showed that macrophages are the first line of defence against *A. fumigatus* conidia and neutrophils are the second line of defence against mycelium (Schaffner et al. 1982). However, studies on the phagocytic defence against *A. fumigatus* remained scarce and results were extremely discording in terms of levels and mechanisms of killing (see Latgé 1999 for earlier references). This lack of understanding of the phagocytic response has been compensated in recent years by a renewed interest in the analysis of the anti-*A. fumigatus* function of the phagocytic cells present in the lung alveola.

Innate immunity more than T cell immunity, is essential to control *A. fumigatus* conidia. For example, athymic nude mice are as resistant as outbred Swiss mice and withstand an inoculum load of 10^8 conidia of *A. fumigatus*. In humans, *A. fumigatus* rarely invades AIDS patients that have an acquired defects in T-cell function but have still an efficient innate immunity response, indicating that T-cells do not play a key role to counteract *A. fumigatus* primoinfection. In the alveola, macrophages and neutrophils are the resident phagocytes. Among these 2 cells, alveolar macrophages are the most potent since a mouse without neutrophils can stand 10^5 conidia without infection (Philippe et al. 2003). In contrast, at higher concentration of conidia (10^7) polymorphonuclear neutrophils (PMN) were essential for the clearance of *Aspergillus* (Balloy et al. 2005a; Balloy et al. 2005b). The third cell of the innate immunity are the epithelial cells that have been now repeatedly shown to be able to engulf conidia (Paris et al. 1997; Wasylnka 2003).

1.1.1. Alveolar macrophages

Alveloar macrophages (AM) are the main phagocytic cells of the innate immunity of the lung. Engulfment of conidia by murine or human alveolar macrophages lasts 2 hours and an average threshold of 2.5 conidia per AM is not regularly overpassed (Philippe et al. 2003) (Fig. 1). Phagocytosis of conidia requires actin polymerisation and phosphoinositol 3 kinase activity (Ibrahim-Granet et al. 2003). Upon stimulation by conidia, MAP kinases of the ERK pathway were rapidly activated reaching a maximum of phosphorylation after 1h of contact (Dubourdeau et al. 2006). Maturation of *A. fumigatus* phagosome results from fusion with the compartments of the endocytic pathway. Inside the phagolysosome, the conidial swelling that is the first stage of germination, is not inhibited. This germination

event is suicidary for the fungus since it induces the intracellular defence reactions and subsequent killing (Fig. 1). Killing of conidia by AM is a very slow process. After a 6 h incubation, *in vitro* killing of resting conidia reached only 7% at a 1 :1 conidia :AM ratio (Philippe et al. 2003). This result showed that the inhibition of germ tube formation in the AM phagosome was mainly fungistatic since the germinative capacity of the swollen conidia that remained inside the AM was only partially affected. Killing is indeed very slow since an immunocompetent mouse required several days to clear the inhaled inoculum. Increasing the killing rate *in vitro* is possible by modifying the conidium : AM ratio and by using germinated swollen conidia, the sensitive stage of the conidium. Reduction in the conidium: AM ratio from 1 :1 to 1 :10 was associated with an increase in killing from 7 to 22%. Up to 47% of swollen conidia were killed after 6h incubation *in vitro* vs 22% of resting conidia at a 1 :10 conidia : AM ratio. Intensification of the phagocytic reaction is associated with the translocation of NFkB that is maximal after 6h of phagocytosis (Dubourdeau et al. 2006). This cellular event results in the secretion of cytokines such as TNFα or IL1α that are important for the recruitment of phagocytes and the enhancement of fungicidal activity of phagocytes.

The contribution of NADPH oxidase and inducible nitric oxide synthase to the conidicidal activity of the alveolar macrophage were studied using alveolar macrophages from KO mice and metabolic inhibitors of these enzymes (Philippe et al. 2003). AM from NADPH oxidase deficient mice were unable to kill *A. fumigatus* conidia. Inhibitors of NADPH oxidase that decreased the production of reactive oxidant intermediates (ROIs), inhibit the killing of *A. fumigatus* without altering the phagocytosis rate. In contrast to NADPH oxidase, nitric oxide synthase does not play a role in the conidial killing. Corticosteroids did not alter the internalization of conidia by AM. In contrast, they inhibit the production of ROI and promote the intracellular germination of the conidia inside the AM (Fig. 1). This impairement of reactive oxidant intermediates production by corticosteroids triggers the development of invasive aspergillosis in immunosuppressed mice.

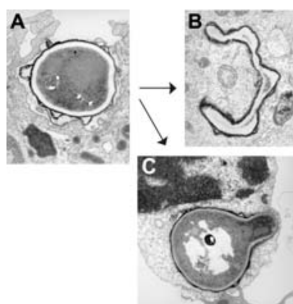


Figure 1. Phagocytosis of *A. fumigatus* conidia by alveolar macrophages. A: conidia phagocytosed; B: dead conidia in the phagolysosome of an immunocompetent mouse; C: germinating conidium in an alveolar macrophage from a mouse treated with cortisone acetate (susceptible to *A. fumigatus* infection)

1.1.2. Neutrophils

Polymorphonuclear neutrophils (PMN) act mainly on hyphae, as a second line of defence (Schaffner et al. 1982). Neutrophils are indeed recruited 24 hours after initial conidial inhalation in mice (Balloy et al. 2005a; Balloy et al. 2005b). It is known that PMNs are also able to ingest and kill resting or swollen conidia that have escaped macrophage phagocytosis (Sturtevant and Latgé 1992). Contact between neutrophils and the fungus triggers a respiratory burst, secretion of reactive-oxygen intermediates, and degranulation (Bellocchio et al. 2004b). In contrast to the killing of conidia by macrophages, hyphal damage by PMN was rapid, in that 50% of the hyphae were killed after a 2 hour incubation. Cellular damages to the hyphae by PMNs have been however poorly characterized. PMN granules contain a set of carbohydrate modifying enzymes including chitinases and proteases, that are able to attack and modify the cell wall structure and permeability of the hypha, very quickly upon release from the granules (Borregaard and Cowland 1997; Latgé 1999). Cationic peptides and defensins have been also reported to play an antifungal role that is not quantified *in vivo* (Levitz et al. 1986; Okamoto et al. 2004). Moreover, antifungal effector activity occurs in a morphotype-specific manner (Bellocchio et al. 2004a) since conidia and hyphae elicit different response but also differ in their susceptibility to microbicidal mechanisms. Conidicidal activity of PMNs mainly proceeds by NADPH oxidase pathway. Interestingly, gliotoxin, a metabolite produced by hyphae (but absent from conidia) of *A. fumigatus* that inhibits NADPH oxidase assembly, blocks conidial killing but not hyphal killing by PMN (Stanzani et al. 2005; Tsunawaki et al. 2004), suggesting a lack of role of this toxin during hyphal invasion of the lung parenchyma. Non-azurophil granules were released more by conidia than hyphae. In contrast, azurophil degranulation exemplified by myeloperoxidase release was more abundant after hyphal stimulation. All the cellular and biochemical events occuring during fungal destruction by the PMNs should be revisited and integrated to reach an overal understanding of the mechanisms responsibles for *A. fumigatus* killing.

1.1.3. Epithelial cells

A. fumigatus can be internalized by cultured A549 pneumocytes and primary lung cells (Paris et al. 1997; Wasylnka 2003). After three hours at 37 °C, A549 cells internalized 30% of bound conidia. In contrast to phagocytes, uptake was specific as polystyrene beads were not internalized by A549 cells. The intracellular processing was the same in A549 cells as in macrophage except trafficking to the lysosome was slower, i.e., 61% of conidia were in acidified LAMP-1-positive compartments 6 h after infection. In one study, 6.5% of the conidia remained viable in A549 cells after 12 h, and 3% after 24h (Wasylnka, 2003). In another study using an *in vitro* model of primary culture of human nasal epithelial cells, only 10% of the conidia loose their viability after 12 h of phagocytosis (Botterel Personnal Communication). Germinating conidia were seen within the A549 phagosomes after 6 and 24 hours. After longer incubation periods (24–36 h), the hyphal tips of some germlings became exposed to the extracellular space while the rest of the germling

remained intracellular. No significant release of host cell lactate dehydrogenase was detected after 24 h, suggesting that the extension of hyphae did not lyse the host cell membrane a phenomenon also seen in macrophage. These data suggest that *A. fumigatus* conidia may be taken up by airway epithelial cells where they survive and germinate, protected from the host immune system (Wasylnka et al. 2005). This hypothesis is reinforced by recent data showing that following phagocytosis by epithelial cells, conidia inhibit apoptosis induced by TNF-a, the major cytokine released after conidial phagocytosis and consequently increase the time life of infected cells (Berkhova et al. 2006).

1.2. Adaptative Immunity

Dendritic cells (DC) initiate induced immunity against *A. fumigatus* (Bozza et al. 2002b; Romani et al. 2002). Respiratory DCs transport conidia or hyphae of *A. fumigatus* from the airways to the thoracic lymph nodes. During transport, DC mature and their contact with CD4+/Tcells in the lymph nodes lead to the induction of selective Th1/Th2 immune response. Phagocytosis of conidia by DC lead to Th1 priming whereas that of hyphae induce Th2 priming. The Th1 response is associated with increased production of inflammatory cytokines such as interferon (IFNγ), IL2 and IL12 and stimulation of macrophages and neutrophils. Th2 response is associated with suppression of antifungal effector cell activity, decreased production of IFNγ and increased concentrations of IL4 and IL10. Experimentally in animals, the suppression of host Th1 $CD4^+$ lymphocyte response and a switch to a Th2 immune response induces the development of invasive aspergillosis (Cenci et al. 1998; Cenci et al. 1997).

In humans, there is an increasing body of evidence that adaptive immune response also plays a critical role in the host defense against *A. fumigatus*. In contrast to neutrophil recovery, which generally occurs within the first 2–3 weeks post-allogeneic stem cell transplantation, the number of functional T-cells and T-cell function increase slowly over the first few months after transplantation (Marr et al. 2002; Wald et al. 1997). It has recently been shown that a significant antigen-specific proliferation of interferon (IFN)-γ-producing T-cells occurred in healthy individuals and in patients surviving IA (Grazziutti et al. 1997; Hebart et al. 2002). Although more data should be obtained with human patients at risk for IA, a favourable outcome of IA seems also associated in humans with a Th1 response.

Table 1 shows the cytokines and chemokines that affect positively or negatively *A. fumigatus* development *in vivo*. It shows an unbalance towards agonists of *A. fumigatus* infection. Is this the reality or a bias of the studies looking more at the positive side of the defense? Microarray analysis are now undertaken by several groups to complete the list and kinetics of expression of cytokines produced in response to *A. fumigatus* (Walsh et al. 2005).

The Th1/Th2 dichotomy is not so clear cut in the immune modulation of *A. fumigatus* infection. Although it is a Th2 cytokine, several studies have shown that IL10 does not have any detrimental effect on *Aspergillus* infection: (1) Roilides

Table 1. Cytokines and chemokines with positive or negative effects on the development of invasive aspergillosis (with relevant references in parenthesis)

Anti-*Aspergillus fumigatus* cytokines
G-CSF (Roilides et al., 1993)
GM-CSF (Roilides et al., 1996)
M-CSF (Roilides et al., 1995)
TNFα (Roilides et al., 1998, Mehrad et al, 1999)
IL6 (Duong et al, 1998, Cenci et al, 2001)
IL10[(1)] (Roilides et al., 1997, 2001)
IL12 (Roilides et al., 1999, Cenci et al, 1998)
IL15 (Winn et al, 2003)
IFNγ (Roilides et al., 1994, Cenci et al, 1997)
IL8(Borger et al, 1999)
IL18 (Cenci et al., 1998, Brieland et al, 2001)
IL23 (Gafa et al., 2006)
TGF-β[(1)] (Montagnoli et al, 2006)
Anti-*Aspergillus fumigatus* chemokines
CXCR2 (Mehrad et al, 1999)
CXCL1/KC (Mehrad et al, 2002)
CCL2/MCP1 (Blease et al, 2001)
CCL3/MIPIα (Mehrad et al, 2000)
Pro-*Aspergillus fumigatus* cytokines
IL4 (Cenci et al, 1999)
IL10[(1)] (Roilides et al., 1997, 2001)
IL5 (Cenci et al., 1997,1998)
TGF-β[(1)] (Montagnoli et al, 2006)

(1) Cytokines reported to have negative or positive effects.

et al. (2001) have shown that patient resisting to infection have high IL10 levels; (2) Increase stimulation of conidial killing *in vitro* following supplementation of serum from different patients, is correlated to high concentrations of IL10 in the serum added to human alveolar macrophages (Philippe et al, unpublished); (3) A protective Th1 reactivity coexisted in mouse with the detection of significant levels of IL10 (Bozza et al. 2003); (4) IL10 mediated T-cell tolerance to *Aspergillus* antigens and plays a role in controling the intensity of the inflammatory T-cell response to *Aspergillus* infection (Casaulta et al. 2003). IL10 could be beneficial through the induction of regulatory (T-reg) cells. These Treg cells seem essential for the host to withstand the non-self, mediating tolerance to alloantigens, inhibiting graft vs host disease and supporting the growth of Treg-preventing donor Th1 alloreactivity (Hori et al. 2002; Mills 2004). Indeed, recovery from infection does not depend only on effector-cell function but also on the resolution of the inflammatory process (Hope et al. 2005). Early in the infection, pro-inflammatory cytokines (TNF, IL1β, IL6 and IL12) plays an essential role in controling the infection. At that time, high levels of IL10 is detrimental for the host. However, later during the course of the infection, high concentrations of IL10 may be beneficial to resolve the inflammatory response (McGuirk et al. 2005). IL10 produced by Treg is responsible for

the establishment of commensalism or fungal latency and persistence. T-reg might compromise fungal clearance but will be also beneficial to the host by limiting infection induced pathology, a concept demonstrated with *Candida albicans* that seems also to be true with *A. fumigatus* (Romani 2004). The link between inflammation, fungal growth and outcome of aspergillosis has been studied in animals submitted to different immunosuppressive regimens (Balloy et al. 2005a; Balloy et al. 2005b; Berenguer J. 1995). In corticosteroid-treated animals, no TNF-α and IL10 are secreted; death results from an exacerbation of the inflammatory response, with a pneumonia with bronchiolitis, hemorragic necrosis with neutrophil infiltrates, increase repiratory distress, and low fungal burden. In contrast, animals treated with vinblastine that do not recruit neutrophils at the site of infection, have a low respiratory distress, have a high mycelial burden and show a good response to Amphotericin B. Such results lead to a new concept in the host defence against *A. fumigatus*: maintaining a high mycelial burden may not be directly associated to death.

1.3. Recognition of *A. Fumigatus* by the Host

In vivo, *A. fumigatus* encounters and interacts with many secreted glycoproteins circulating in the biological fluids of the host or with membrane-bound receptors on the surface of the immune cells. These proteins have recently received increasing attention since they can act as primary immunomodulators in the alveolar space.

1.3.1. Circulating molecules

Pentraxin 3, a member of the Pentraxin superfamily of animal lectins has been associated to increased circulating levels in different infection conditions. PTX3 binds to *A. fumigatus* conidia and efficiently oppose to *A. fumigatus* growth as shown in PTX $3^{-/-}$ mice that are extremely susceptible to *Aspergillus* infection (Garlanda et al. 2002; Gaziano et al. 2004). It is supposed that limitation of fungal infectivity by PTX3 results from a quicker and more efficient handling of conidia by resident mononuclear cells. Conidia but not hyphae bind to PTX3. C-reactive proteins another pentraxin that belong to the short pentraxin family, has been also associated to *Aspergillus* infection (Richardson et al. 1991). However, its role during *Aspergillus* infection has not been investigated. Another acute phase protein, fibrinogen that has been correlated with *A. fumigatus* infection and that binds to conidia and hyphae has not been investigated immunologically (Bouchara et al. 1997; Caillot et al. 1997; Coulot et al. 1994).

Collectins belong to the superfamily of C-type lectins (van de Wetering et al. 2004). Three of them, mannan binding lectin (MBL), surfactant protein A (SPA) and surfactant protein D (SPD) have shown to play a protective role during IA (Kishor et al. 2002; Madan et al. 2005). After binding, SPA and SPD enhance phagocytosis and killing of conidia by neutrophils and alveolar macrophages (Madan et al. 1997). Binding is calcium dependant and occurs through their lectin domain (Madan et al. 2005). Direct killing of fungi by SPD has been also reported, probably due to an increase cell permeability, as suggested for *Histoplasma* (McCormack et al. 2003).

Recombinant SPD and MBL increased the survival rate of IA mice. This result were confirmed in SPD deficient corticosteroid-treated mice that are more susceptible to infection (Madan et al. 2001). Astonishingly, SPA that enhances killing *in vitro* in presence of phagocytes, does not have any role *in vivo*. MBL acts more as promoting complement deposition. A significant correlation has been seen between MBL alleles and IA susceptibility (Crosdale et al. 2001).

Finally, the last circulating molecules that interact with *Aspergillus* are antibodies. They have been used and recognised as efficient markers of the development of the fungus in the host but their role if any, during infection has been poorly studied. Antibodies have been shown to be important as opsonins for increase phagocyte recognition since blockade of Fc-Receptor inhibits hyphal phagocytosis by dendritic cells (Ishibashi et al. 2005; Kurup 1984) and could be active in the initiation of the T-cell response (Yang and Brunham 1998).

1.3.2. Receptors

The role of Toll-like receptors has been analysed in *A. fumigatus* by several research groups, especially since *Drosophila* lacking the Toll proteins (after which the name of TLR was coined) are prone to *Aspergillus* infection (Lemaitre et al. 1996). In contrast to arthropods, studies of TLRs in mammals lead to conflicting results (Balloy et al. 2005b; Braedel et al. 2004; Mambula et al. 2002; Marr et al. 2003; Meier et al. 2003; Netea et al. 2003) (Table 2). Discrepancies between the conclusions of these different studies can be mainly explained by the low impact of TLRs on the innate immune response against *A. fumigatus* since TLR2, TLR4 and Myd88 deficient animals survive pulmonary *A. fumigatus* infection as wild type (Balloy et al. 2005b; Marr et al. 2003). This finding implies that Myd88 independent pathways are the most efficient host defense against inhaled conidia. Obtaining a global picture of the role of the TLRs in the defense of the host is also difficult to assess since different cells (naïve peritoneal or bronchoalveolar macrophages, neutrophils or transfected cells) from various hosts (mouse, human) as well as different fungal morphotypes have been used in the published studies. In addition, mice have to be immunosuppressed to show a difference between TLR KO and wild type mice whereas all *in vitro* studies used immunocompetent cells (Balloy et al. 2005b). Although the data are controversial, there are sufficient to conclude that Myd88-dependent TLR2 and TLR4 plays a role although limited, in orientating the phagocytic activities and the release of proinflammatory cytokines (TNFα) by macrophages and dendritic cells. TLRs favour conidicidal activity of neutrophils. Myd88 is also essential for adaptative immunity and the establishment of a Th1 response. Although no rule can be established between specific TLRs and fungal morphotypes, it is clear that the individual TLRs are activated differently by conidia or hyphae.

Modulation of TLRs is also extremely complex. Signalling through TLR2 occurs in combination with Dectin1, whereas TLR4 and CD14 act cooperatively at least in humans to recognize conidia (Gantner et al. 2003; Wang et al. 2001). The role of TLR9 is also unclear. TLR9$^{-/-}$ mice were incapable of mounting an

Table 2. Contribution reported in the litterature of TLRs in the immune responses against *Aspergillus fumigatus*

Reference	Phagocytic cell	TLR involved	Fungal morphotype	Parameters tested
Wang et al. (2001)	Human Mϕ	TLR4	H(1)	TNFα, IL6, IL1β release
Mambula et al. (2002)	Peritoneal murine Mϕ	TLR2	RC, H>SC(2)	TNFα release
	Human Mϕ, Tranfected HEK293	TLR2	no difference	NFkB activation
Marr et al. (2003)	Bone marrow derived murine Mϕ	No role of Myd88		Phagocytosis, Intracellular killing TNFα release
Meier et al. (2003)	Transfected HEK293,	TLR4 (TLR2)(3)	H > RC	NFkB activation, IL6
	Peritoneal murine Mϕ	TLR4 TLR2 + TLR4	H > RC	TNFα release neutrophil recruitment, MIP2 synthesis
Netea et al. (2003)	Peritoneal mouse Mϕ, Tranfected 3E10	TLR4 TLR2 TLR2	RC > H RC = H H	TNFα, IL1α, ILIβ TNFα, IL1α, ILIβ IL10
Braedel et al. (2004)	Bone marrow derived murine DCs	TLR2+TLR4	H(4)	IL6, IL12 release
Bellochio et al. (2004a, b)	Mouse	TLR2, TLR4(5) TLR2 (negative) No TLR(6) TLR4		Reduction of growth in the lung after infection TNFα release Neutrophil recruitment Survival
	PMN	TLR4 (TLR2) TLR2 TLR4	RC > H RC = H	Killing (phagocytosis) activity TNFα release
	DC	TLR2		Decrease in IL12, increase in IL10
	T-cell	Myd88		Adaptative immunity, Th1 response
Balloy et al. (2005b)	Mouse, alveolar Mϕ	TLR2		Survival, TNFα, MIP2 release

(1) Conidia were not tested; H: hyphae
(2) RC: resting conidium; SC: swollen conidium; binding is TLR independent; a > b = a give a better response than b
(3) TLR in parenthesis have some effect but lower than the TLR preceeding the parenthesis
(4) Intracellular and extracellular mycelial extracts activate differently TLR2 and TLR4
(5) TNFα release was increased in $TLR2^{-/-}$ mice
(6) Neutrophil recruitment was not TLR-dependent.

Ag- specific Th1 response whereas they were extremely resistant to *A. fumigatus* infections (Bellocchio et al. 2004b). Signalling through TLR2 promoted the fungicidal activity of PMN through oxidative pathways involving extracellular release of non-azurophil granule components and proinflammatory cytokines. TLR4 favoured the oxidative pathway through myeloperoxidase. Other effectors such as Amphotericin B, can also modulate the anti-microbial activity of PMNs through TLRs. The increased antifungal activity of neutrophils in presence of amphotericin B has been explained by the interaction of the drug with TLRs. Deoxycholate Amphotericin B upregulated the expression of TLR2 and decreased that of TLR4. In contrast, liposomal AmB seemed to favour the expression of TLR4 over TLR2 and reduced the proinflammatory effects of deoxycholate amphotericin B (Bellocchio et al. 2005; Sau et al. 2003).

Carbohydrate directed receptors (Dectin1, DC-SIGN and mannose receptor) have also been shown to bind to *Aspergillus* (Taylor et al. 2005). However, again the effect of these receptors on phagocyte activity has not been fully dissected and like TLRs, is associated to conflicting results. Dectin1, a type II transmembrane protein that belongs to the NK-like C type lectin receptor family recognizes β1, 3 glucans (Brown et al. 2003; Brown et al. 2002). Upon recognition of germinated conidia by dectin1, alveolar macrophage are able to induce an inflammatory response characterized by the production of various cyto- and chemokines such as TNFα, IL1α, IL1β, IL6, CXCL2, CCL3, G-CSF, GM-CSF (Steele et al. 2005). However, Dectin1 is not involved in conidium phagocytosis. Binding and internalization of conidia has been correlated with DC-SIGN cell surface expression levels on dendritic cells and alveolar macrophages (Serrano-Gomez et al. 2004). DC-SIGN contains a mannan-binding lectin domain that has been shown to bind to cell wall galactomannan of *A. fumigatus*. This result suggests that circulating antigen may bind to DC-SIGN and reduce the T-cell stimulatory activity of DCs. Mannose receptor has been shown to be essential for murine pulmonary DC and human Langerhans cells (Bozza et al. 2002a; Bozza et al. 2002b). In contrast, Persat et al. (2003) and Serrano-Gomez et al (2004) has shown that CD 206 (mannose receptor) did not play a role during phagocytosis by DC and alveolar macrophages.

Besides the lectin-type receptors recognizing PAMPs, other "ancient"receptors such as CR3 (CD11b/CD18) and FCγRII and III receptors have been forgotten in recent years (Romani 2004). Their importance is today minimized although they both have been shown to play a discriminatory role during hyphae uptake in earlier studies (Kan and Bennett 1991; Kurup 1984).

How important are these receptors for phagocytosis ? With *A. fumigatus* similar internalization is seen with $TLR2^{-/-}$, $Dectin1^{-/-}$ phagocytes. Moreover, no opsonization is required for efficient conidial engulfment by alveolar macrophages. Finally, no difference is seen in phagocytic indeces when conidia are replaced by latex beads. The specificity of the recognition can be also questioned. For example it has been reported that galactomannan (GM) is inhibiting the binding of conidia to various host effectors. However, inhibition data obtained with GM may not be such relevant: doses of 50 μg GM/ml are used for the inhibition in one study whereas

nanogram quantities are circulating in the biological fluids. Moreover, three studies uses the Sigma carob galactomannan that has a composition completely different of the *Aspergillus* GM: in the case of the carob, it is a β1, 4 mannan backbone to which are attached through an a 1,6 linkage single residues of α galactopyranose; in the case of *A. fumigatus*, the mannan core has a linear configuration containing α(1–2)- and (1–6)-linked residues in a ratio of 3:1, and the side chains, branched on two α(1–2)-linked mannose residues, are composed of β (1–5) galactofuranosyl residues with an average degree of polymerization of 4 (Latgé et al. 1994).

The important molecules in the interaction host cell and *A. fumigatus* cells are the transmembrane receptors inside the intracellular phagosome that are able to sense the changes of the fungus inside the cell. Dectin1 could be one of them (Herre et al. 2004). Our knowledge of the events occuring intracellularly remains very limited.

1.4. Escape to Host Antifungal Effectors

Mechanisms used by *A. fumigatus* to counteract the phagocyte defense mechanisms and the hostile lung environment are shown in Fig. 2.

Reactive oxidant intermediates are essential components of the phagocytes in the killing of *A. fumigatus* conidia. In addition of the experimental data shown above, one clinical line of evidence supports the role of ROI : invasive aspergillosis is the primary cause of death in patients suffering from granulomatous chronic disease

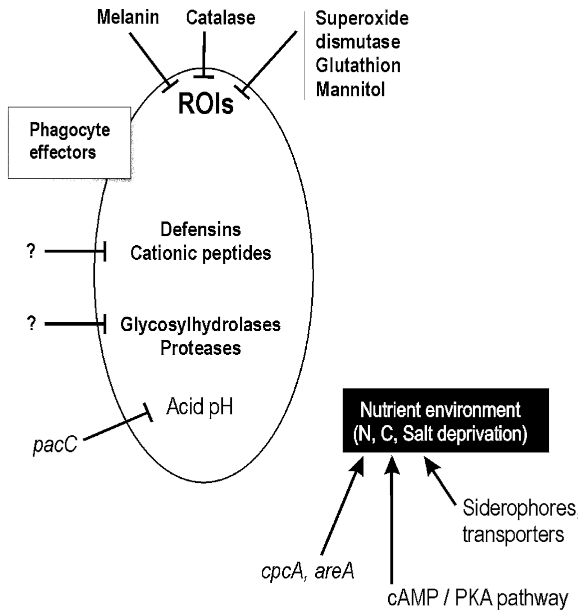


Figure 2. Molecules used by *A. fumigatus* to counteract the killing mechanisms of the phagocytes or the hostile nutritional environment of the host lung

accounting for over one-third of all deaths in the U.S registry. This disease is characterized by a genetic defect in the NADPH oxidase that prevents respiratory burst. Resistance to respiratory burst is then critical for fungal survival. *A. fumigatus* has not developed specific mechanisms to counteract the phagocytic response but features constitutively inherent to this species are important for protection. The first one is pentaketide-melanin that is present on the surface of the conidia (Tsai et al. 1999). A *pksP*(= *alb*1) mutant or wild type strains producing pigmentless conidia showed a reduced virulence. The *pksP* mutant of *A. fumigatus* is significantly more sensitive to hydrogen peroxide and sodium hypochlorite than the wild-type strain. Also, the mutant strain is more susceptible to damage by murine macrophages *in vitro* since it was shown that melanin-containing conidia are able to quench ROS derived from human granulocytes. Based on these data, it was possible to increase the level of killing in vitro using white conidia (Jahn et al. 2000; Jahn et al. 1997; Sarfati et al. 2002): > 85% of swollen white conidia (vs 22% of resting green conidia) are killed after 6 hours of phagocytosis by alveolar macrophages (Fig. 3).

A. fumigatus is also producing catalases that are able to scavenge H_2O_2. Catalase mutants are indeed more sensitive to H_2O_2 but in an experimental IA mouse model these mutants are indeed poorly affected in virulence with only a slight delay in fungal development (Paris et al. 2003a; Paris et al. 2003b). This result suggests that H_2O_2 is not the most efficient oxidative mechanism against *A. fumigatus*. Not a single oxidant is representative of the general oxidative stress encountered by the fungus. On supplement of H_2O_2, at least two more oxidants as $O_2^{\cdot -}$ that

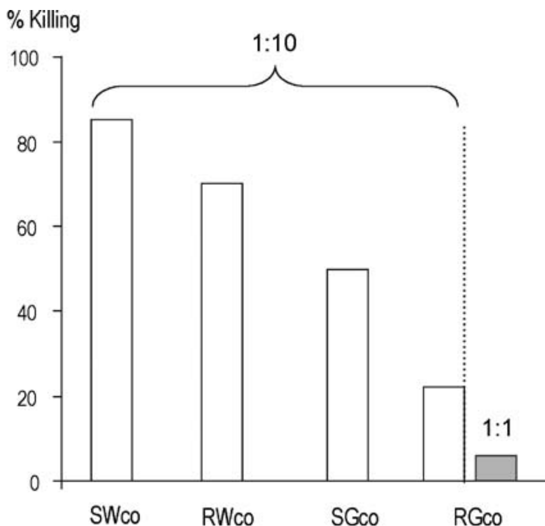


Figure 3. Experimental tricks to increase the conidial killing by alveolar macrophages; R:resting, S: swollen; G: green; W:white; Conidium(co): alveolar macrophage (AM) ratio of 1:1 or 1:10 are indicated

can be produced in vitro by menadione or products of lipid peroxidation such as linolexacid hydroperoxide or malondialehyde can have antifungal activities (Tekaia and Latgé 2005; Temple et al. 2005). Knowing which reactive oxidant intermediate is involved in the killing is important to fully understand the nature of the damage and how cells respond to it. For example in yeast, deletants that are sensitive to H_2O_2 show an over representation of mitochondrial function, whereas deletants of the pentose phosphate pathway (and NADPH) are sensitive to $O_2^{\cdot -}$ and mutant affected in the peroxisome function are sensitive to linolexacid hydroperoxide. Similar studies have not been pursued in *A.fumigatus*. In spite of the presence of superoxide dismutase secreted by *A. fumigatus in vivo* (Holdom et al. 2000), this species is extremely sensitive to menadione since it required 10–100x less amount of this compound to kill *A. fumigatus* than *C. albicans* (Chauhan et al. 2003; Latgé et al, unpublished). No superoxide dismutase mutant has been constructed that would allow the analysis of the role of this oxidant during killing. Other molecules such as mannitol are produced by *A. fumigatus* during stress and could also play an anti-oxidative function. Moreover, the role of the tripeptide glutathione that has been emphasized in several systems as antioxidant, has not been considered in *A. fumigatus* while comparative genomic analysis have shown that the entire metabolic pathway is present in *A. fumigatus* (Burns et al. 2005; Tekaia and Latgé 2005).

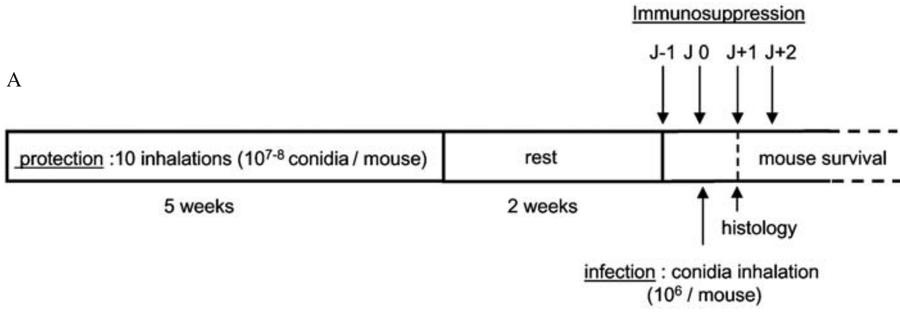
As mentioned above, defensins, cationic peptides produced by neutrophils, enzymes and especially glycosylhydrolases and proteases affect fungal growth and cell wall integrity. Antagonistic molecules or mechanisms used by the fungus to escape these aggressins have not been studied.

Without being toxic, nutrient deprivation can limit fungal growth *in vivo*. *A. fumigatus* possessed efficient mechanisms to grow in nutritionnally hostile or deficient environments (Bignell et al. 2005; Krappmann et al. 2004; Schrettl et al. 2004; Vicentefranqueira et al. 2005). Regulators controling the nitrogen (*cpcA*, *areA*) and carbon sensing (*gpaA,B*) and import are essential to sustain growth in the lung. *A. fumigatus* produces siderophores to counteract iron sequestration, a very common and efficient host mechanism to control the growth *in vivo* of numerous pathogens (Schrettl et al. 2004). The physico-chemical environment is also playing a role since it has been shown recently that transcription factors of the pacC cascade that controls the adaptation of the fungus to the environmental pH has been essential for the virulence of the fungus (Bignell et al. 2005). Finally, all genes controlling thermophilly are essential determinant of growth *in vivo*.

1.5. Orientating Adaptative Immunity

The first successful vaccination trial concerned the protection of turkeys against *A. fumigatus* (Richard et al. 1991; Richard et al. 1982). Since then, it has been repeatedly shown that vaccination with *A. fumigatus* extracts or conidia can confer protection against subsequent challenges with *A. fumigatus* conidia, even in immunosuppressed mice (Cenci et al. 2000; Ito and Lyons 2002). In our laboratory, the protocol used is shown in fig. 4. Three criteria were followed to define this protocol: (i) phagocyte cell

A



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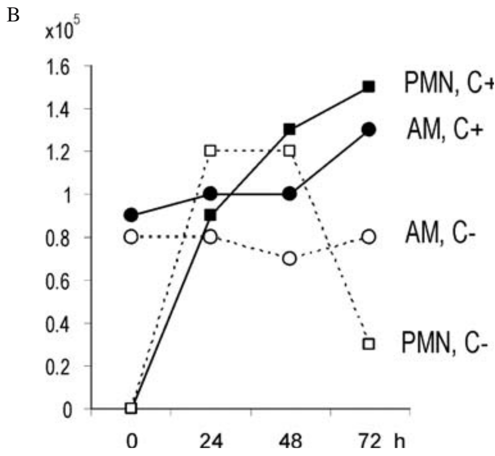

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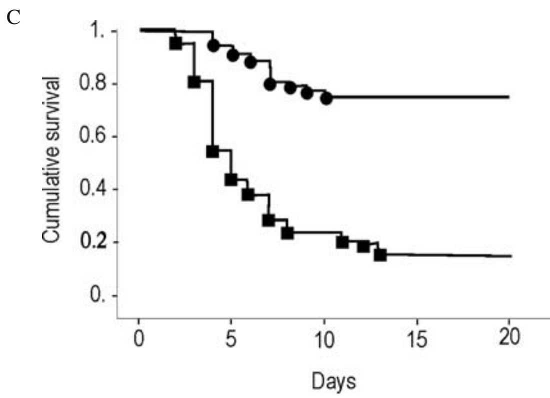

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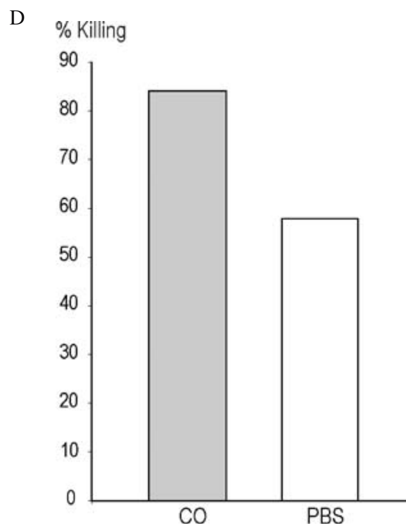

Figure 4. (Continued) A: Protocol used to induce immune resistance to infection in an immunocompromised animal; B: cell population ($\times 10^5$ per ml of BAL) kinetics in bronchoalveolar lavages of mice following inoculation of 10^8 conidia; PMN: neutrophils; AM: alveolar macrophages; C+: cortisone-treated animal; C− naïve animal; C: survival of mouse treated as in protocol A (circles: animals that received 10 times 10^8 conidia; squares: animals injected with PBS; D Killing capacities of AM from primed animals (filled bar, co) vs naïve animals (open bar, PBS)1:10 Co:AM ratio was used with swollen conidia

populations in the BAL, (ii) level of antibody, (iii) resistance to infection in immunocompromised mice assessed by mouse survival and reduction of fungal burden in the lung monitored by histopathology. Analysis of cells in the bronchoalveolar lavages showed that following inhalation of conidia (10^7/mouse) an increase of macrophage and neutrophil peaked 1 or 2 days after conidial inhalation (Fig. 4). This increase decreased after 3 days and resolved after 1 week. That is why our experimental protection protocol was twice a week. Under this strategy, a level of phagocytic cells higher than control could be continuously maintained for at least 30 days.

Inoculation of conidia to an immunocompetent mouse protects the mouse against an infection even after immunosuppression (Fig. 4). A month period of immunization is required to obtain maximal protection. Repeated conidial inoculation before immunosupression induced an increased conidial killing by AM (Fig. 4). A significant increase in phagocytic and antifungal activity of PMNs has been also seen. Protection was attained in all mouse strains tested (DBA2, Balb/C, C57Bl/6, C3HEN) with the exception of nude mice (unpublished data).

1.5.1. Do antibodies play any role in protection?

Maximal immunoprotection assessed in mouse was correlated to the maximal level of anti-*A. fumigatus* antibodies. During repeated inoculation of conidia to naive mice, antibodies directed against 2 major antigens of *A. fumigatus* were identified.

Interestingly, sera reacted almost monoclonally to these 2 antigens, that were the dipeptidylpeptidase (DPP)V and the catalase Cat1 (Beauvais et al. 1997; Calera et al. 1997) (Fig. 5). Maximal level of antibodies was obtained in outbred mice as well as in inbred C57Bl/6 mice, after 10 inoculations (5 weeks, twice a week, 10^8 conidia per inhalation). Higher is the concentration of conidia inoculated and more repeated are the inoculations, higher is the amount of antibodies produced (Fig. 5). Ig isotypes produced were mainly IgG1, IgG2a and IgG2b (Fig. 5). A rise in the level of antibodies was also seen in bronchoalveolar lavages of primed mice. IgG1 and IgA were the prominent antibodies observed in the BALs of C57Bl/6 and

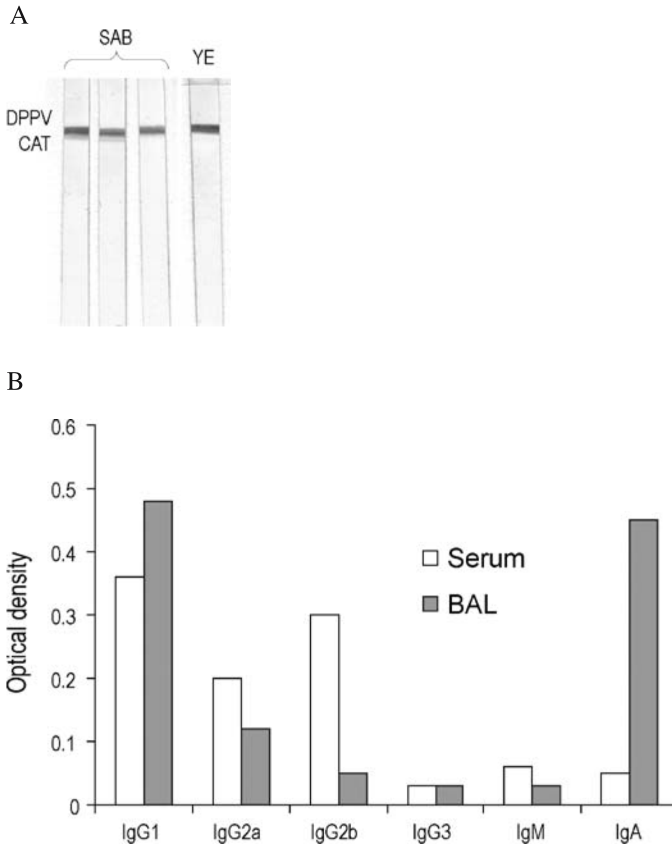

Figure 5. Antibody response of mice resistant to infection following repeated intranasal inoculations A: Western blot showing that sera (1:100 dilution) from mice primo-infected with conidia reacted specifically with the doublet catalase 1/dipeptidylpeptidase V(migrating with very close Mr and seen as a single wide band in the fig). The crude antigenic extract electrophoresed is an ethanol precipitate of a 24 hour shaken culture of *A. fumigatus* in a 1% yeast extract (YE) or 2% glucose + 1% peptone liquid medium (SAB) Four mice sera are shown. B: Anti-DPV IgG isotypes present in the serum and BAL of OF1 mice inhaled 10 times with 10^7 conidia (similar data were obtained with C57 BL6 inbred mice)

outbred mice. As in sera data, the highest antibody level in BAL was obtained after 10 inoculations.

The DPPV (Beauvais et al. 1997) is a glycoprotein with a molecular mass of 88 kDa that releases Ala-Ala, His-Ser and Ser-Tyr dipeptides from proteins at neutral pH. *In vitro*, this enzyme is secreted in large quantities when the medium contains proteins or protein hydrolysates in absence of other carbon and nitrogen source. This enzyme can bind collagen, hormones and cytokines and degrade them. Since DPPV (also known as the "chymotrypsic" antigen) was one of the major antigens recognized in sera of immunocompetent patients with aspergilloma, it was hypothesized that this molecule could lead to some humoral protection since these aspergilloma patients control the development of the fungus. Moreover, antibodies have been claimed to be responsible for a protective response in several fungi (Casadevall and Pirofski 2003). To test the putative protective response of antibodies during the *A. fumigatus* infection, two types of methods have been used to induce the production of antibodies: the injection of a pure protein or the transfer *in vivo* of recombinant DNA. The advantage of DNA vaccines is that they mimic the effects of live attenuated vaccines in their ability to induce major histocompatibity complex class I restricted $CD8^+$ T-cell responses (Gurunathan et al. 2000).

A recombinant DPPV was produced in *Pichia pastoris* as described (Beauvais et al., 1997) and used directly for mice experiment. A recombinant DNA was constructed, containing the entire DPPV gene in the mammalian expression vector pCi (Fig. 6). The expression of DPPV was tested *in vitro* by transfecting neuroblast (Neuro 2A) cells using the FuGene ™6 transfection reagent of Boehringer Mannheim. As shown in Fig. 6, the DPPV was expressed and released in the medium after 24h transfection and expression increased overtime.

The production of specific antibodies recognizing the DPPV was followed in a mouse model *in vivo*. The recombinant DPPV (rDPPV) (20 μg) or *A. fumigatus* conidia (4.10^7) were inhaled five to ten times. Alternatively, the recombinant DNA carrying the DPPV gene with or without the signal peptide (pCi-DPPV) (100 μg) was injected one to four times intramuscularily. The results showed that specific antibodies were produced against the DPPV only using the rDPPV, the *A. fumigatus* conidia and the pCi-DPPV with the signal peptide. No antibodies were produced when the pCi-DPPV without the signal peptide was injected. The secretion of the protein is therefore an absolute requirement for the production of antibodies. Maximum antibody production was obtained to a similar extent when the mice received 10 inhalation of rDPPV or *A. fumigatus* conidia, or 1 injection of pCi-DPPV (Fig. 6).

To test the ability of the DPPV antibodies to protect mice against aspergillosis infection, the mice were immunosuppressed and infected by *A. fumigatus* conidia (10^6) after maximal level of antibody was reached. The level of protection was estimated by histological studies of lung sections and *in vitro* killing assays. Protection was observed only when the animals received condia, in spite of a high antibody production in the three type of vaccination (rDPPV, pci-DPPV, *A. fumigatus* conidia). High level of anti-DPPV antibodies which are present in the protected mice are not able to protect mice against aspergillosis. Earlier studies

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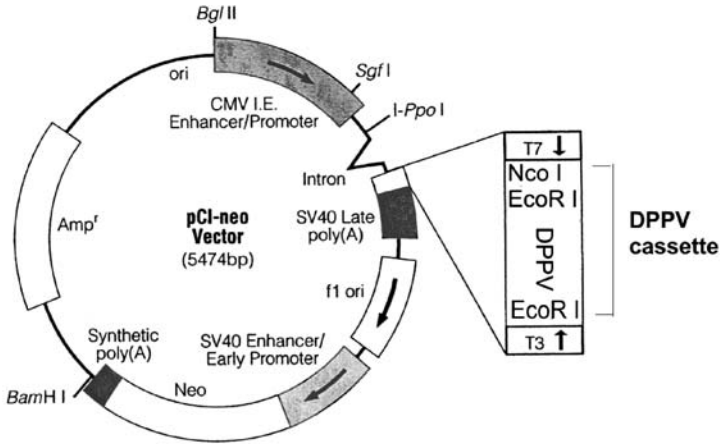

Vector Map Notes:

1. Sequence reference points:

a.	cytomegalovirus immediate-early enhancer	1-659
b.	cytomegalovirus immediate-early promoter	669-750
c.	chimeric intron	890-1022
d.	T7 promoter	1067-1085
e.	multiple cloning site	1085-1137
f.	SV40 late polyadenylation signal	1067-1388
g.	phage f1 region	1438-1938
h.	neomycin selectable marker	
i.	SV40 enhancer and early promoter	2002-2420
j.	SV40 minimum origin of replication	2318-2383
k.	coding region of neomycin phosphotransferase	2465-3259
l.	synthetic polyadenylation signal	3323-3371
m.	β-lactamase (Amp^r) coding region	2316-3176

Neo = neomycin phosphotransferase.

DPPV cassette =

Nco I EcoR I signal Peptide of DPPV

CC**ATG** GGG ATC TTG AAT TCT GCG GCC GCC CGCTGGCTCTCCATTGCCGCTGCGGCATCGACTG

DPPV cDNA

CTTTGGCT CTTACA.......................AAC**TAG** AGGAATGCTACCTTAACCTAGTGGAATACTGTA

EcoR I

TAGTGTACTTCAGGTTGCAACTACCAAGAAAAGAACCCTTTTATCCATTCCCGCCGGCGTCTTAAG

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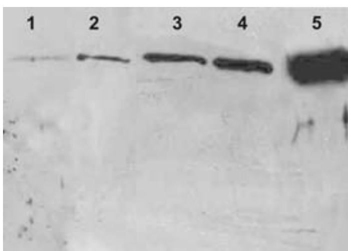

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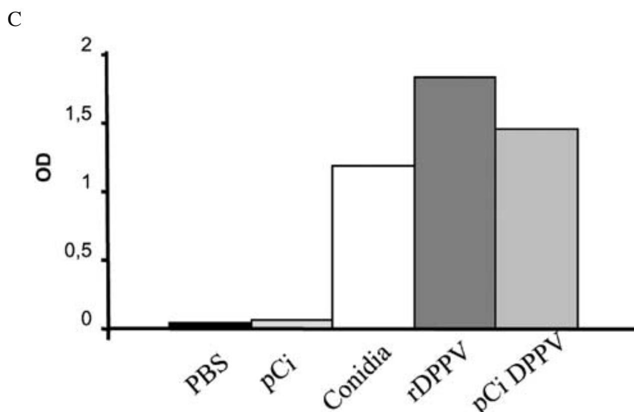

Figure 6. Production of antibodies by DNA vaccination: A: Mamalian pCi expression vector of the secreted DPPV of *Aspergillus fumigatus* (Promega, kind gift of Y. Jacob) used for DNA vaccination; B: Immunoblot analysis of DPPV produced by neuroblast cells (1–4) using a 0.1% dilution of a mouse anti-DPPV antiserum and anti-mouse peroxidase conjugate or by *Pichia pastoris* (5). Lane 1–4, 21 hours (lane 1), 38 hours (lane 2), 45 hours (lane 3) and 62 hours (lane 4) expression of DPPV in neuroblast cells; lane 5, Control: DPPV expressed in *P. pastoris*.; C: ELISA data obtained after immunization of mice by either a recombinant rDPPV or the pCi-DPPV. Controls are animal injected with conidia, PBS or the pCi without the DPPV

by Montagnoli et al. (2003) have shown that resistance to primary or secondary infections was significantly increased in $B^{-/-}$ mice. The authors suggested that the lack of efficacy of the antibodies may be due to wrong epitope specificity. Our results with the DPPV showed that antibodies directed against a major antigen and associated to resistance to infection were not protective in *A. fumigatus*. This result confirms that antibodies do not generate a memory antifungal immunity and their role during infection remains unknown.

This result does not mean that antibodies cannot have a direct protective role during *Aspergillus* infection. For example, anti-idiotypic Mabs mimiking a killer toxin recognizing βglucans inhibit early conidial germination. This inhibition even transient can cure IA in mice (Cenci et al. 2002). This result suggests that anti-βglucans natural antibodies known to be present in patients could favour the initial host antifungal defence in the lung. Anti-*A. fumigatus* MAbs have been shown recently to have a direct protective efficacy in murine experimental aspergillosis (Chaturvedi et al. 2005).

1.5.2. T-cell manipulations and Dendritic cell vaccines

In patients undergoing stem cell transplantation, adaptive cell immunity with antigen-specific T-cells has been already shown to restore protection against cytomegalovirus or Epstein Bar viruses (Einsele 2003; Einsele et al. 2002). A similar approach has been recently undertaken to fight *A. fumigatus* infections. Human activated T-cells can indeed be produced upon stimulation with a cellular

extract of *A. fumigatus* (Ramadan et al. 2005a; Ramadan et al. 2005b; Ramadan et al. 2004) (Lehrnbecher, personal communication). Transfusion of *Aspergillus*-specific T-cells significantly increased *A. fumigatus* hyphal damage either directly or through the activation of human neutrophils. *Aspergillus*-specific T-cells produced IFNγ- and IL-2 upon stimulation with intracellular extracts. In contrast, no significant production of IL-4 or IL-10 was observed, indicating that a Th1 response was induced. Most interestingly, CD4^{+} T-cell mediated alloreactivity of generated *Aspergillus*-specific T-cells was reduced compared with that of the original cell population. Therefore, *Aspergillus*-specific T-cell immunity, transferred through the infusion of *ex vivo*-generated, donor-derived, *Aspergillus*-specific T-cells might be beneficial for recipients of allogeneic transplant patients.

The induction of a Th1 response by dendritic cells (DC) after conidium phagocytosis has been exploited by several groups to develop a dendritic cell vaccine that is efficient in an immunocompromised and even more specifically allogeneic hematopoietic transplantation background (Bozza et al. 2003; Shao et al. 2005). For that purpose, DC were activated by live conidia or conidial total RNA. Transfusion of conidium activated DC induced a Th1 priming in animal and humans and increase resistance to the infection. Such vaccines have shown their efficacy in animals and start to be used in humans (Perruccio et al. 2004; 2005). This opens new perspectives for reducing transplant related mortality after allogeneic hematopoietic transplantation.

Many questions remain to be answered before the immunoprotective *A. fumigatus* molecules can be identified. How is the RNA processed by DCs ? In animals, it was shown that in contrast to conidia, activation of DC by hyphae do not protect against *A. fumigatus* infection. Why is then a mycelial culture filtrate inducing protection? Bozza et al. (2002) have shown that in presence of CpGs, DCs primed by an hyphal antigen (Aspf9 in Crameri's nomemclature = Aspf16 in Kurup's nomemclature = homolog of *S. cerevisiae* Crh1p) can induce resistance whereas other proteins such as a fibrinogen binding protein, an unknown protein induced in low concentration of Zn, a peroxisomal membrane protein and an hsp90 are totally inefficient (Kurup 2005). Since protection seems specific of the antigen used, what is the epitope family that is important for protection without exacerbating allergy? Moreover, injection of conidia of different fungal species result also in protection against *A. fumigatus* (unpublished data), asking the question of the pan fungal origin of the dendritic cell stimulator. p-formaldehyde fixed conidia do not protect showing that molecules must be released by the fungus to induce protection. This is in agreement with the requirement of conidial swelling inside the macrophages to induce an immune reaction. More work remains to be done to identify the molecule activating positively or negatively DC and T cells.

2. CONCLUSIONS AND PERSPECTIVES

IA is a disease of the immunocompromised host. The immunological status of this host has not been analysed as extensively as the immunocompetent one. For example, the number of resident phagocytes or the level of destruction of the lung

epithelia in these patients has not been precisely studied. Moreover, these patients are submitted to multiple and various types of immunosuppressive regimens and drugs: cytotoxic drugs inducing neutropenia or cyclosporin/tacrolimus reducing T cell reactivity or corticosteroids to fight Graft versus Host disease, all leading to different immunological impairments. A kinetic factor is also involved since the recovery of neutrophils and T cells is different overtime in the transplant patient at risk for IA. Finally, some of the phagocytic events are impaired whereas others are not : conidial engulfment is not perturbed whereas the pathways associated to killing (ROI production, MAPkinase phosphorylation, NFkB translocation, cytokine synthesis) are down-regulated in the immunocompromised host. It must also to be taken into account that data obtained in our pet mouse cannot be always translated to humans. For example, in our hands the corticosteroids dexamethasone and methyl-prednisolone that are used in humans to treat the GvH disease that increased the risk for IA, do not have any immunological impact in mouse and cannot be used to produce a murine IA whereas cortisone acetate, a drug never used in humans does promote experimental animal IA. Many of the anti-*A. fumigatus* immunological responses of the host, summarized in Fig. 7 have been well characterized in the immunocompetent host. Integrating all these data with the immunological impairments of the transplant or leukemic patient at risk for IA has not been completed yet.

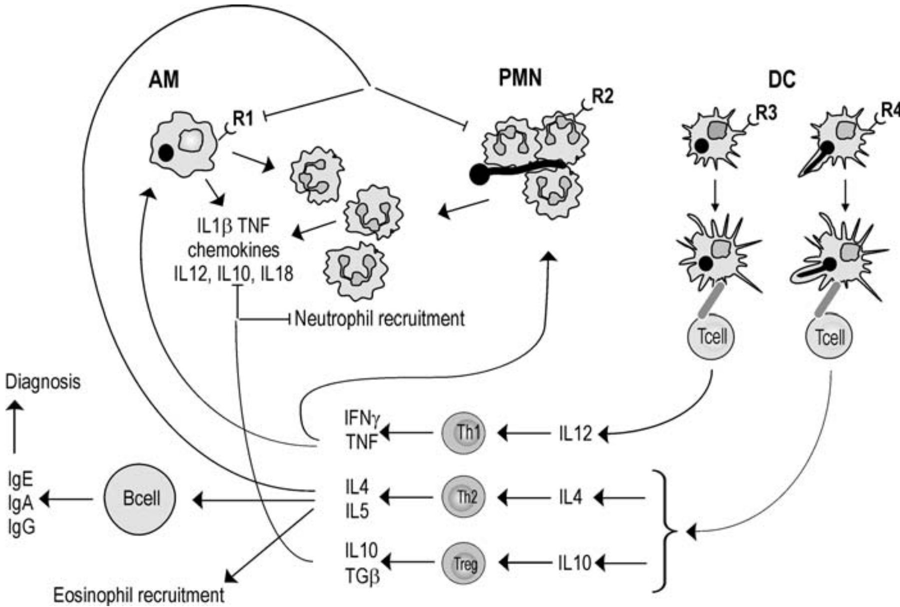

Figure 7. Cellular events occuring during the host immune response against *Aspergillus fumigatus* (adapted from Romani, 2004 and Mills, 2004). R1-R4: receptors (TLR2, TLR4, DC-sign, mannose receptor, Dectin1, CR3, FCγR,...) specific of each phagocyte and each fungal morphotypes: AM: alveolar macrophage; PMN: neutrophil; DC: dendritic cell

One of the conclusion emerging from recent immunological data is the correlation between the type of immune response and the fungal morphotypes. This is seen both for the induction of a Th1/Th2 response following phagocytosis by dendritic cells, for the killing mechanisms developed by neutrophils or for the receptor (TLR, Dectin-1, DC-SIGN) recognized or activated. Changes occuring in the composition between conidia and hyphae especially at the cell surface that is sensed by host receptors, can be responsible for orientating the immune response. These immunological reactive molecules specific of each fungal morphotypes (resting conidium, swollen conidium and mycelium) have not been investigated yet and are a wide research avenue. It is indeed known that the composition of the conidial and mycelial cell wall are different (Bernard and Latgé, 2001). One of the putative candidate to react with the immune system, is the rodlet hydrophobins (Paris et al. 2003a). These proteins that are on the conidial surface and are covalently bound to cell wall polysaccharides through a GPI-remnant, are absent in the mycelial stage. Other molecules to investigate will result from the exploitation of proteome data obtained with the 3 fungal morphotypes, a research now feasible since the completion of the sequence of the *A. fumigatus* genome. Astonishingly, hyphae are inducing a non-protective response. An explanation for this is that the explosion of the inflammatory reaction would be more detrimental to the host than fungal growth itself. Similar results were found with *Candida albicans* (Romani 2004). Morphological switching may not be then important per se but because each fungal stages induces a different immunological regulation. Host cannot be seen now only as counteracting the fungal growth but also modulating actively fungal virulence. It also indicates that the host can withstand important mycelial growth without any significant damage. This concept is practically largely applied in IA management since an efficient antifungal treatment will not kill entirely the fungus but will circumvent its development until the recovery of the immune response that will slowly eliminate the fungus. Progress in IA will require that studies of Mycology and Immunology become more integrated to better understand the different outcomes of the infection: persistence of the fungus, resolution of the infection with excessive collateral damage or resolution with limited immunopathology associated to the development of immune memory.

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CHAPTER 16

CANDIDA

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Abstract: This chapter deals with the yeast *Candida,* one of the increasingly important human pathogens, and addresses multiple factors that result in either immunity to or infection with this micro-organism. Factors contributing to the pathogenicity, antigenicity and type of infections caused by the genus *Candida* and its' species are discussed. An overview of immunity to *Candida* includes previous and recent data on innate and adaptive immune responses, focusing on recognition and sensing of *Candida* via pattern recognition receptors (Toll-like receptors, Dectin-1) and their role in directing the ensuing cascade of cytokine production, that leads to protective or non-protective immunity mediated by cellular and humoral adaptive immune responses. The disease Chronic Mucocutaneous Candidiasis (CMC), which includes a subgroup of patients with the APECED syndrome (Autoimmune PolyEndocrinopathy Candidasis Ectodermal Dystrophy), where patients show a selective susceptibility to infections with *Candida,* is discussed in detail. The role of cell-mediated and humoral immunity in impaired protection against *Candida* is presented, as well as recent data demonstrating dysregulated cytokine production in response to *Candida,* which is suggested could be the underlying immune defect in these patients. The importance of CMC as a non-conventional primary immune deficiency, manifesting as narrow susceptibility to infection with weakly pathogenic microbes is stressed, given that these unique human "models" have the potential of hugely increasing our understanding of basic immune mechanisms involved in protection against *Candida* and other fungi

Keywords: *Candida,* immunity, Toll-like receptors, cytokines, Chronic Mucocutaneous Candidiasis, APECED

1. IMMUNITY TO *CANDIDA*

1.1. *Candida*

The genus *Candida* comprises around 200 species of both sexual and asexual ascomycetous or ascomycetous-like fungi, the majority of which are non-pathogenic to humans, growing as environmental saprophytes. However, a number of species

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have been isolated from sites of disease, though causation has not been proven in all cases (Calderone, 2002). The principle pathogen is *Candida albicans*, but a number of other species, notably *Candida dubliniensis, Candida glabrata, Candida krusei, Candida parapsilosis* and *Candida tropicalis*, have emerged as significant agents of disease in the last 20–30 years, causing 35–65% of candidaemias (Krcmery & Barnes, 2002).

Most *Candida* species grow as yeasts, but the majority also exhibit a filamentous growth form e.g. pseudohyphae, where the junctions between cells are marked by invaginations, and in the case of *C. albicans* and *C. dubliniensis* by true hyphae, where cellular junctions maintain parallel sided cell walls (Merson-Davies & Odds, 1989). The pathogenicity of the different forms is still debated, although it is of note that in the majority of infected tissue *C. albicans* produces a mixture of budding yeasts, psudohyphae and true hyphae suggesting that all these growth forms play a role in survival and proliferation in vivo.

The various *Candida* species show considerable antigenic similarities, while antigenic variability of serotypes has been related to mannan side-chains (Nelson et al 1991). Mannoproteins of the cell wall contain major immunodominant antigens such as a well characterised 65-kD (MP65) component (Cassone et al 1998 and 2005), readily eliciting immune responses in humans and animals, although interestingly, these responses are mostly directed to the protein moiety of the MP (Cassone et al 1998).*Candida* species share antigenic determinants with members of other yeast genera and cross-react, albeit weakly, with fungi such as *Saccharomyces cerevisiae, Aspegillus fumigatus* and *Trichophyton rubrum* (Cassone et al 1998, Odds 1988).

1.2. Infections with *Candida*

This chapter will deal with host immunity to these pathogenic fungi, in particular *C. albicans* and *C. glabrata*, but before that we will briefly introduce the diseases caused by these organisms.

C. albicans is a commensal yeast and as such colonises the skin and/or mucosa of the majority of healthy humans without causing tissue damage. However, it and other *Candida* species, as opportunistic pathogens, can establish disease in a variety of permissive circumstances. Oral thrush and its association with debilitating disease has been recognised since antiquity, indeed the generic name *Candida* was proposed by Berkhout (1923) and derives from the Latin name *toga candida* for the white robe worn by roman senators, and probably refers to the clinical appearance of oral thrush. Since then *Candida* infections of virtually every organ in the body have been reported. Infections can be superficial, involving the oral and aural cavities, genitalia, skin and nails, which tend to arise in locally moist conditions created by maceration and occlusion in intertriginous areas; systemic *Candida* infections involve all other sites. *Candida* infections are on the increase, perhaps as a consequence of rapidly expanding usage of antibiotics and immunosuppressive agents for cancer management, organ and bone-marrow transplantation, treatment

of autoimmune diseases or in patients with HIV-AIDS (Dismukes 2006). Patients with chronic mucocutaneous candidiasis (CMC) are a separate, small but significant patient group with persistent, selective *Candida* infections without a known underlying cause.

1.3. Animal Models of Candidiasis

A large amount of data on *Candida* immunity as been collected from work performed in animal models of disease. We will therefore briefly review this area. Although various species including rats, rabbits, guinea pigs etc. have been used as experimental models, murine models of candidiasis are generally perceived as the most informative (de Repentigny 2004). Contrary to humans, in whom *Candida* colonisation occurs shortly after birth (Odds 1988), mice are not normally colonised by *Candida* and must therefore be experimentally infected. However, even though the mouse model does not fully mimic the onset and progression of candidiasis in humans, systemic *C. albicans* infection can be induced in non-immunosuppressed mice, with different strains demonstrating different susceptibilities. Infection with *C. glabrata* generally requires immunosuppression to be established, in order for disease to occur (Kamran et al, 2004). Mucosal/oropharyngeal candidiasis in mice is more difficult to induce than systemic disease, and necessitates various immunosuppressive pre-treatments (steroid administration, irradiation etc.) or underlying immune defects such as in *nu/nu* or severe combined immune deficiency (SCID) mutant mice that harbour major dysfunctions of cellular immunity. More recently specific knock out mice have been used to study *Candida* infection. Interleukin (IL)-12 (IL-12), tumour necrosis factor (TNF)/lymphotoxin (LT)α and IL-6 deficient mice are highly susceptible to *Candida* infection, mice deficient in IL-10 are more resistant while lack of IL-4 had little effect (Romani 1999). Interestingly, findings for interferon (IFN) and IL-1α/β deficient mice are controversial, showing both increased susceptibility and no effect (Ashman et al 2004, Romani 1999, Vonk et al 2006). It is likely that as more knock out mice become available the role of specific components of the immune system in the prevention of *Candida* infections will be elucidated.

It is of note that a spontaneous mouse model of CMC is not available (de Repentigny et al 2004, Ashman and Papadimitriou 1995). Remarkably, in mice with a targeted mutation of the *Aire* gene, representing a mouse model of the human Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy (APECED) syndrome and a subgroup of CMC patients (vide infra), overt autoimmune disease and susceptibility to *Candida* infections has not been documented (Ramsey et al 2002, Anderson et al 2002, Pontynen et al 2006).

1.4. Immunity to *Candida*

Whether exposure to *Candida* evolves into tolerance or infection depends largely on host immunity. The majority ($\sim 80\%$) of healthy humans harbour *Candida* spp,

in particular *C. albicans*, as a commensal microbe on a mucosal surface, especially the gastrointestinal and genital mucosa with no ill effect and readily demonstrate in vivo and in vitro cell-mediated immunity that keeps it under control (Odds 1988). Conversely, *Candida* has developed numerous sophisticated virulence mechanisms (Navarro-Garcia et al 2001, Haynes 2001) aimed at overcoming these defences with which it constantly probes the integrity of the immune system. Even a minimal break in defence ranks, such as maceration of the skin or change in pH, not to mention immunosuppressive therapy, HIV/AIDS or underlying immune defects of the host, will shift the balance in favour of the microbe. This elaborate balancing act is a complex, continuous process and, from the hosts' point of view, involves multiple facets of both the innate and adaptive immune systems.

Immunity to fungi and *Candida* in particular has been extensively reviewed (Odds 1988, Bodey 1993, Ashman 2004). However, over the last few years, new and exciting data unravelling the role of the innate immune system in recognition, protection or susceptibility to *Candida* has emerged, changing our understanding of fungal immunity in a major way.

1.4.1. Phagocytes and immunity to Candida

The innate immune system has only recently come into the spotlight from years of neglect and has claimed its' rightful place, not only as the first line of defence but as a crucial instructor and orchestrator of the subsequent adaptive immune response. In antifungal immunity, the innate immune system provides multiple effector mechanisms that are directly involved in elimination of the fungus which are crucial both as first line of defence and in protection against systemic spread of the organism. The role of phagocytic cells such as macrophages and particularly neutrophils is paramount, which becomes painfully clear in neutropaenic patients who readily succumb to fungal, in particular *Candida* and *Aspergillus* infections, often with a lethal outcome (Ashman et al 2004). Functional phagocytic defects such as inborn errors of reactive oxygen radical production, leading to defective intracellular killing, as is the case in patients with chronic granulomatous disease (CGD) make these host easy targets for *Candida* (Notarangelo et al 2006). Neutrophils and macrophages can ingest and kill yeast cells and hyphae, by both oxidative and non-oxidative mechanisms, and also collaborate to effect extracellular killing. Soluble mediators with opsonic activity such as complement and collectins enhance binding and phagocytosis of fungi, although recent data suggest that binding through the complement C3 receptor may actually undermine production of protective cytokines (Odds 1988, Romani et al 2002, Shoham and Levitz 2005).

1.4.2. Cytokines and immunity to Candida

Although the role of direct fungicidal mechanisms provided by the innate immune system cannot be overemphasized, an even more important aspect is its instructive role in orchestrating the subsequent adaptive immune response. This role is mediated by expression of co-stimulatory molecules and production of chemokines and cytokines, soluble mediators produced by most cells of the innate immune system,

but most importantly by dendritic cells (DCs) that are currently believed to be the conductors of this complex symphony. DCs activate T cells through expression of co-stimulatory molecules and produce cytokines that will skew the adaptive immune response either toward a cellular, T helper (Th)1 or a humoral Th2 type response (Colonna et al 2006). Although this scenario is arguably oversimplified, it nevertheless illustrates how the two most important effector arms of the immune system are activated. The importance of Th1 cytokines in protection against *Candida* was demonstrated in a series of elegant experiments in mice by Romani and others (reviewed by Romani 1999 and 2004, Ashman et al 2004). Efficient control and eradication of fungi including *Candida* relies on the generation of a protective Th1 response that is initiated by IL-12 secreted by DCs and phagocytic cells.IL-12, together with IL-18 induces T cells and NK cells to produce IFNγ, a key activator of effector cells (neutrophils and macrophages) and ensures prolonged responsiveness of CD4 + Th1 cells to IL-12 stimulation. As opposed to this, generation of a Th2 response is not only unhelpful as happens with IL-4 production that leads to antibody synthesis, but can actually be detrimental as happens when IL-10 is produced (Romani et al 2006), which is a potent immunosuppressive cytokine and leads to down-regulation of pro-inflammatory cytokine synthesis including IL-1, IL-6, TNFα and IL-12. Obviously the later scenario is undoubtedly beneficial and necessary to downscale and end an ongoing protective Th1 response once it has fulfilled its role. However, it is already clear that the role of Th1 versus Th2 cytokines in generating protective immunity to *Candida* is far more complex, as has been demonstrated by the necessity of IL-4, IL-6 and IL-10 availability at specific time-points and quantities for mounting optimal protection against primary *Candida* infection and preventing reinfection (Mencacci et al 2000). The complexity of the roles of Th1/Th2 cytokines in protection against *Candida* is also exemplified by the controversial findings in mice deficient in specific cytokines, as discussed above.

This notwithstanding, the absolute requirement for an orchestrated cytokine response to *Candida* infection has been highlighted in recent work with a *C. glabrata ace2* mutant. Infection of immunosuppressed CD1 mice with *C. glabrata ace2* cells results in a massive over-stimulation of the pro-inflammatory arm of the innate immune system, compared to that seen during infection with wild type fungi, resulting in a septic shock-like response and death within 24 hours (Kamran et al 2004).

1.4.3. Toll-like receptors and immunity to Candida

The question of how the immune system “knows” when to respond and which cytokines to produce upon encounter of foreign microorganisms has only recently become clearer with the unveiling of an elaborate recognition mechanism employed by cells of the innate immune system, first proposed by Charles Janeway Jr and published by Medzhitov et al (1997). The groundbreaking discovery of Toll-like receptors (TLRs) led to the recognition of their role as receptors for conserved pathogen associated molecular patterns (PAMPS). Since then, 10 TLRs in humans

and 13 in mice have been reported (Iwasaki and Medzhitov 2004) as well as other non-TLR pattern recognition receptors (PRRs) (Brown 2006).

Different TLRs are activated by different microorganisms and initiate predetermined signalling pathways, leading to differential production of proinflammatory cytokines and co-stimulatory molecule expression, conferring specificity to the innate immune responses triggered by particular microorganisms (Iwasaki and Medzhitov 2004). It was initially reported that TLRs control activation of adaptive immunity leading to Th1 but not Th2 type responses (Schnare et al 2001). However, subsequent reports demonstrated differential activation of human DCs via TLR4 and TLR2 wherein TLR4 agonists specifically promoted production of the Th1 inducing cytokines IL-12p70 and chemokine-IFNγ-inducible protein (IP-10), while TLR2 stimulation did not induce these cytokines, but lead to production of IL12p40, IL-8 and p19/IL23 instead, producing conditions that favour Th2 development (Re and Strominger 2001). Agrawal et al (2003) reported that the underlying mechanism for inducing distinct Th responses in human DCs by different TLRs, was differential engagement of signalling pathways.

The importance of TLRs for fungal immunity was recognised from the beginning, as it was the observation in *Drosophila* that the Toll/Dorsal signalling pathway participated in an anti-fungal immune response, that prompted subsequent research into TLRs (Medzhitov et al 1997). Recognition of fungal structures by TLRs was first reported for zymozan and a TLR2/6 heterodimer (Underhill et al 1999). Subsequent studies investigating the role of TLRs in *Candida* infections demonstrated their huge significance in determining host resistance or susceptibility (reviewed by Netea et al 2004b). Studies in TLR deficient mice demonstrated that $TLR4^{-/-}$ C3H/HeJ mice showed increased susceptibility to disseminated candidiasis, while TNFα and IL1β production was dependent on TLR2, thus demonstrating recruitment of distinct TLRs by a single pathogen (Netea et al 2002a). Mice deficient in MyD88, a crucial molecule in the TLR signalling pathway, showed impaired resistance to both *C. albicans* and *A.fumigatus*, while $TLR2^{-/-}$ mice produced more IL-12 and less IL-10 and were not increasingly susceptible to *Candida* infection (Bellocchio et al 2004). An interesting study reported that TLR2 activation actually suppresses immunity to *Candida* through the induction of IL-10 and the generation of regulatory T cells (Netea et al 2004a), although opposite findings had previously been published (Villamon et al 2003). It was also demonstrated that TLRs and non-TLR PRRs could pair up to provide collaborative recognition as was shown for TLR2 and Dectin1 in the recognition of *Candida* (Gantner et al 2003). A recent report suggests that this can be used by fungi to avoid host protective responses (Dillon et al 2006). Manipulation of the immune response by fungi is also achieved through different fungal morphotypes, as is shown by differential activation of TLRs and subsequent cytokine production by *Candida albicans* hyphae and yeast (Chantal et al 2005). A study demonstrating simultaneous involvement of lectin-type receptors and TLRs in the recognition of *Candida albicans* highlights the complexity of interactions between the innate immune system and fungal pathogens (Netea et al 2006). Utilising mutants defective in various aspects of glycosylation, these

authors demonstrated that the various layers of the *C. albicans* cell wall activate the innate immune system through different effector mechanisms. *C. albicans N*-linked mannan was recognised by the mannose receptor, *O*-linked mannan by TLR4 and β-glucan by the TLR2/Dectin-1 complex. The authors concluded that *C. albicans* recognition by monocyte/macrophage cells, and subsequent cytokine and chemokine production is mediated by three distinct recognition systems.

In addition to TLR's, a recently discovered PRR, Dectin-1, that can bind β-glucans has been described. This C-type lectin receptor can, following ligand binding, activate diverse cellular responses that may initiate anti-*Candida* effector mechanisms, including cytokine production, phagocytosis and stimulation of the pro-inflammatory burst (Brown 2006). Indeed dectin-1 has been shown to mediate the uptake and killing of *C. albicans* (Gantner et al 2005).

1.4.4. Dendritic cells and immunity to Candida

DCs are at the interface between the innate and adaptive immune system and translate signals received through PRRs into soluble mediators, that guide the adaptive immune response toward a cellular (Th1) or humoral (Th2) outcome (Colona et al 2006). DCs express TLRs, and it is believed that fungal pathogens are recognised by myeloid DCs expressing TLR1, 2, 4 and 6, which, when activated will generally drive production of type-1 inducing cytokines, such as IL-12. DCs can phagocytose both *Candida* hyphae and yeast, which leads to DC maturation and activation (Romagnoli et al 2004). However, it has been reported that yeast and hyphae lead to differential activation of DCs, in that hyphae inhibit IL-12 production while inducing IL-4 (d'Ostani et al 2000). This data could now be interpreted in the light of differential activation of TLRs expressed on DCs by yeasts and hyphae and consequent differential activation of cytokine production as discussed above (Chantal et al 2005). It has been suggested that co-ligation of CR3 with FcγR by *Candida* hyphae will induce IL-4 and IL-10 production and Th2 type cell activation, while phagocytosis through the mannose receptor would promote Th1 type anti-fungal responses (Romani et al 2002).

DC's also express dectin-1 and this, at least in part, is responsible for directing a Th-1 type response in these cells to *S. cerevisiae* and *Pneumocystis carinii* β-glucans (Carmona et al 2006). If this is also the case for *Candida* infections remains to be determined.

1.4.5. Adaptive immunity to Candida

Protective immunity to *Candida* relies on the induction of cellular immunity, cytokines and effector phagocytic cells. Generation of a vigorous Th1-type cellular response initiated by IL-12 and other type-1 inducing cytokines (IL-18 and IL-23) is required for host resistance to *Candida* (Romani 2004). *Candida*-specific T cells can readily be demonstrated in healthy, sensitized individuals, who demonstrate in vivo delayed-type hypersensitivity (DTH) reactions in skin tests and in vitro lymphocyte proliferation to *Candida* antigens (Odds 1988, Bodey 1993, Kirkpatrick 2001). CD4+ T cells play a crucial role in anti-fungal defences, recruiting and

activating monocyte/macrophages and neutrophils to perform their role in killing and eliminating the infection. CD8+ T cells are believed to exert direct anti-fungal activity, lysing yeast cells as well as yeast-containing phagocytes (reviewed by Romani 2004).

The role of humoral immunity in protection against *Candida* is less clear as earlier studies suggest that *Candida*-specific antibodies do not have a protective role (Odds 1988, Bodey 1993). Indeed, high-titer anti-*Candida* antibodies are regularly found in patients that cannot clear *Candida* (Lilic et al 1996a), or in patients that succumb to systemic candidiasis (Odds 1988). Recently, there has been renewed interest in antibody-mediated protection against fungal infections, with focus of different *Candida* antigens targets, such as mannans, glucans and heat-shock proteins, with the aim of producing monoclonal or recombinant antibodies as a prospectively new treatment (Magliani et al 2005, reviewed in Cassone et al 2005). Encouraging data has been reported on the generation of a new vaccine containing β-glucan – a major *Candida* cell wall component – which provided protection against systemic candidiasis in an animal model (Torosantucci et al 2005). A recombinant human anti-mannan antibody was also demonstrated to confer protection when administered passively to mice with disseminated candidiasis (Zhang et al 2006). Antibodies to fungal heat-shock protein hsp90 were originally observed in patients recovering from systemic candidiasis (Matthews 1992) and are currently considered targets for immunotherapy by a genetically recombinant antibody (Burnie et al 2006).

2. CHRONIC MUCOCUTANEOUS CANDIDIASIS

2.1. Definition and Classification of CMC

CMC is a descriptive, clinical diagnosis and as such is often confusingly used to denote all patients with persisting mucosal *Candida* infection, irrespective of whether the underlying cause or predisposing factors are known (such as denture stomatitis or HIV/AIDS). However, it has long been recognised that there are patients with primary susceptibility to mucosal *Candida* infections (Forbes 1909) for which the name "chronic mucocutaneous candidiasis" was first proposed by Chilgren et al (1967). In this chapter, the name CMC is used to describe a heterogeneous group of patients with a common problem of primary, unexplained susceptibility to persistent, recurring, debilitating and distressing infections of the skin and mucous membranes with *Candida*.

As the underlying defect remains unknown, attempts to classify CMC have been descriptive and numerous, mostly based on time of onset, familial occurrence, mode of inheritance and co-occurrence of autoimmune endocrinopathy (Kirkpatrick et al 1971 and 2001, Higgs and Wells 1972). In the most recent international update on primary immunodeficiency diseases (PIDs), CMC is classified as a "Well Defined Immunodeficiency Syndrome" with an autosomal dominant, recessive or sporadic mode of inheritance while the subgroup of CMC patients who have APECED are classified under "Diseases of Immune Dysregulation" (Notarangelo et al 2006). The

Online Mendelian Inheritance in Man (OMIM) database currently recognises the following major subgroups:

- OMIM *240300 Autoimmune Polyendocrinopathy Syndrome Type 1 (APS1), also coined the Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy (APECED) Syndrome (Ahonen 1985). Recently the gene responsible was identified (Nagamine et al 1997 and Finnish-German APECED Consortium 1997) and designated *AIRE* (autoimmune regulator) (OMIM *607358). The mode of inheritance is classically autosomal recessive, although autosomal dominant is also recognised by OMIM. Patients typically present with organ-specific autoimmunity involving endocrine and other organs (hypoparathyroidism, adrenal failure, ovarian/testicular failure, diabetes mellitus, hepatitis, keratoconjunctivitis, intestinal dysfunction, vitiligo, alopetia etc.) as well as mucocutaneous candidiasis, recently reported to have occurred in all adults in a long-term follow-up study of 91 APECED patients in Finland (Perheentupa 2006). The auto-antigens have been identified as key intracellular enzymes present in the affected organs (steroidogenic enzymes 21-hydroxylase (21-OH), side-chain cleavage enzyme (SCC) and 17α-hydroxylase (17α-OH) in the adrenal cortex and gonads, glutamic acid decarboxylaze 65 (GAD65) and tyrosine posphatase-like protein IA-2(IA-2) in the pancreas, cytochromes P450 1A2 (CYP1A2) and 2A6 (CYP2A6) in hepatitis, tryptophan hydroxylase (TH) in intestinal dysfunction, tyrosine hydroxylase (TH) in alopetia and calcium-sensing receptor (CaSR) in the parathyroid) (Soderbergh et al 2004).
- OMIM *606415 Familial CMC with thyroid disease was reported as a separate syndrome by Coleman and Hay (1997), distinguished from APECED by it's autosomal dominant mode of inheritance, association with thyroid disease and lack of other endocrine organ involvement. A candidate linkage region was mapped to chromosome 2p (Atkinson et al 2001).
- OMIM *11458 Familial CMC, with autosomal dominant inheritance and no endocrine organ involvement including thyroid disease is recognised as a separate entity. Some patients have recurring infections and loss of teeth (Higgs and Wells 1972).
- OMIM *212050 Familial CMC with autosomal recessive inheritance and no endocrine organ involvement may have a late onset and is associated with iron deficiency in some patients (Higgs and Wells 1972).

Sporadic CMC is also recognised (Kirkpatrick 2001, Odds 1988), as well as CMC associated with IgG subclass deficiency (Bentur et al 1991), selective antibody deficiency (Kalfa et al 2003) and ICAM-1 deficiency (Zuccarello et al 2002), but whether these represent separate CMC subgroups remains to be confirmed.

With the exception of the APS1/APECED syndrome, the underlying genetic defects in CMC are unknown. Intense research into the function of the *AIRE* gene has elucidated its' crucial role in central tolerance induction, which can explain the organ-specific autoimmunity seen in APS1/APECED patients and mutant "knock-out" mice lacking a functional AIRE gene. However, very little is known about AIREs' role in generating immune responses and how *AIRE* mutations are linked

to this unusual selective susceptibility to *Candida*. The pathogenesis of chronic infections with *Candida* in APS1/APECED and other patients with CMC remains unknown and it is tempting to speculate that the different groups of CMC patients may have immune defects involving different sites of a complex but common pathway normally leading to protection against *Candida*. Such scenarios have been seen in other PIDs, where defects of different genes that are involved in a common immune pathway produce similar disease phenotypes in spite of diverse underlying defects. This is the case in the IL12-dependent IFNγ pathway deficiency, where different defects affecting IL-12p40 or receptor chains IL-12/23Rβ, IFNγR1 or R2 result in the same phenotype, with increased susceptibility to *Mycobacteria* and *Salmonella* infections (Ottenhoff et al 1998).

New data discussed above has increased our understanding of immune mechanisms involved in normal responses to pathogens such as *Candida* and – together with scarce but precious data on immunity in CMC patients – has enabled us to come closer to understanding what the immune defect(s) underlying the inability to fight *Candida* might be.

2.2. Immune Defects in CMC

2.2.1. Cell-mediated immunity in CMC

The early evidence for an association between candidiasis and deficient cellular (T cell) immunity came from clinical observations in patients with primary immunodeficiency syndromes such as SCID, Di George Syndrome and subsequently in patients with secondary T cell deficiency such as in HIV/AIDs.

The initial reports on immune abnormalities in CMC patients (Chilgren et al 1967, Kirkpatrick et al 1971, Valdimarsson et al 1973) consistently found defects in in vivo and in vitro correlates of cell-mediated immunity in response to *Candida* antigens. Kirkpatrick et al (1971) reported that 8 of the 12 CMC patients studied did not produce an in vivo DTH reaction in skin tests to *Candida* extracts and did not show in vitro lymphocyte proliferation or produce lymphokines (migration inhibitory factor- MIF) upon stimulation with *Candida*. Responses to other antigens and mitogens were mostly normal. No defects of polymorfonuclear leukocyte phagocytosis or oxidative killing of *C. albicans* were found. Total haemolytic complement activity and titers of anti-*Candida* antibodies were normal or increased. In a study of a large group of 26 CMC patients, Valdimarsson et al (1973) demonstrated a diversity of immune abnormalities ranging form normal to complete lack of both in vivo and in vitro responses, mostly confined to *Candida*. Antibody levels were normal and no abnormality of phagocytic cells was found.

These and subsequent publications largely confirmed that immune abnormalities in CMC patients were present in various degrees, and if present affected the cellular arm of the immune response, manifesting as absent DTH in vivo and lack of lymphocyte proliferation and lymphokine production in vitro, mostly selectively in response to *Candida* antigens. No major alterations in total lymphocyte and lymphocyte subsets counts were reported. Innate immunity, including complement

function, phagocytosis, intracellular killing, chemotaxis, reactive oxygen radical production and natural killer cell activity were mostly reported as being intact (reviewed by Odds 1988, Bodey 1993, Lilic and Gravenor 2001, Kirkpatrick 2001), although subtle impairment of macrophage function (Bortolussi et al 1981) and defective handling of mannan were noted and linked to direct suppressive activity and induction of *Candida*-specific suppressor cells (Fisher et al 1982). However, the argument that mannan was responsible for the immune defects and indeed the susceptibility to *Candida* in CMC patients was disproved by reports that eradication of *Candida* by antifungal treatment did not improve defective in vivo and in vitro responses to *Candida* (Mobacken et al 1987).

2.2.2. Humoral immunity in CMC

Humoral responses in general and specifically to *Candida* were repeatedly shown to be intact (Kirkpatrick et al 1971, Valdimarsson et al 1973). In 10 patients with CMC, we demonstrated normal serum total IgA and IgM levels with increased total IgG. Total IgG subclass IgG1, IgG2 and IgG4 levels were normal while IgG3 was markedly increased. Assessment of *Candida*-specific antibodies showed markedly increased *Candida*-specific IgG1 and IgA antibodies in all patients (Lilic et al 1996a). These findings did not suggest humoral immune deficiency. However, as mentioned above, association of CMC with IgG subclass deficiency (Bentur et al 1991) and selective antibody deficiency (Kalfa et al 2003) has also been reported. It is of note that several studies on larger numbers of CMC patients report increased susceptibility to encapsulated bacteria and other pathogens, resulting in frequent, often severe respiratory tract, skin and other infections in the majority of patients, even though immunoglobulin levels were reported to be normal (Kirkpatrick et al 1971, Herrod 1990). Patients with antibody deficiencies such as combined variable immune deficiency (CVID) or X-linked agammaglobulinemia (XLA) are not particularly susceptible to *Candida* infections (Notarangelo et al 2006).

Taken together, the above findings led to a firm belief that the underlying immune defect in CMC was a T cell defect; it was suggested that CMC patients lacked *Candida*-specific T cells, which were removed during thymic selection resulting in a “hole in the repertoire” for *Candida* antigens (Kirkpatrick et al 1971, Odds 1988, Bodey 1993).

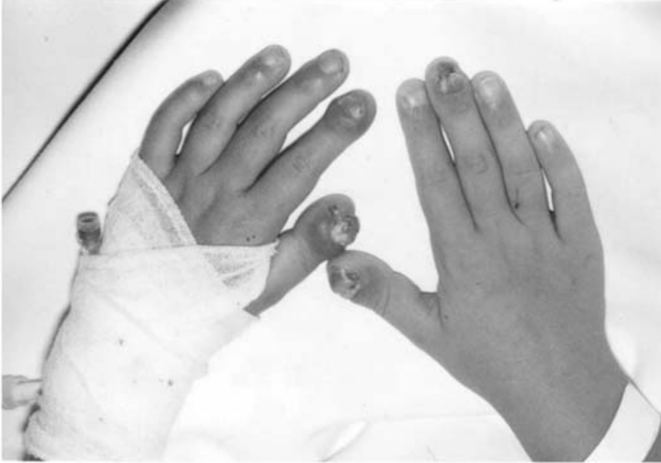
2.2.3. Cytokines in CMC

With increasing knowledge of the role of cytokines in generating immune responses, it was soon shown that stimulation of healthy human peripheral blood mononuclear cells (PBMCs) by *Candida* mannoproteins leads to cytokine production including IL-1 β, TNFα, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN γ (Ausiello et al 1993). The crucial role of cytokines in generating a protective response to *Candida* was demonstrated in mice (reviewed by Romani 1999, Ashman et al 2004) in a series of experiments which confirmed that a cell-mediated, T helper 1 (Th-1) type response was essential for protection from and eradication of the fungus (vide supra). These findings begged the question of

cytokine production in CMC patients, and our group (Lilic et al 1996b) and others (Kobrynski et al 1996) demonstrated for the first time altered cytokine production in CMC patients in response to *Candida* antigens. In 10 CMC patients, upon in vitro stimulation with *Candida* antigens, we found low or absent IL-2, increased IL-6 and either absent or increased IFNγ levels. Responses to other antigens and mitogen were not altered. Interestingly, these alterations were most prominent upon stimulation with a polysaccharide-rich as opposed to a protein-rich *Candida* antigen. In 8 CMC patients, Kobrynski et al (1996) demonstrated increased IL-4 with unaltered IL-10 and IFNγ production; they also found a decrease in CD4+ CD29+ T-helper inducer cells. Subsequently, impaired lymphocyte proliferation and cytokine production was reported by another group (de Moraes-Vasconcelos et al 2001) who found low IL-2 and IFNγ production and unaltered levels of IL-4 and IL-10 while responses to mitogen stimulation were intact. Van der Graff et al (2003) reported *Candida*-specific IFNγ deficiency and increased production of IL-10, while TNFα and IL-1 β production were unaltered. Because of the equivocal finding of both decreased and normal IFNγ production in CMC patients and its recognised role in enhancing macrophage candidicidal activity, we administered IFNγ therapy in a 6 year-old girl with severe, debilitating CMC (Fig 16.1) associated with recurrent pulmonary infections who had not responded to conventional treatment (Abinun et al, 1994). Even though some improvement was noted in in vitro cytokine production to *Candida* antigens, IFNγ therapy showed little if any clinical benefit during a 6-month trial.

The above findings suggested (Lilic 2002) that in CMC patients, the susceptibility to and impaired clearance of *Candida* could be the result of inadequate cytokine production i.e. lack of type-1 or, more likely type-1-inducing cytokines, given that our previous results demonstrated the impairment was most marked in response to the *Candida* carbohydrate fraction. To assess this hypothesis, in 24 CMC patients, we studied production of a range of inflammatory, anti-inflammatory, type 1 and type 1-inducing cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, and IFNγ) in response to 5 different fractions of *C. albicans* (purified mannan, carbohydrate and protein-rich) as well as non-*Candida* protein (tetanus toxoid) and polysaccharide (pneumococcal polysaccharide) antigens. Our findings demonstrated for the first time in CMC patients a severe impairment of IL-12 production which was most marked in response to purified *Candida* polysaccharide (C-PS), but was also found for other antigens and mitogen (Fig 2A). As opposed to this, IL-10 and IL-6 production was hugely increased to the same *Candida* fraction (C-PS) but not to non-*Candida* polysaccharide or mitogen (Fig 2B and C). Levels of IFNγ were borderline low or normal in response to various antigens tested, while expression of type 1 cytokine receptors (IFNγR1 and IL-12Rβ1) was normal in all CMC patients. TNFα production was unaltered. Interestingly, IL-4 and IL-5 levels were low but comparable to control values for all antigens tested. In this cohort, 2 patients had *AIRE* gene mutations but their results were similar to the rest of the group (Lilic et al 2003). Recently, high-titer autoantibodies to type I interferons (IFNλ and ω) were demonstrated in all APECED patients tested (Meager et al

A



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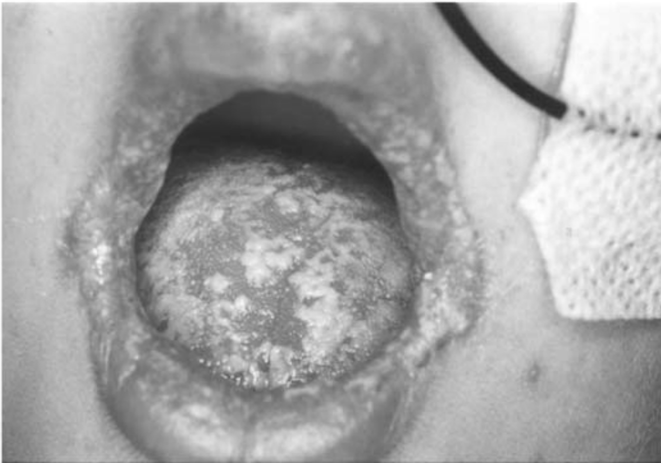

Figure 1. Severe, debilitating CMC of the nails (A) and mouth (B) in a six-year old girl. Reprinted with permission from Dr Mario Abinun and Professor Andrew J Cant (see color section)

2006), suggesting that altered function of this cytokine might be involved in the pathogenesis of this disease.

2.2.4. *T regulatory cells in CMC*

To investigate whether T regulatory cells were affected, we studied 4 patients with confirmed *AIRE* mutations underlying the APECED syndrome associated

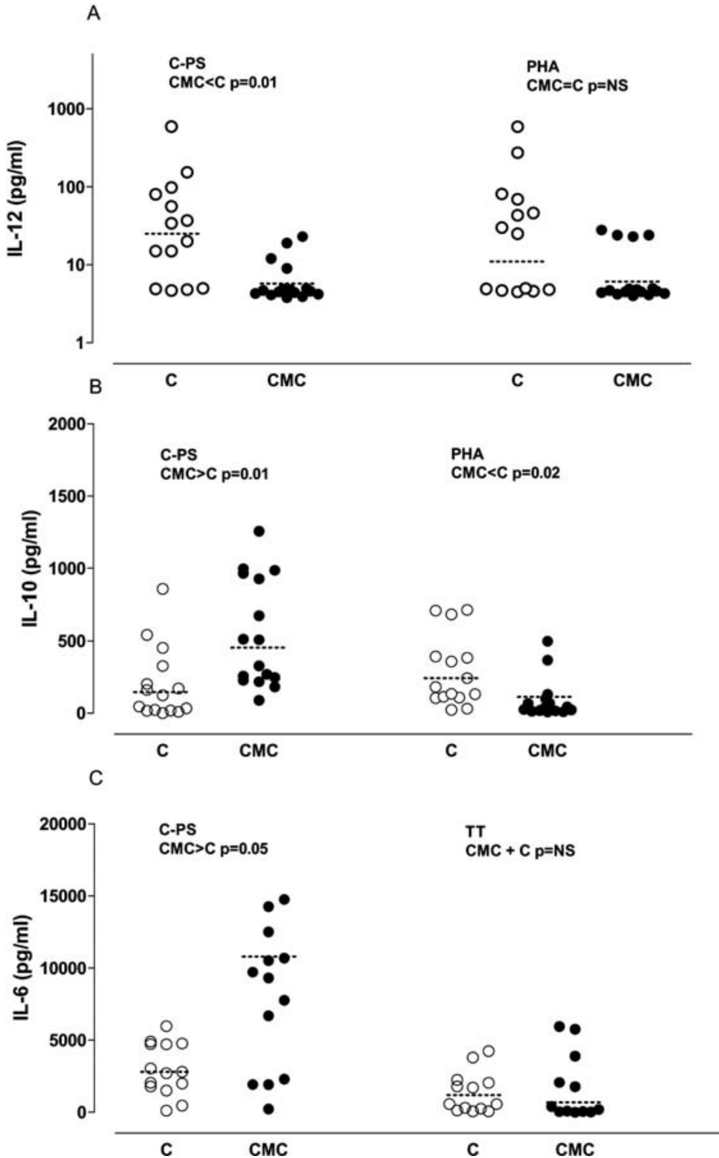

Figure 2. Differences in cytokine production in response to *C.albicans* and non-*Candida* antigens in patients with CMC and healthy controls. A. IL-12, B. IL-10, C.IL-6. Whole-blood cultures from patients and controls was stimulated with *C.albicans* polysaccharide fraction (C-PS) or non-*Candida* antigens (TT= tetanus toxoid and PHA= phytohaemaglutinin) for 48h. Supernatants were collected and cytokine levels assessed by ELISA. Each symbol indicates cytokine levels for an individual patient (CMC, solid circle) or control (C, open circle). Note Log scale for IL12; NS=non-significant; lines represent median values. Paritally reprinted from Lilic et al, Infect Immun (2003) 71:5690–5699, with permission form the American Society for Microbiology

with CMC. Previous work in mice had demonstrated that the *Aire* gene regulates ectopic expression of auto-antigens in thymic epithelial cells (Anderson et al 2002) and induces deletion of autoreactive thymocytes (Liston et al 2003). At the same time it was independently shown that naturally occurring T regulatory (Treg) cells, characterised by $CD4^{+}CD25^{hi}$ FOXP3 expression, were generated in the thymus on ectopically expressed agonist ligands (Apostolou et al 2002), suggesting that in addition to ensuring negative selection of thymocytes, AIRE might have a role in generating Treg cells. In our APECED patients we studied peripheral blood T cells expressing antigens characteristic of natural Tregs including $CD4^{+}CD25^{hi}$, glucocorticoid-induced TNF receptor (GITR), $CD45RB^{lo}$, L-selectin ($CD62L^{hi}$) as well as the FOXP3 transcription factor. Our findings demonstrated a markedly reduced proportion of T cells bearing these markers (Fig 3), suggesting that APECED patients were deficient in Treg cell. However, the level of FOXP3 mRNA in isolated CD4+ cells was similar in patients and controls (Ryan et al 2005). Interestingly, it was recently shown (Bacchetta 2006) that patients with mutations of the *FOXP3* gene and the autoimmune disease **I**mmune dysregulation, **P**olyendocrinopathy, **E**nteropathy **X**-linked (IPEX) unexpectedly have normal numbers and phenotype of FOXP3 + CD4 + CD25 + T regulatory cells, in sharp contrast to findings in *foxp3* deficient mice. These findings underpin the need for further studies for conclusive results on T regulatory cells in APECED patients, both natural Tregs and induced-T regulatory (Tr1) cells, which mediate suppression by IL-10 secretion – a cytokine whose levels have repeatedly been found to be increased in CMC patients (vide supra). Recently, Anderson et al (2005) demonstrated that *Aire* does not have a crucial role in thymic selection of natural T regulatory cells, at least in the mouse model. A very interesting

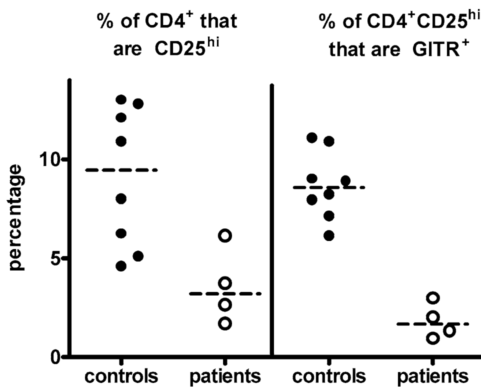

Figure 3. CD4+CD25+ T-regulatory cells are decrease in patients with APECED and CMC. Peripheral blood CD4+ cells from *AIRE*$^{-/-}$ patients and age-matched controls were assessed by flow cytometry for cell surface markers CD4/CD25 or CD4/GITR. Lines represent median values. Paritally reprinted from Ryan et al, J Allergy Clin Immunol 116:1158–1159 (2005), with permission form American Academy of Allergy Asthma and Immunology

recent study demonstrated a major reduction in invariant natural killer T (iNKT) cells in *Aire*$^{-/-}$ mice. NKT cells are known to be potent regulators of immune responses, including autoimmunity; these findings suggest that *Aire* is required for the development of iNKT cells and may have a role in the APECED/APS1 (Mi et al 2006). However, it must be stressed that the *Aire*$^{-/-}$ mouse does not develop overt autoimmune disease nor spontaneous *Candida* infections (Ramsey et al 2002, Anderson et al 2002, Pontynen et al 2006) as do patients with *AIRE* mutations, suggesting important differences between the mouse model and human disease. In extrapolating conclusions from "mice to men", it is of utmost relevance that major immune defects can remain unnoticed until exposed to their specific challenge, usually in the form of a specific pathogen (e.g. IL-12 and IFNγ efficiency and exposure to *Mycobacteria*) (Ottenhoff et al 1998). Laboratory animals are not routinely exposed to environmental pathogens which may markedly affect the presentation of the immune defect they harbour. Consequently, it is surprising that there have been no studies reported on *Candida* infection in *Aire*$^{-/-}$ mice.

A very recent publication demonstrating that antigen presentation by *Aire*$^{-/-}$ murine DCs is altered, leading to T cell hyper-responsiveness (Ramsey et al 2006) suggests that the link between *AIRE* mutations and the increased susceptibility to *Candida* in APECED patients might be at the level of DCs which could be defective in instructing T cell responses.

2.3. On the Pathogenesis of CMC

Our results confirme that CMC patients have seriously deregulated cytokine production, particularly in response to *Candida* carbohydrates (mannan, glucan, chitin). The markedly impaired IL-12 production strongly points to a defect in the type 1-inducing cytokine arm of the response i.e. to the innate immune system that directs T cell responses, rather than the T cell itself. A likely candidate to harbour the defect would be the DC, that instructs the type of adaptive T cell response to follow. A flood of new and exciting data has recently unveiled a crucial role for PRRs such as TLRs and C-type lectins on DCs in determining the type of cytokines produced upon interacting with *Candida* antigens (vide supra), making them prime candidates for studies in patients with CMC, as is the focus of ongoing research in our laboratory. It is of note that a recent report did not support a role for genetic polymorphisms of TLR2 and TLR4 in CMC patients (Van der Graff et al 2003).

Alternatively, deregulated cytokine production may be a consequence of abnormal regulatory cell activity, which could target selected cytokines such as IL-12, IL-2 of IFNγ. Indeed, the repeatedly reported high levels of IL-10 in CMC patients could originate from induced-T regulatory (Tr1) cells, which exert their regulatory function through production of the suppressive cytokine IL-10 (Romani and Puccetti 2006). The role of naturally occurring CD4+CD25+ T regulatory cells remains another possibility that needs to be clarified, particularly in APECED patents with the *AIRE* gene mutation, which may be involved in the generation of these

cells (Anderson et al 2002). In these patients, *AIRE* mutations could also affect the antigen-presentation function of DCs, leading to altered T cell activation and responsiveness to *Candida* antigens.

In both scenarios, in the absence of type 1-inducing cytokines, T cell-mediated cellular responses would not be generated and would explain the repeated findings of absent cell-mediated responses to *Candida* antigens in CMC patients. As previously mentioned, it is possible that different groups of CMC patients may have immune defects involving different sites of a complex but common pathway normally leading to protection against *Candida*.

2.4. Summary and Significance of CMC

In summary, the accumulating data regarding immune defects in CMC has highlighted markedly deregulated cytokine production which results in an inability to mount efficient, protective cell-mediated responses to *Candida*. The underlying defect may be at the level of DC activation by *Candida* and impaired production of type 1-inducing cytokines, suggesting involvement of PRRs such as TLRs and C-type lectin receptors, which are known to be crucially involved in initiating cytokine production. Alternative possibilities are impaired antigen presentation by $AIRE^{-/-}$ DCs or defects of at the level of T regulatory cells. It is likely that different defects resulting in the same phenotype will be found in the different subgroups of CMC.

The importance of understanding the aetiology and pathogenesis of CMC extends beyond the disease itself for several reasons:

- CMC is a good example of a non-conventional PID, manifesting as a narrow susceptibility to infection with weakly pathogenic microbes (as opposed to conventional PIDs, typically manifesting as a broad susceptibility to multiple organisms) (Casanova et al 2005). Non-conventional PIDs are becoming the focus of research as they can ultimately explain individual susceptibility to common infections as inborn errors of immunity. Deciphering the genetic basis of impaired immunity to specific infections is a phenotype-to-genotype approach ("forward genetics") as opposed to the currently favoured genotype-to-phenotype approach, achieved by targeted mutations/disruptions of certain genes ("reverse genetics") (Casanova et al 2002). Forward genetics identifies rare natural mutants with increased susceptibility or resistance to specific pathogens and has the potential to hugely increase our understanding of how the immune system works.
- Secondly, CMC is an unusual PID in which the main defect seems to be deregulated cytokine production, which is not currently recognised as a separate group of PIDs. Cytokine deregulation (Chehimi et al 2001) and an imbalance of Th1/Th2 cytokines (Netea 2002b) was previously also reported in the hyper-IgE syndrome, another PID without a known cause. It remains to be confirmed whether cytokine deregulation is the primary or secondary defect in these PIDs.

- Lastly, the subgroup of APECED patients with CMC represent a human *AIRE* gene "knock-out" causing both autoimmunity and immune deficiency. Understanding the common denominator underlying these two exceptionally important conditions in human disease will be a huge step forward in elucidating fundamental aspects of the immune system and in applying our knowledge to benefit human health.

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Note added in proof: The manuscript by Mi et al. has been retracted.

CHAPTER 17

IMMUNOLOGY OF INFECTIONS WITH *CRYPTOCOCCUS NEOFORMANS*

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Abstract: *Cryptococcus neoformans* is largely an opportunist, causing infection when host defences are breached. During the past two decades, invasive cryptococcal infections have emerged as a major threat to these immunocompromised hosts, especially to non-treated HIV patients. Also patients with neoplastic diseases are at significant risk for infections as a result of their underlying illness and its therapy. The outcome of infections differs, depending upon which aspect of immunity is impaired. This article reviews the current understanding of the role and relative importance of innate and adaptive immunity to *Cryptococcus neoformans.* An understanding of the host response to this organism is important in decisions regarding use of currently available anti-fungal strategies and in the design of new therapeutic modalities.

1. CRYPTOCOCCUS NEOFORMANS

Cryptococcus neoformans is an encapsulated fungus that mainly causes infections of the central nervous system in immunocompromised individuals with T-cell defects (e.g., AIDS, lymphoproliferative disorders, immunosuppressive therapy), but which can also infect immunocompetent individuals (Dromer et al., 1996; Mitchell et al., 1995). The incidence of disseminated cryptococcosis has increased considerably in the last decades because of the AIDS pandemic, and still rises in developing areas such as Sub-Saharan Africa and Asia were highly active anti-retroviral therapy (HAART) and anti-fungal therapy are not readily available (Mwaba et al., 2001; Imwidthaya and Poungvarin, 2000; Hajjeh et al., 1999).

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Currently, two varieties are recognized, *C. neoformans* var. *grubii* and var. *neoformans* (Franzot et al., 1999), which are predominantly found in temperate regions. Based on genetic characterization it has recently been recognized that the former third variety (*C. neoformans* var. *gattii*), which prevails in (sub)tropical regions, is a different species and was renamed *Cryptococcus gattii* (Trilles et al., 2003). The varieties differ somewhat in virulence and in the clinical course of infection (Mitchell et al., 1995). The most frequent occurring serotype in AIDS-associated cryptococcosis is the *C. neoformans* var. *grubii*, whereas var. *neoformans* causes a minor percentage of infections (Dromer et al., 1996; Shimizu et al., 1986). *C. gattii* has a relative predilection for the immunocompetent host (Speed and Dunt, 1995).

Encounters between the human host and cryptococci will be frequent due to the common presence of these fungi in our environment (soil, avian excreta and eucalyptus trees (Levitz, 1991)). It is generally accepted that infection is initiated by inhalation of poorly encapsulated or desiccated cryptococci from the surroundings. Usually, the initial pulmonary infection is asymptomatic. From the lungs, the cryptococci might enter the bloodstream and preferentially spread to the brain by means still largely unknown, leading to an often life-threatening meningoencephalitis with a high relapse-rate (Mitchell and Perfect, 1995). The second most frequent site of infection are the lungs, other less common sites of infection include the skin, prostate, lymph nodes, adrenal glands, heart, bones and the gastro-intestinal tract. Symptoms of cryptococcal meningitis usually develop insidiously over weeks, and might consist of a fever, headache, nausea, signs of increased intracranial pressure or neurological deficits, but the clinical picture might be difficult to recognize. The diagnosis is usually made by detection of cryptococcal polysaccharides in serum or cerebrospinal fluid (cryptococcal antigen test), but the fungus might also be detected by Indian Ink staining and microscopic evaluation of samples or by culture (blood, CSF and respiratory secretions). The choice of therapy consist depends on the location and extensiveness of the cryptococcal infection as well as the immune status of the host, and consists of anti-fungal agents such as fluconazol, amphotericin B/flucytosine (Saag et al., 2000).

2. THE HOST RESPONSE DURING CRYPTOCOCCOSIS

Cell-mediated immunity is the cornerstone of the host's defense mechanisms against cryptococci. Intact phagocytosis (innate immunity) (Monga, 1981), T-cell function (adaptive immunity; reviewed in (Murphy, 1998)) and the subsequent production of cytokines (Aguirre et al., 1995; Cross and Bancroft, 1995; Decken et al., 1998; Hoag et al., 1997; Huffnagle et al., 1996; Olszewski et al., 2001) and chemokines (Huffnagle 1999; Huffnagle et al. 1999; Huffnagle et al. 1995) have proven to be essential for protection against cryptococci and the prevention of dissemination.

2.1. Innate Host-Defense Mechanisms

2.1.1. *Innate chemical defense*

2.1.1.1. Surfactant As infection with *C. neoformans* starts in the lung, the pulmonary innate immune system is the first line of defense encountered by this pathogen. Surfactant protein D (SP-D), present in the alveolar lining fluid, is part of this local innate immune system (Crouch and Wright, 2001; Lawson and Reid, 2000; Mason et al., 1998; McCormack and Whitsett, 2002). Binding of microorganisms by SP-D can result in their aggregation (Lawson and Reid, 2000; Mason et al., 1998; McCormack and Whitsett, 2002), which is thought to stimulate their removal by enhancing their mucociliary clearance (Crouch and Wright, 2001). Furthermore, binding of SP-D to microorganisms can stimulate (Bufler et al., 2003; Madan et al., 1997; Ofek et al., 2001) as well as inhibit (Ferguson et al., 1999; van Rozendaal et al., 2000) their subsequent uptake and killing by phagocytes, depending on the specific pathogen involved.

Although SP-D can bind both encapsulated and acapsular strains of *C. neoformans*, the amount of SP-D bound per encapsulated cell is much lower than that binding to acapsular *C. neoformans* (Dromer et al., 1988). Furthermore, despite the observation that SP-D binds to encapsulated cryptococci, it does not cause aggregation of these strains. In a study analyzing binding of SP-D to the capsule components glucuronoxylomannan (GXM), galactoxylomannan (GalXM), mannoproteins-1 (MP1) and MP2, SP-D binds with highest affinity to GXM and MP1. This was surprising, since GXM is the major constituent of encapsulated cryptococci and is not present in acapsular strains. These results suggest that the assembly of GXM in the capsule may be important in lowering the affinity for SP-D and thereby preventing aggregation. GalXM and MPs are present on the outside of acapsular *C. neoformans*. Therefore, these carbohydrate structures were thought to represent likely ligands for SP-D. Clearly, MP1 is the ligand for SP-D in acapsular or poorly encapsulated strains. As the cryptococcal cell wall underneath the capsule also contains ß(1–6) glucans (James et al., 1990), which have been shown to be bound by SP-D with high affinity (Allen et al., 2001), these structures may represent other important ligands for SP-D on the cell wall of not yet fully encapsulated yeast cells. The presence of high GXM levels in the epithelial lining fluid may result in the protection of not yet fully encapsulated and thereby unprotected *C. neoformans* cells from SP-D-induced aggregation and subsequent removal by mucociliary clearance. This may lead to the exacerbation of the infection with a greater risk of dissemination throughout the body.

2.1.1.2. The Complement system The most important element of nonspecific humoral immunity involved in anti-cryptococcal defense is the complement system, which provides opsonins (mainly C3) for phagocytosis and chemotactic factors (mainly C5a) for the recruitment of inflammatory cells. Nonencapsulated cryptococci are known to activate complement through both the antibody-dependent classical pathway and the alternative pathway (Kozel et al., 1991;

Kozel et al., 1992). Specific antibodies to fungal antigens are a prerequisite for the activation of the classical pathway, and these are not always present in patients (Keller et al., 1994; Casadevall and Perfect, 1998). Encapsulated yeasts exclusively activate complement through the alternative pathway, presumably since their thick capsule is able to prevent the binding of antibodies to epitopes in the cell wall (Kozel et al., 1991; Kozel et al., 1992; Wilson, 1992). Although encapsulated cryptococci are able to activate the complement pathways, purified GXM appears to be a weak stimulator *in vitro* (Laxalt and Kozel, 1979). Experimentally induced complement deficiency in guinea pigs revealed the relevance of this phenomenon: C4 deficiency (i.e. impairment of the classical pathway) did not lead to an increased susceptibility for cryptococcal infection, whereas the mortality of cryptococcosis is increased in C3-deficient animals (i.e. with both impaired alternative and classical pathways) (Diamond et al., 1974).

2.1.2. Innate cellular defense

In the nonspecific cellular response, cryptococci are attacked and phagocytosed by alveolar macrophages (Monga, 1981), later assisted by neutrophils (Miller and Mitchell, 1991), monocytes (Levitz and Farrell, 1990), natural killer cells (Murphy, 1993), and lymphocytes (T-cells, B-cells) (Levitz et al., 1995; Murphy et al., 1993; Muth and Murphy, 1995a), which are capable of killing or inhibiting cryptococci. In most of their functions, cells of the innate immune system are helped by local and systemic defense systems (i.p. surfactant proteins and the complement system).

2.1.2.1. Macrophages Macrophages are the predominant cells in the process of phagocytosis and killing of cryptococci (Cross and Bancroft, 1995; Granger et al., 1986; Keller et al., 1994; Kozel et al., 1989; Levitz and Tabuni, 1991). This has been shown by the experimental ablation of macrophages, which augments the host's vulnerability to cryptococcosis (Monga, 1981) and, reversely, by the activation of macrophage function, which enhances the clearance of cryptococci (Goldman et al., 1994). Macrophages are also involved in granuloma formation, which usually characterizes an efficient host response to cryptococcosis. Macrophages are able to bind, phagocytose and, upon appropriate stimulation, kill cryptococci. Phagocytosis occurs with or without opsonization, but successful clearance of the yeasts does require opsonization of the fungi, since the polysaccharide capsule is able to mask antigens on the cryptococcal cell wall (McGaw and Kozel, 1979; Laxalt and Kozel, 1979; Kozel and Hermerath, 1984; Retini et al., 1998). Serum-independent phagocytosis occurs via mannose and β-glucan receptors on macrophages (Cross and Bancroft, 1995), whereas cryptococci opsonized by antibodies or complement bind to Fc receptors or complement receptors (CR1, CR3 and CR4), respectively. Additionally, macrophages produce proinflammatory cytokines and chemokines that are able to recruit inflammatory cells as well as to stimulate phagocyte function. Macrophages also promote T-lymphocyte proliferation by presenting antigens of phagocytosed cryptococci to T-cells through HLA class II DR molecules. In the

brain, microglia, which are macrophage-like cells, and astrocytes additionally participate in local cellular anti-cryptococcal defense (Aguirre and Miller, 2002; Blasi et al., 1992; Lee et al., 1994). Further, microglia, which are macrophage-like cells, and astrocytes participate in local anti-cryptococcal defense in the brain (Casadevall and Perfect, 1998; Blasi et al., 1992; Aguirre and Miller, 2002).

Dormant infections. For years, it was assumed that inhaled cryptococci would either be cleared immediately after entrance by the physical and innate defense mechanisms in the airways of an immunocompetent host, or lead to subsequent infection in an immunosuppressed host. Cryptococcal lesions in patients dying from cryptococcosis often consist of massive extracellular collections of organisms (Casadevall and Perfect, 1998). The combination of the presence of an anti-phagocytic capsule and the histological evidence for extracellular localization in tissue has led to the commonly held assumption that *C. neoformans* causes disease by extracellular growth in the tissues of susceptible hosts (Casadevall and Perfect, 1998). However, several observations from both *in vitro* and *in vivo* studies in experimental infection of rats and mice suggest that this view of *C. neoformans* pathogenesis is incomplete.

There are several strong lines of evidence that suggest *C. neoformans* meets the criteria for definition as a facultative intracellular pathogen. Since the 1950s, studies of pulmonary pathology have noted the presence of intracellular *C. neoformans* in lung tissue (Schwartz, 1988; Baker and Haugen, 1955). Studies with isolated phagocytic cells had demonstrated that intracellular survival and replication occur in some systems, suggesting that *C. neoformans* can be a facultative intracellular pathogen *in vitro* (Lee et al., 1995; Diamond and Bennett, 1973; Karaoui et al., 1977; Bulmer and Tacker, 1975). *In vivo*, similar results demonstrate the biological relevance of these findings. Analysis of primary *C. neoformans* pulmonary infection in mice shows that intracellular yeast are present within macrophages at all stages after infection (Feldmesser et al., 2000). Evidence that intracellular replication occurs is provided by: (1) the percentage of budding cells among intracellular yeast is approximately five times that among extracellular yeast; (2) with increasing time after infection, phagosomes contain increasing numbers of yeast per phagosome, which are increasingly heterogeneous in size; and (3) yeast at all stages of budding are found inside phagosomes. Interestingly, survival inside macrophages is associated with profuse accumulation of intracellular capsular polysaccharide and, possibly, the synthesis of melanin-like pigment in the cell wall. This capsular polysaccharide secretion inside macrophages appears to represent a novel mechanism for intracellular pathogenesis.

In addition to the studies that relate to events following initial pulmonary infection, studies in a rat pulmonary model provide further support for the classification of *C. neoformans* as a facultative intracellular pathogen. Normal rats can control pulmonary infection with large inocula but cannot eradicate the organism and remain persistently infected (Goldman et al., 2000). Long-term persistence of *C. neoformans* in a rat model of latent pulmonary infection is associated with residence inside macrophages. Granuloma formation appears to be the effective tissue response for control of *C. neoformans* infection in normal hosts, but

ingestion of *C. neoformans* cells by macrophages does not necessarily result in killing. Consequently, persistence and latency are associated with intracellular parasitism.

The view that *C. neoformans* is a facultative intracellular pathogen is in agreement with the understanding which has emerged that the development of cell-mediated immunity, a response more commonly associated with intracellular pathogens, is required for control of this infection, and could explain, in part, why the role of adaptive humoral immunity has been difficult to establish conclusively.

2.1.2.2. Granulocytes The role of neutrophils (polymorphonuclear cells; PMN) is somewhat controversial. Even though neutropenia is not a proven risk factor for cryptococcosis, PMN are often found in inflamed tissues early in infection in close association with cryptococci (Perfect et al., 1980). Furthermore, they participate in the early phase of granuloma formation (Kilgore et al., 1997). *In vitro*, PMN are more potent than macrophages or monocytes in killing cryptococci (Miller and Mitchell, 1991); however, susceptibility to killing differs considerably between cryptococcal strains.

Evidence from a few reports suggests that eosinophils are possible effector cells against *C. neoformans*, as they have been found surrounding cryptococci in pulmonary infections (Feldmesser et al., 1997; Goldman et al., 1994; Kagaya et al., 1985) and are capable of antibody-dependent phagocytosis of the fungal cells in vitro (Feldmesser et al., 1997). High virulent cryptococcal strains have been associated with the development of eosinophilic pneumonia via the induction of a non-protecting Th2-type cytokine response (Olszewski et al., 2001).

2.1.2.3. Natural Killer (NK) cells Another cell type involved in anticryptococcal defense is the natural killer (NK) cell, which can bind and kill cryptococci by the release of cytotoxic factors (Levitz et al., 1994a). Although several studies have indicated that a lack of NK cells can lead to a decreased clearance of cryptococci (Kawakami et al., 2001), NK cells appear to be less fungistatic *in vitro* than monocytes and cell lysis ensues relatively slowly (Levitz et al., 1994a).

2.1.2.4. Endothelial cells Although the role of endothelial cells in cellular defense against cryptococci is still largely unclear, the penetration of and subsequent migration through the endothelial barrier is necessary for cryptococcal dissemination. Ibrahim (Ibrahim et al., 1995) demonstrated that cryptococci bind to endothelium in the presence of serum and acapsular strains adhere better and cause more damage to the endothelium than encapsulated cells, suggesting that poorly encapsulated cryptococci are responsible for dissemination. There is, however, evidence that endothelial cells can enhance the phagocytosis and killing of cryptococci by neutrophils, theoretically based on the ability of the endothelium to produce stimulatory cytokines (Roseff and Levitz, 1993).

2.2. The Adaptive (Specific) Cellular Defense

The critical importance of cell-mediated immunity in anticryptococcal defenses has been convincingly demonstrated in clinical and experimental settings. Disseminated cryptococcosis is usually associated with CD4 T cell deficiency, such as in HIV-associated or HIV non-associated CD4 T cell lymphopenia (Duncan et al., 1993). The important role of T cells in a murine experimental model in which mice had congenital or acquired T cell deficiency has been previously reported (Graybill et al., 1979; Graybill and Mitchell, 1979). Requirement of CD4+ lymphocytes for both optimal clearance of *C. neoformans* and survival of mice infected with *C. neoformans* in a murine model of cryptococcosis has been evidenced (Mody et al., 1990).

Adaptive T helper–cell responses are classified as Th1 and Th2, and have different patterns of cytokine secretion as well as different patterns in development of specific response (Mosmann and Coffman, 1989; Mosmann and Sad, 1996). The differentiation of T helper cells along a Th1 or Th2 pathway is an essential determinant for the host susceptibility or resistance to fungal infections including cryptococcosis (Koguchi and Kawakami, 2002; Shoham and Levitz, 2005; Antachopoulos and Roilides, 2005).

As mentioned above, cryptococcosis usually occurs in T cell deficient patients, it is noteworthy that under these conditions, T cell response could be further impaired by *C. neoformans* and in particular by its capsular material. The mechanisms involved in the immunosuppressive activity have been partly elucidated. In particular, it has been observed that capsular material down-regulates a series of biological functions of antigen presenting cells (APC) such as monocytes, macrophages and dendritic cells (DC) and consequently T cell response (Vecchiarelli, 2000b). Furthermore it has been demonstrated that *C. neoformans* interacts directly with T cells (Murphy et al., 1993) and human activated lymphocytes have been shown to inhibit growth of *C. neoformans* in vitro (Levitz and Dupont, 1993) through direct activity against the fungus (Muth and Murphy, 1995a; Muth and Murphy, 1995b; Levitz et al., 1995). Nevertheless T cells play a direct role in anti-cryptococcal activity, a critical step in predicting the development of T cell protective or non protective response is represented by APC interaction with this fungus. Given that the differentiation of Th1 type response is considered protective against *C. neoformans* infection, the impact of *C. neoformans* with APC could be decisive for driving Th1 or Th2 type response.

On the other hand, the role of DC, that are the quintessence of APC, in determining whether protective or non-protective anti-cryptococcal cell-mediated responses develop, has been elucidated. In particular APC efficiency is dependent on several factors including the type of DC subsets (Bauman et al., 2000), as well as the cryptococcal antigen engaged (Vecchiarelli, 2000a; Vecchiarelli, 2000b). Certain types of DC subsets determine whether protective or non-protective anti-cryptococcal cell-mediated immune (CMI) responses develop. Myeloid DC are needed for induction of the protective response, conversely lymphoid DC, are negative regulators of CMI responses (Bauman et al., 2000). Moreover

C. neoformans antigens such as mannoproteins (MP) stimulate APC function (Pietrella et al., 2005; Pietrella et al., 2001a; Pitzurra et al., 2000), and consequently protective response (Mansour et al., 2004; Mansour et al., 2002; Levitz et al., 2001). The negative impact of the major component of capsular material, glucuronoxylomannan (GXM) on APC function has been well studied (Retini et al., 1998; Vecchiarelli et al., 1994). In particular the inhibition of DC maturation (Vecchiarelli et al., 2003), the induction of Il-10 by APC (Vecchiarelli et al., 1996; Retini et al., 2001), and inhibition of IL-12 (Vecchiarelli, 2000b; Retini et al., 1999) are among the best studied regulatory activities of GXM. These effects result in exacerbation of *C. neoformans* infection associated to down regulation of T cell- response, in helping differentiation of normal CD4(+) T cells into a Th2 phenotype, and in limitation of development of protective Th1 protective response (Retini et al., 1998; Retini et al., 2001; Almeida et al., 2001).

An additional negative regulation of GXM on T cells response has been described in terms of diminution of T cell number (Chiapello et al., 2001), the mechanism could be ascribed to the capacity of GXM-loaded APC to induce apoptosis of activated T cells (Monari et al., 2005).

Many deleterious effects exerted by capsular material on T cell response could be bypassed or reversed by using Mab to GXM (Syme et al., 1999; Vecchiarelli et al., 1998b; Yuan et al., 1997). However for passive antibody–mediated protection against *C. neoformans* a series of conditions are required, including the presence of $CD4^{+}$ T cells and IFN-γ (Murphy et al. 1998), Th1 and Th2 –associated cytokines (Beenhouwer et al., 2001) and B cells (Rivera et al., 2005).

Given that the portal entry of *C. neoformans* is usually the respiratory tract, particular attention has been devoted to the local immune defenses at lung level. Here pulmonary clearance of *C. neoformans* requires CD4 cells (Hill and Aguirre, 1994) and development of Th1 type immunity (Huffnagle et al., 1996; Hernandez et al., 2004).

Several factors could contribute to regulation of local response. In particular, the expression of C-C chemokine receptor 2(CCR2), primary receptor for monocyte chemoattractant protein-1, a chemotactic factor for monocytes and T lymphocytes, is required in inducing Th1 response, and the lack of CCR2 results in a switch to a Th 2 type response (Traynor et al., 2000). Another critical factor influencing Th1 response is TNF-α that in the afferent phase of immune response, is essential for induction of IL-12 and IFN-γ. In addition the neutralization of early TNF results in a Th2 shift (Herring et al., 2002). Also the lack of IFN-γ receptor influences the ability to resolve the infection at lung level. (Chen et al., 2005). Furthermore IFN-γ is considered an important factor that contains the chronic infection avoiding the enhancement of Th2 cytokine production and a switch from chronic to progressive pulmonary cryptococcal infection (Arora et al., 2005).

T cell response to *C. neoformans* is influenced by costimulatory molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and CD40 ligand (CD40L), expressed on T cells. In an “in vitro” system the augmented expression of costimulatory molecule CTLA-4 was observed on purified CD4 T cells treated with encapsulated

with respect to acapsular strain (Pietrella et al., 2001b). Indeed CTLA-4 has been extensively described as responsible for transmitting negative regulation on T cells (Chikuma and Bluestone, 2003), and this could be an additional mechanism used by *C. neoformans* to elude host defense Another co-stimulatory molecule involved in regulating T cell response is CD40L (O'Sullivan and Thomas, 2003). In an experimental system, by using mice genetically lacking CD40L, it has been observed that the expression of CD40L on T cell is required for obtaining an efficient T cell response against *C. neoformans* (Pietrella et al., 2004).

Besides CD4, CD8 T cells also play a role in anticryptococcal activity (Mody et al., 1994a; Mody et al., 1994b; Syme et al., 1997; Ma et al., 2002) and several studies have been performed to elucidate their role in immune response at lung level. An involvement of CD8 T cells in clearing *C. neoformans* from the lung, and in promoting recruitment of cells mediating inflammatory response, have been demonstrated (Mody et al., 1993; Huffnagle et al., 1994). Furthermore the role of CD8 has been recently underlined by demonstrating that CD8 T cell function is independent of CD4 T cells and that IFNγ production from CD8 T cells is involved in limiting survival inside macrophages during pulmonary *C. neoformans* infection (Lindell et al., 2005).

In summary, although a direct role of T lymphocytes against *C. neoformans* has been documented, the capacity of T helper cells to differentiate in Th1 phenotype remains a critical factor for the development of protective response. It is noteworthy that this is true for controlling systemic, as well as pulmonary, infection, even though CD8 seems to have a complementary role in resistance to pulmonary infection. The central role of APC functional status in inducing Ag-specific T cell protective or non protective responses highlights a new target for sustaining protection.

Efforts have been devoted to obtaining fungal antigens to elicit a protective T cell response, and in this line, MP could be considered a possible candidate, but additional studies are needed to better understand how MP conveys positive information to immune cells, in order to ameliorate its protective efficacy.

3. EFFECTS OF THE CRYPTOCOCCAL CAPSULAR COMPONENTS ON THE HOST-RESPONSE

Several cryptococcal virulence factors, including melanin, phospholipase B, urease and capsular polysaccharide have been identified that contribute to the infectivity of the fungus and favor its survival in the host (Buchanan and Murphy, 1998; Casadevall and Perfect, 1998). *C. neoformans* is unusual among the pathogenic fungi in that it has a polysaccharide capsule that is anti-phagocytic *in vitro*. Numerous studies have established that the capsule inhibits phagocytosis and that phagocytic cells cannot ingest *C. neoformans* cells *in vitro* in the absence of opsonins (Kozel et al., 1996; Kozel et al., 1988). The capsule is formed soon after entrance in the host and its main component is the large polysaccharide glucuronoxylomannan (GXM). Other minor polysaccharides are galactoxylomannan (GalXM) and the mannoproteins (MPs). Several lines of research have associated the presence of

limited inflammatory responses or more severe infection with heavy encapsulation or highly virulent strains Farmer and Komorowski, 1973; Rippon, 1988; Gutierrez et al., 1975; Fromtling et al., 1982; Kwon-Chung and Rhodes, 1986; Attal et al., 1983; Kagaya et al., 1985; Kawakami et al., 1999). The relevance of the capsule for virulence has been further clarified by the identification and targeted disruption of several genes that are involved in capsule synthesis (Chang and Kwon-Chung, 1994; Chang et al., 1996; Chang and Kwon-Chung, 1999; Chang and Kwon-Chung, 1998; Wilder et al., 2002). Moreover, cryptococci are able to change their capsule size and composition during infection (phenotypic switching), leading to changes in polysaccharide composition and resulting in enhanced virulence and lethal outcome in murine models of infection (Goldman et al., 1998; Fries et al., 1999; Fries et al., 2001).

Several mechanisms have been described by which the capsule and its polysaccharides affect the host response (reviewed in (Buchanan and Murphy, 1998; Casadevall and Perfect, 1998; Murphy, 1999; Vecchiarelli, 2000b; Ellerbroek et al., 2004c). First, the capsule and its main polysaccharide GXM are able to prevent the phagocytosis of cryptococcal cells by acting as a mechanical shield that masks cell wall antigens or bound opsonins (McGaw and Kozel, 1979; Kozel and Gotschlich, 1982) and interferes with the presentation of antigens (Kozel and Mastroianni, 1976; Kozel and Gotschlich, 1982; McGaw and Kozel, 1979; Collins and Bancroft, 1991; Retini et al., 1998). Second, the capsular polysaccharides are abundantly shed into the blood and other body fluids of the patient during infection (Diamond and Bennett, 1974; Eng et al., 1983; Eng et al., 1986; Metta et al., 2002) from where they modulate several aspects of the host-response (reviewed in (Ellerbroek et al., 2004c). High serum titers of polysaccharides have been associated with progressive disease in humans (Diamond and Bennett, 1974) - which is not merely due to a higher burden of organisms (Murphy, 1989).

The effects of cryptococcal polysaccharides on the host-response are diverse. Cryptococcal polysaccharides (mainly GXM) have been demonstrated to interfere with the migration of neutrophils toward chemotactic stimuli and inflammatory sites in vitro (Dong and Murphy, 1995a; Lipovsky et al., 1998a; Coenjaerts et al., 2001) as well as in vivo (Dong and Murphy, 1995b; Lipovsky et al., 1998b; Lipovsky et al., 2000; Mirshafiey et al., 2000a; Mirshafiey et al., 2000b; Mirshafiey et al., 2002; Tissi et al., 2004; Ellerbroek et al., 2004a). Two recognized mechanisms underlying this inhibitory effect of GXM on neutrophil migration are interference with chemokinesis and interference with neutrophil adhesion to and transmigration of the endothelium (Ellerbroek et al., 2002; Ellerbroek et al., 2004b). In addition, in vitro all capsular polysaccharides (GXM, GalXM and MPs) induce proinflammatory cytokine production (IL-12, TNFα, IFN-γ, IL-1β, and IL-6) by monocytes and PMN in the presence of active complement or mannose binding protein (hMBP) (Levitz et al., 1994b; Delfino et al., 1996; Delfino et al., 1997; Retini et al., 1996; Levitz and North, 1997; Chaka et al., 1997b; Vecchiarelli et al., 1998a; Walenkamp et al., 1999; Pitzurra et al., 2000; Ellerbroek et al., 2004c; Pietrella et al., 2005). Additionally, cryptococci and their polysaccharides induce chemokine

production (MCP-1, MIP-1α, MIP-1β, RANTES and Il-8) by peripheral blood cells and microglial cells (Lipovsky et al., 1998a; Vecchiarelli et al., 1998a; Huang, 2000; Goldman et al., 2001).

However, depressing effects of cytokines, chemokines and their receptors have also been reported, such as the shedding of TNF-receptors from the surface of neutrophils by GXM and mannoprotein-4 (Dong and Murphy, 1996; Coenjaerts et al., 2001) and GXM-induced Th2-related cytokine responses and IL-10 production (Levitz et al., 1996; Vecchiarelli et al., 1996; Levitz et al., 1997) possibly leading to down regulation of TNFα and IL-1β responses (de Waal et al., 1991; Vecchiarelli et al., 1995; Levitz et al., 1996). Interestingly, cryptococci inhibit TNFα-stimulated production of the chemokines MCP-1 and IL-8 by endothelial cells, which is possibly related to the production of fungal prostaglandins (Mozaffarian et al., 2000; Noverr et al., 2001). In addition, cryptococci down regulate the C5a receptor (C5aR) on the surface of neutrophils (Monari et al., 2002). However, due to the variability of the experimental conditions as well as the complexity of cytokine production, which depends partly on auto regulation, the results are difficult to interpret and extrapolate to the *in vivo* situation (Chaka et al., 1997a). Several *in vivo* studies have demonstrated cytokine and chemokine responses in lung fluid, blood, and cerebrospinal fluid during cryptococcosis in patients and murine models but the order of magnitude of the *in vivo* cytokine responses appears to depend highly on strain virulence (see (Huffnagle and Lipscomb, 1998) for further reading).

The polysaccharide capsule and its major component GXM have also been demonstrated to suppress T-cell mediated immunity. First, interference with antigen presentation by phagocytes leads to the inhibition of lymphocyte proliferation (Mody and Syme, 1993; Syme et al., 1999). Second, GXM induces T-suppressor cell responses, which are associated with repressed delayed-type hypersensitivity reactions to cryptococcal antigens (Murphy and Moorhead, 1982; Murphy et al., 1983; Murphy and Mosley, 1985; Khakpour and Murphy, 1987; Blackstock et al., 1987), decreased clearance of cryptococci during infection (Murphy, 1989), inhibition of phagocytosis and differentiation towards non-protective Th2-type cytokine responses by lymphocytes (Buchanan and Murphy, 1994; Almeida et al., 2001).

In contrast, however, mannoproteins enhance several aspects of cell-mediated immunity. Mannoproteins are strongly immunogenic and are probably the main inducers of the delayed-type hypersensitivity phenomenon observed in human and experimental cryptococcosis (Murphy et al., 1988). Further, mannoprotein-1 and -2 have been shown to promote proliferation of lymphocytes and mononuclear cells in response to *Cryptococcus neoformans* (Pitzurra et al., 1997) and are strong inducers of IL-12 production resulting in protective Th1 type cytokine responses by lymphocytes (Pietrella et al., 2001a; Pietrella et al., 2002). Both mannoprotein-88 and -98 activate T-cells (Huang et al., 2002; Levitz et al., 2001).

GXM affects antibody responses in different ways. On one hand, purified GXM has been shown to be poorly immunogenic in animals and to induce antigen-specific

immunogenic tolerance at high doses (Breen et al., 1982; Murphy and Cozad, 1972), which might be caused by the induction of T-suppressor cells (Breen et al., 1982). This antibody unresponsiveness to cryptococcal polysaccharide has been demonstrated in cured cryptococcosis patients as well as in GXM-primed mice (Breen et al., 1982; Henderson et al., 1982; Henderson et al., 1986; Kozel et al., 1977; Murphy and Cozad, 1972; Sundstrom and Cherniak, 1993). On the other hand, vaccination with GXM-protein conjugates has been shown to elicit an antibody response (Devi et al., 1991; Casadevall et al., 1992) and passive immunization with anti-GXM antibodies in experimental cryptococcosis has resulted in improved survival, enhanced clearance of cryptococci (Dromer et al., 1987; Mukherjee et al., 1993a; Mukherjee et al., 1993b; Mukherjee et al., 1994b; Mukherjee et al., 1994a; Shapiro et al., 2002 and enforcement of anti-fungal therapy (Gordon and Casadevall, 1995; Mukherjee et al., 1995a). These antibodies have been shown to increase phagocytosis and killing (Griffin, Jr., 1981; Mukherjee et al., 1995b), to activate complement (Ikeda et al., 1984), to assist in GXM clearance from tissues (Goldman et al., 1995; Lendvai et al., 1998), and to modulate cellular responses (Griffin, Jr., 1981; Vecchiarelli et al., 1998b). The protective nature of these anti-GXM antibodies has raised the interest in the production and the application of a vaccine in human cryptococcosis (Casadevall et al., 1998; Casadevall and Pirofski, 2001; Fleuridor et al., 2001). Even though galactoxylomannan elicits an antibody response (Reiss et al., 1984; van de Moer et al., 1990), it appears to play a minor role in virulence.

Finally, cryptococcal polysaccharides enhance *in vitro* HIV replication by inducing TNFα secretion as well as by TNFα independent mechanisms (Pettoello-Mantovani et al., 1992; Pettoello-Mantovani et al., 1994; Orendi et al., 1994; Orendi et al., 1997; Harrison et al., 1997).

4. CELLULAR RECEPTORS FOR CRYPTOCOCCI AND ITS CAPSULAR POLYSACCHARIDESS

Phagocytosis of cryptococci by macrophages occurs both in the absence or presence of opsonisation, although successful clearance of cryptococci by phagocytosis requires opsonisation of the fungi by antibodies or complement. Cryptococci opsonicated by antibody or complement bind to Fc receptors and complement receptors (CR1, CR3 and CR4; expressed upon stimulation), respectively (Griffin, Jr., 1981; Levitz and Tabuni, 1991; Levitz et al., 1997), whereas serum-independent phagocytosis occurs via mannose and β-glucan receptors on macrophages (Cross and Bancroft, 1995; Syme et al., 2002). Mannose receptors have specifically been shown to bind certain mannoproteins present in the capsule of cryptococci (Mansour et al., 2002).

The induction of cytokine production during cryptococcal infection is presumably initiated by binding of cryptococci to certain receptors on leukocytes, one example is the binding of cryptococci to mannose receptors (Cross and Bancroft, 1995). Another group of receptors involved in pattern recognition and cytokine production

is the family of toll-like receptors (TLR). TLRs are expressed on a variety of cells and are important mediators of proinflammatory cytokine release. *In vitro* studies indicate that cryptococcal GXM binds to TLR-2- and TLR-4 (Shoham et al., 2001). The relevance of TLRs was investigated using models of cryptococcal infection in knockout-mice These studies showed that TLR-2 and the TLR- associated adaptor molecule MyD88 play an important role in the host-response to cryptococci, whereas TLR-4 seemed to play a less important role (Yauch et al., 2004; Biondo et al., 2005). Additionally, the pattern-recognition receptor CD14, a co-receptor of TLR-4, appears to be involved in the phagocytosis and binding of cryptococci (Lipovsky et al., 1997; Monari et al., 2003) and mannoproteins (Chaka et al., 1997b; Chaka et al., 1997c) and might mediate some of the effects of cryptococcal polysaccharides on leukocyte migration and cytokine production (Ellerbroek et al., 2004b). Finally, another candidate receptor for GXM is CD18. GXM binds to CD18 on PMN (Dong and Murphy, 1997) and CD11b/CD18 and CD11c/CD18 were shown to be involved in the phagocytosis of cryptococci (Taborda and Casadevall, 2002). It was further demonstrated that GXM binds to and is subsequently taken up by neutrophils and monocytes, which could be partially prevented by blocking CD14 and CD18 (Monari et al., 2003).

Although potential receptors have been described for GXM and MP, the precise mechanism by which they exert their effects on the immune system has not been clarified. Yet, an unbiased, holistic study specifically designed to detect cellular receptors for GXM has never been performed.

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CHAPTER 18

HISTOPLASMA CAPSULATUM

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Abstract: The human pathogen *Histoplasma capsulatum* is a major endemic dimorphic fungi, existing in a mycelial form in the environment and as a yeast during infection. Exposure to *H. capsulatum* produces a range of host responses, from asymptomatic infection to rapidly fatal disease. This chapter will review the current knowledge of the microbial characteristics, pathogenesis, epidemiology, clinical manifestations, current diagnostic and therapeutic approaches, and host immunity to *H. capsulatum*. In particular, the host response is determined by the outcome of the complex interactions between innate immunity, adaptive immunity and *H. capsulatum* virulence factors

1. INTRODUCTION

Histoplasma capsulatum var. *capsulatum* is a dimorphic fungus primarily acquired via respiratory exposure that is responsible for approximately 500,000 infections in the USA each year, which makes it the most prevalent cause of fungal pulmonary disease (Bradsher 1996; Retallack and Woods 1999; Cano and Hajjeh 2001). The fungus is endemic worldwide (Kwon-Chung and Bennett 1992), though there are areas with a high incidence of disease [Figure 1]. For instance, histoplasmin [culture filtrate from mycelial *H. capsulatum*] skin testing has shown that up to 90% of adults in areas along the Ohio and Mississippi River Valleys in the USA have been exposed to the fungus (Furcolow 1963; Edwards, Acquaviva et al. 1969; Wheat 1992; Bradsher 1996). In nature and at ambient temperatures, *H. capsulatum* is generally mycelial. In mammalian hosts and at temperatures at or greater than 37 °C, the organism typically transforms into a yeast-like, unicellular form that reproduces by budding. Immunity to *H. capsulatum* requires effective cellular responses that rely heavily on cytokine-dependent processes.

2. ETIOLOGY

In 1906, Darling established the genus *Histoplasma* when describing three fatal cases of disseminated histoplasmosis while working in Panama (Darling 1906). He reported "a protozoön general infection producing pseudotubercules in the lungs

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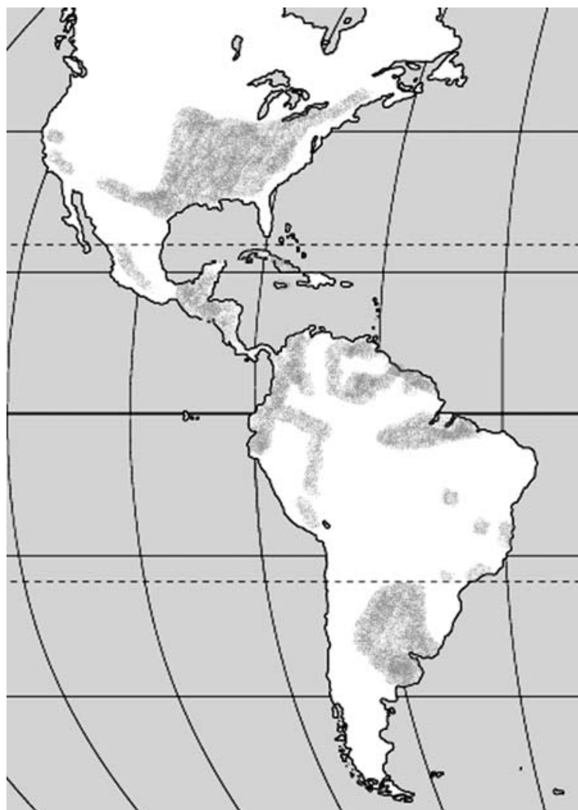

Figure 1. The prevalence of histoplasmosis in North and South America. The shaded areas indicate regions with high endemicity of *H. capsulatum*

and focal necroses in the liver, spleen and lymph nodes". Figure 2 depicts the typical appearance of *H. capsulatum* in tissue. Darling incorrectly thought that the small [1–4 μm], intracellular oval bodies were protozoa and compared them to *Leishmania sp*. In 1912, da Rocha-Lima, after a detailed histological comparison of *Histoplasma* and protozoa, correctly identified *H. capsulatum* as a fungus (da Rocha-Lima 1912; da Rocha-Lima 1912–1913). However, it was not until 1934 that a pre-mortem diagnosis of histoplasmosis was made with the organism being isolated from the blood of the patient and also subsequently from autopsy tissue by De Monbreun (De Mondbreun 1934). De Monbreun provided a detailed description of the dimorphic nature of *H. capsulatum* and reproduced the clinical and pathological characteristics of the disease in mice, puppies, and rhesus monkeys after intravenous infection. In 1972, Kwon-Chung established that *H. capsulatum* was heterothallic and described the perfect state identifying the mould as an ascomycete and naming it *Emmonsiella capsulata* (Kwon-Chung 1972).

Subsequent to the identification of *H. capsulatum*, Duncan (Duncan 1947; Duncan 1958) and Vanbreuseghem (Dubois, Janssens et al. 1952) recognized a new form of

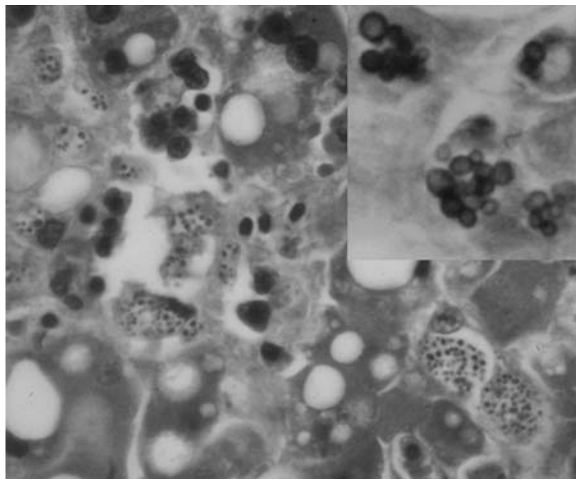

Figure 2. H. capsulatum in tissue. Micrographs of a liver depicting swollen and vacuolated hepatocytes and sinusoidal Kuppfer cells filled with *H. capsulatum* yeast cells. The liver section has been stained with Haematoxylin and eosin with the inset showing Gomori's methenamine silver staining. Original magnification, X400 (see color section)

histoplasmosis in Africa that was histopathologically different from classic disease in that the yeast-like cells were typically larger, 8–15 μm in diameter. Although initially named *H. duboisii*, isolates were able to mate with *H. capsulatum* strains resulting in the taxonomic treatment of *H. duboisii* as a variety of *H. capsulatum, H. capsualatum* var. *duboisii* (Kwon-Chung 1975). There is a third variety, *H. capsulatum* var. *farciminosum*, which initially was described as *Cryptococcus farciminosum* in 1873 by Rivota (Rivolta 1883). Unlike the other varieties that cause disease in diverse mammals, *H. capsulatum* var. *farciminosum* is known to cause disease only in equines.

3. MICROBIAL CHARACTERISTICS

H. capsulatum is most often isolated from soil high in nitrogen, often organic material enriched with guano of various avian species or bats. The mould was first isolated in nature in soil from a chicken house in northern Virginia (Emmons 1949). However, presumably due to their high body temperature, the fungus does not cause disease in fowl. In contrast, bats can develop disease, generally with intestinal lesions (Shacklette, Diercks et al. 1962; Diercks, Shacklette et al. 1965; Emmons, Klite et al. 1966; Shacklette 1969), and *H. capsulatum* has been isolated from more than 25 species of bats (Disalvo, Bigler et al. 1970).

In the laboratory, *H. capsulatum* produces moderately growing, white colonies on agar at 25 to 30 °C and the colonies often become a light brown over time. By electron microscopy, hyphal elements are 1.25–2.0 μm in diameter (Maresca 1989). The hyphal elements have a bilaminate wall approximately 20 nm thick and

the hyphal tips are either uninucleate or binucleate. *H. capsulatum* produces two types of conidia below 35 °C. There are large [8 to 15 μm], thick walled, oval, or occasionally oblong macroconidia with finger-like [tuberculate] projections. Microconidia [2–5 μm] are produced singly at the tips of short, narrow conidiophores (Garrison and Boyd 1977; Garrison and Boyd 1978). Both conidial forms are produced singly at the tips of narrow, short conidiophores that branch at right angles to vegetative hyphae.

The mechanism controlling the switch from the mycelial to the yeast form of *H. capsulatum* is complex, but is largely dependent on a shift in temperature and availability of nutrients (Howard 1967; Retallack and Woods 1999; Woods 2002). Although many isolates do not completely convert to yeast-like forms in the laboratory, the addition of blood to the agar or animal passage typically results in conversion to thin walled, oval 2–5 μm cells. On agar, colonies of the yeast-like form are smooth or wrinkled and range from a cream to beige color that becomes light grey to brownish over time. The cells reproduce by polar budding with a narrow isthmus between the mother and daughter cell. Importantly, despite the identifier "capsulatum" in the fungi's name, the cells lack a capsule or slime layer.

4. PATHOGENESIS AND CLINICAL MANIFESTATIONS

Infection by *H. capsulatum* presumably is initiated after inhalation and deposition of microconidia [2–5 μm] within alveoli. This event is followed by conversion of microconidia to the yeast form (Medoff, Kobayashi et al. 1987). The phase transition begins within several hours to a few days (Kimberlin, Hariri et al. 1981). During primary infection, the yeast cells are phagocytosed into the endosomal compartment of phagocytes [Figure 3] which then migrate to local lymph nodes and subsequently disseminate hematogenously (Fojtasek, Sherman et al. 1993). The incubation period

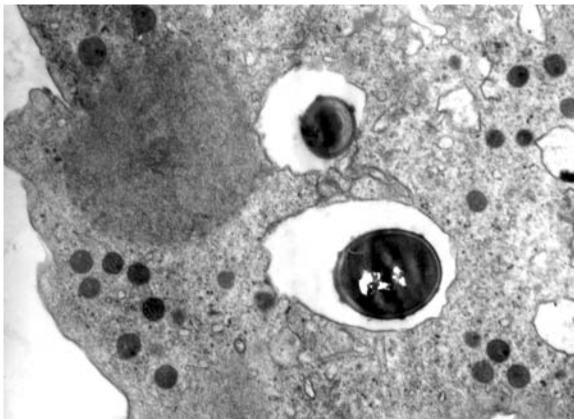

Figure 3. H. capsulatum yeast cells are phagocytosed by and survive within macrophage. Transmission electron micrograph depicting two intracellular yeast cells, magnification X12,000

for the disease is typically 8 to 17 days, though heavy exposure may result in disease in as little as 3 days (Goodwin, Loyd et al. 1981).

The severity of histoplasmosis is associated with the number of spores inhaled, the virulence of the infecting strain, and the immunological status of the exposed individual. The data suggests that low inoculum infection results in 1% of individuals developing self-limiting disease, while 99% have subclinical infection (Wheat 1997). High inoculum infection causes symptomatic disease in 50–100% of people (Wheat 1997). Hence, infection usually results in a mild, often asymptomatic respiratory illness, but may progress to life-threatening systemic disease, particularly in individuals with AIDS (Graybill 1988; Johnson, Khardori et al. 1988; Wheat, Connolly-Stringfield et al. 1990; Wheat 1996). In an individual with HIV infection, disseminated histoplasmosis has been considered an AIDS-defining illness (Centers for Disease Control and Prevention 1987). The prevalence of histoplasmosis in individuals with AIDS is particularly high, occurring in 2–5% of patients from endemic regions of the USA (Wheat, Connolly-Stringfield et al. 1990 Wheat 1996; Wheat 1997). The prevalence may be less at present given the apparent protective effect of improved antiretroviral therapy as seen with the decreasing incidence of cryptococcosis in the USA (Mirza, Phelan et al. 2003). Historically, the prevalence of histoplasmosis in individuals with AIDS has reached 25% in cities highly endemic for the fungus (Wheat, Connolly-Stringfield et al. 1990). In Latin America, the rate is lower [5–8%] (Arango, Cano et al. 1990; Arechavala, Robles et al. 1993; Murillo and Castro 1994). In contrast to immunocompetent individuals where dissemination is uncommon, disseminated disease occurs in 95% of individuals with AIDS (Wheat, Connolly-Stringfield et al. 1990; Wheat 1996). After infection, 85% percent of patients with AIDS develop disease requiring hospitalization, with an overall fatality rate of 12% (Hajjeh, Pappas et al. 2001). Even with administration of amphotericin B, the fatality rate in severe disease [eg. shock, respiratory failure] is 47–70% (Wheat 1996; Wheat, Chetchotisakd et al. 2000). Furthermore, significant morbidity and mortality in patients with AIDS persists despite the use of highly effective anti-HIV medications in this population (Hajjeh, Pappas et al. 2001).

In general, *H. capsulatum* is not contagious via the person-to-person route (Lenhart, Schafer et al. 2004). However, transmission can occur in the setting of organ transplant with reactivation of *H. capsulatum* in the implanted organ (Watanabe, Hotchi et al. 1988; Kwon-Chung and Bennett 1992; Wong and Allen 1992; Shallot, Pursell et al. 1997; Limaye, Connolly et al. 2000). Reactivation disease is also important in individuals previously exposed to *H. capsulatum* in whom the fungus previously established a latent infection. However, the risk of reactivation during immunosuppression is very low [$< 0.5\%$], even in high risk groups such as renal or bone marrow transplant patients (Davies, Khan et al. 1978; Vail, Young et al. 2002). It is also worth noting that the myclial growth of *H. capsulatum* is extremely hazardous to laboratory workers and that the ability to disperse large numbers of spores makes this organism a potential bioweapon. The ability of the spores to widely disseminate and cause disease has recently been highlighted by a report of epidemic disease due to poor filtration in buildings within a medical

school in Texas (Luby, Southern et al. 2005). Biosafety level 3 precautions are indicated when processing *H. capsulatum* mould cultures, soil or other material potentially contaminated with conidia. In the laboratory, biosafety 2 precautions are appropriate for the yeast-like form.

As described, the most common result of exposure to *H. capsulatum* is an asymptomatic infection. However, the fungus produces a broad spectrum of disease ranging from a mild influenza-like illness to a disseminated form that may involve virtually any tissue (Bradsher 1996; Retallack and Woods 1999; Cano and Hajjeh 2001). Symptomatic histoplasmosis usually consists of a flu-like illnesses characterized by a rapid onset of fever, chills, headache, myalgia, non-productive cough, and chest pain. Patients typically recover over several weeks and antifungal therapy is indicated for persistent symptoms or in the setting of significant immunosuppression (Wheat, Sarosi et al. 2000). In acute disease, 10% of patients have rheumatological symptoms, such as arthritis or severe arthralgia accompanied by erythema nodosum (Rosenthal, Brandt et al. 1983). Pericarditis can also occur in approximately 5–10% of patients after severe disease. Mediastinal fibrosis is a rare post-infectious complication that is perceived to be due to an abnormal inflammatory response to residual *H. capsulatum* antigens. In individuals with long-standing lung diseases, such as emphysema or chronic obstructive lung disease, a syndrome of chronic histoplasmosis can occur. Chronic histoplasmosis is characterized by indolent, progressive lung infiltrates, fibrosis and cavitation (Goodwin, Owens et al. 1976; Wheat, Wass et al. 1984). Most patients present with complaints of fever, weight loss, increasingly severe cough, and dyspnea.

Disseminated disease most commonly occurs in individuals with pre-existing immunosuppression, largely due to malignancy, steroid use, or AIDS (Goodwin, Shapiro et al. 1980; Wheat 1994; Wheat 1996). Prior to HIV, disseminated disease occurred in approximately 0.05% of acute infections (Sathapatayavongs, Batteiger et al. 1983). Clinical manifestations of disseminated histoplasmosis can vary from indolent to fulminant disease. Patients typically present with fever, weight loss, and respiratory symptoms. Patients often have hepatomegaly and/or splenomegaly. Cutaneous and mucous membrane lesions are not uncommon, and patients should be considered to have disseminated disease if *H. capsulatum* is isolated from these sites. Patients with acute disease often have anemia, thrombocytopenia, leucopenia and abnormal liver function tests. Central nervous system involvement occurs in up to 20% of cases (Wheat, Batteiger et al. 1990). Acute progressive disease is lethal without treatment.

5. DIAGNOSIS

The gold standard for diagnosis is the growth of the organism, however cultures typically take 2–4 weeks to grow (Kaufman 1992; Williams, Fojtasek et al. 1994). The likelihood of a positive culture depends on the disease, ranging from 10 to 85% (Williams, Fojtasek et al. 1994) with the best yields in individuals with disseminated or chronic pulmonary disease. Direct examination of tissues or body

fluid can facilitate diagnosis (Kaufman 1992), particularly of bone marrow in acute disseminated disease where the yeast form can be identified in up to 50% of cases. However, due to the similarity in size to other yeast and even intracellular parasites, such as *Toxoplasma gondii* and *Leishmania donovani*, visualization is not sufficient to confirm the diagnosis of histoplasmosis. Serological methods are commonly used for diagnosis. The three main tests in current use include immunodiffusion and complement fixation for antibody detection and a radioimmunoassay that detects a polysaccharide antigen of the fungus (Wheat and Kauffman 2003).

6. THERAPY

The appropriate treatment of a patient with histoplasmosis depends on the severity of illness and the immunological status of the individual. In an individual with HIV infection, histoplasmosis should always be treated with antifungal therapy and treatment should be continued life-long or until the individuals immune system recovers sufficiently in the setting of appropriate anti-retroviral therapy (Goldman, Zackin et al. 2004). Treatment is also indicated in patients with acute pulmonary disease with hypoxemia, symptoms of acute pulmonary disease lasting more than 1 month, chronic pulmonary disease, and disseminated disease (Wheat, Sarosi et al. 2000). Amphotericin B remains the drug of choice for severely ill patients, though itraconazole can be used in certain patients (Wheat, Sarosi et al. 2000).

7. IMMUNITY

The interplay between innate immunity, adaptive immunity and fungal virulence factors affects the outcome of disease in an individual host (Casadevall and Pirofski 2001; Casadevall and Pirofski 2002; Pirofski and Casadevall 2002). The current paradigm for control *H. capsulatum* infection is based on activation of cellular immunity in concert with innate responses. Progressive disease with dissemination occurs in the absence of intact cellular immunity (Allendorfer, Brunner et al. 1999). In addition to more severe disease in the setting of impaired cellular immunity, reactivation of previously controlled foci of infection can occur (Davies, Khan et al. 1978). In particular, individuals with AIDS are at high risk for reactivation disease (Minamoto 1988). Reactivation disease has also been documented in liver transplant recipients with disease originating from latent infections in the transplanted organs (Limaye, Connolly et al. 2000). Reactivation histoplasmosis has increasingly occurred in patients receiving anti-cytokine therapies (Lee, Slifman et al. 2002; Wood, Hage et al. 2003; Deepe 2005; Ehlers 2005).

7.1. Cytokines

Among the many innate elements engaged in augmentation of protective immunity to *H. capsulatum* are several cytokines, including IL-12, tumor necrosis factor [TNF]-α, granulocyte macrophage colony-stimulating factor [GM-CSF], and IFN-γ.

The ability of lymphocytes and phagocytes to produce these cytokines constitutes a major effector mechanism of host resistance (Romani, Mencacci et al. 1992). The absence of these cytokines in mice exposed to *H. capsulatum* often is associated with overwhelming infection and subsequent death. There are characteristic differences in primary and secondary responses to infection with *H. capsulatum*.

In primary sub-lethal infection, immunity to *H. capsulatum* is characterized by the development of a brisk Th1-like cytokine response (Cain and Deepe 1998). During the acute phase of the infection, the principal cytokines released are IL-12, TNF-α and IFN-γ. There are rapid increases in IL-12 and TNF-α levels in the first days after infection followed by increased levels of IFN-γ. The release of these cytokines coincides with an expansion in the number of myeloid cells in the lungs followed by T and B cells. IL-12 dependent production of IFN-γ is crucial for protection against *H. capsulatum* (Allendoerfer, Biovin et al. 1997; Zhou, Sieve et al. 1997). This finding is similar to requirements for protection from other intracellular pathogens such as *Listeria monocytogenes* (Tripp, Gately et al. 1994) and *Leishmania major* (Heinzel, Schoenhaut et al. 1993). Also, TNF-α is critical for control of histoplasmosis (Zhou, Miller et al. 1998; Allendorfer, Brunner et al. 1999).

In primary disease, the induction of the Th1-like response is primarily due to IL-12. This cytokine is required for the generation of protective immunity in naive mice, since neutralization of endogenous IL-12 results in death of infected mice (Zhou, Sieve et al. 1995; Allendoerfer, Biovin et al. 1997). Interestingly, neutralization of IL-12 reduces IFN-γ levels, but TNF-α responses are not significantly altered (Allendoerfer, Biovin et al. 1997). Neutralization of IL-12 after the third day of disease does not affect the outcome, which is consistent with the requirement for IL-12 induction of IFN-γ during initial interactions between the host and pathogen. The import of IL-12 has been confirmed using recombinant IL-12 in SCID and immunocompetent mice, where administration of IL-12 prolongs survival through the induction of IFN-γ (Zhou, Sieve et al. 1995; Zhou, Sieve et al. 1997).

IFN-γ is required for protection. Typically sub-lethal infections are fatal when this cytokine is neutralized or in IFN-γ knockout mice (Allendoerfer and Deepe 1997; Zhou, Miller et al. 1998). IFN-γ production is not required for the induction of IL-12 and TNF-α, since the levels of these cytokines are similar between challenged wild-type and IFN-γ deficient mice (Allendoerfer and Deepe 1997). However, the generation of nitric oxide is lower in the absence of IFN-γ (Zhou, Miller et al. 1998). IL-4 levels impact IFN-γ responses. Neutralization of IL-4 protects mice from lethal infections and survival correlates with increased levels of IFN-γ (Zhou, Sieve et al. 1995).

TNF-α production is induced rapidly after primary infection. Neutralization of TNF-α increases the fungal burden and mortality of mice infected with *H. capsulatum* (Allendoerfer and Deepe 1998; Zhou, Miller et al. 1998). Lack of TNF-α does not affect the levels of IFN-γ, IL-12, and GM-CSF in infected mice. Similar to neutralization of IFN-γ, the levels of nitric oxide are reduced in the absence of TNF-α.

GM-CSF is important in maintaining a Th1-like response. Neutralization of GM-CSF results in a reduction in the levels of IFN-γ, TNF-α, and nitric oxide while the levels of IL-4 and IL-10 concomitantly increase (Deepe, Gibbons et al. 1999). This results in increased fungal burden and greater mortality. Neutralization of IL-4 or IL-10 in the absence of GM-CSF significantly increases survival.

The route of infection appears to have a greater impact on secondary infection compared to primary infection. Several studies have elucidated differences in cytokine requirements after systemic versus pulmonary infection in secondary diseases. Importantly, TNF-α is vital in regulating secondary immunity both in the pulmonary and systemic models (Allendoerfer and Deepe 1998; Zhou, Miller et al. 1998), but the relative importance of TNF-α in systemic infection is apparent only in the absence of IFN-γ. In the pulmonary model, neutralization of TNF-α abrogates protection in wild-type mice. Neutralization of TNF-α results in significant increases in IL-4 and IL-10, and neutralization of these Th2 associated cytokines re-establishes a protective response. In pulmonary infection, neutralization of GM-CSF increases the fungal burden in the lung, but it does not affect mortality (Deepe, Gibbons et al. 1999). Interestingly, neutralization does not influence the production of other cytokines in secondary infection. Mice deficient in IFN-γ are more susceptible to pulmonary infection (Allendoerfer and Deepe 1997), but not to systemic infection (Zhou, Miller et al. 1998). As opposed to primary infection, neutralization of IL-12 does not affect the outcome of either pulmonary or systemic *H. capsulatum* infection (Zhou, Miller et al. 1998).

7.2. T Cells

T cells are vital to host resistance against *H. capsulatum* (Allendorfer, Brunner et al. 1999; Lin and Wu-Hsieh 2004). Protective immunity is characterized by the induction of the production of cytokines by T cells, which subsequently activates phagocytic cells. As described, increased levels of IFN-γ and TNF-α are vital to the control of primary and secondary infection and T cells are largely responsible for the induction of cytokines in tissues infected with *H. capsulatum* (Gomez, Cain et al. 1998; Allendorfer, Brunner et al. 1999).

In primary disease, cellular immunity typically develops after ten days, with cellular proliferation occurring primarily in the lungs and the mediastinal lymph nodes followed by stimulation of cells throughout the reticuloendothelial system (Fojtasek, Sherman et al. 1993). The increase in cells is typified by lymphocytes activating macrophage fungicidal activity (Allendoerfer, Biovin et al. 1997). Mice depleted of $\alpha\beta$T cells, SCID and nude mice are more vulnerable to disease with *H. capsulatum* (Williams, Graybill et al. 1978; Zhou, Sieve et al. 1997; Allendorfer, Brunner et al. 1999). Furthermore, naïve mice lacking CD4+ T cells that are infected with *H. capsulatum* have accelerated mortality (Gomez, Bullock et al. 1988; Allendorfer, Brunner et al. 1999). Protection can be achieved by adoptive transfer of CD4+ T-cells reactive to *H. capsulatum* (Allendoerfer, Magee et al. 1993). CD8+ T cell depletion does not affect survival, but is associated with increased

fungal burden (Deepe 1994; Allendorfer, Brunner et al. 1999). Hence, protective responses in primary histoplasmosis requires $CD4^+$ T cells, whereas $CD8^+$ T cells are necessary for optimal clearance.

The TCR repertoire has been analyzed in primary pulmonary infection and $V\beta4^+$ cells are over-expressed (Gomez, Cain et al. 1998). The increase in the number of $V\beta4^+$ cells corresponds to the initiation of the cell-mediated immune response and declines as the intensity of the infection wanes. Elimination of $V\beta4^+$ cells impairs fungal clearance independent of IFN-γ. Furthermore, the polarization of the TCR response strongly suggests a restricted antigen repertoire in the lungs.

In re-exposure histoplasmosis, the presence of either $CD4^+$ or $CD8^+$ T cells is sufficient for mice to survive secondary infection (Allendorfer, Brunner et al. 1999). However, depletion of $CD4^+$ T cells delays the clearance of *H. capsulatum*. Pulmonary infections are lethal in mice depleted of both $CD4^+$ and $CD8^+$ T cells. Interestingly, the disease is not progressive in the lungs; rather mice succumb to extra-pulmonary infection. In secondary histoplasmosis, $V\beta4^+$ and $V\beta6^+$ T cells work in concert to resolve infection (Gomez, Woodward et al. 2001).

7.3. Phagocytes

The primary effector cells in host resistance to *H. capsulatum* are macrophage. This protective role is complex since they may initially provide a protective environment for the fungus. In the lung, *H. capsulatum* binds to CR3 receptors on macrophages, is phagocytosed, and replicates intracellularly (Bullock and Wright 1987; Newman, Bucher et al. 1990). The fungus survives in the phagolysosomes of macrophages by regulating vacuolar pH (Eissenberg, Goldman et al. 1993; Strasser, Newman et al. 1999) and, in some instances, inhibiting phagolysosomal fusion (Eissenberg, Schlesinger et al. 1988; Strasser, Newman et al. 1999). The maintenance of a pH of 6.5 facilitates the recovery of iron from transferin and the prevention of phagolysomal fusion reduces the exposure of *H. capsulatum* to hydrolytic enzymes (Strasser, Newman et al. 1999). The fungus has also adapted to escape the effects of toxic oxygen radicals, which are a major factor in host defense against diverse pathogenic microbes. Human macrophages generate an oxidative burst response after ingestion of *H. capsulatum*, yet the fungus is not significantly affected by these molecules (Bullock and Wright 1987). In murine macrophages, an oxidative burst does not occur and *H. capsulatum* actively inhibits the generation of toxic oxygen species (Eissenberg and Goldman 1987; Wolf, Kerchberger et al. 1987; Wolf, Abegg et al. 1989).

T cell activation in histoplasmosis is associated with enhanced macrophage fungicidal activity, but macrophage response depends on host species and the organ location. In human monocytes or monocyte-derived macrophages, intracellular growth is reduced by the addition of colony stimulating factors or interleukin-3 for 7 days prior to challenge (Newman and Gootee 1992). However, exposure of these cells to IFN-γ or TNF-α with or without a priming agent, such as lipopolysaccharide [LPS], did not affect intracellular replication (Newman and Gootee 1992).

In contrast, three day macrophage cultures exposed to IFN-γ were able to significantly inhibit intracellular growth via a superoxide anion-dependent mechanism (Brummer, Kurita et al. 1991). Murine peritoneal macrophages develop activity against *H. capsulatum* upon IFN-γ stimulation (Wu-Hsieh and Howard 1987). In contrast, splenic marcrophage require the addition of LPS with IFN-γ to similarly impact the growth of *H. capsulatum* (Lane, Wu-Hsieh et al. 1993) and IFN-γ does not affect the activity of alveolar macrophages (Allendoerfer and Deepe 1998). The hypothesis for the beneficial activity of IFN-γ on macrophage results from the production of nitric oxide by the macrophage (Lane, Wu-Hsieh et al. 1994). The nitric oxide serves to chelate iron, an essential nutrient for *H. capsulatum*, and the inhibitory effect of IFN-γ and nitric oxide can be prevented by the addition of excess iron (Lane, Wu-Hsieh et al. 1991).

Dendritic cells can kill ingested *H. capsulatum* yeast cells (Gildea, Morris et al. 2001). Phagocytosis of *H. capsulatum* by dendritic cells is mediated by binding of cytophilin [Dr. Simon Newman, personal communication] to the fibronectin receptor very late antigen-5 [VLA-5] (Gildea, Morris et al. 2001). Furthermore, it has been shown that dendritic cells can serve as a key antigen-presenting cells for the initiation of cell-mediated immunity (Lelouard, Gatti et al. 2002). Dendritic cells presenting *H. capsulatum* antigens can stimulate specific $CD8^{+}$ T cells to effectively control fungal infection (Lin, Yang et al. 2005).

Neutrophils inhibit *H. capsulatum* growth *in vitro* (Newman, Gootee et al. 1993; Zhou, Miller et al. 1998). Both human and murine neutrophils effectively inhibit the fungus (Kurita, Brummer et al. 1991; Newman, Gootee et al. 1993; Zhou, Miller et al. 1998). The azurophil granules are responsible for this fungistatic effect (Newman, Gootee et al. 1993), which is primarily due to neutrophil defensins [HNP-1, HNP-2, and HNP-3], cathepsin G and bactericidal-permeability-increasing protein. Interestingly, *H. capsulatum* elicits an oxidative burst and phagolysosomal fusion occurs in neutrophils, but the organism is capable of short term intracellular survival (Kurita, Terao et al. 1991). Depletion of neutrophils is associated with increased mortality in primary, but not secondary histoplasmosis (Zhou, Miller et al. 1998).

7.4. Humoral Immunity

The classical activities of antibody pertain to opsonization, toxin and viral neutralization, complement fixation, and antibody-dependent cellular cytotoxicity [ADCC]. However, antibodies can interact and modify the actions of the other arms of the immune system. In fact, recent advances have revealed that a major function of antibody is the regulation of cell-mediated responses, which affects the host inflammatory response to infection (Casadevall and Pirofski 2004; Casadevall and Pirofski 2005).

In human infection, IgM antibodies to *H. capsulatum* are evident by 2 weeks, followed by rising titers of IgA and IgG (Chandler, Smith et al. 1969). The IgG fraction contains complement-fixing and precipitating antibodies (Chandler, Smith

et al. 1969). In mice, antibody levels to *H. capsulatum* peak by day 21 after infection (Fojtasek, Sherman et al. 1993). The number of B cells in the lungs of infected mice increases in the first week of infection, albeit to a lesser degree than other myeloid cells lines (Fojtasek, Sherman et al. 1993; Cain and Deepe 1998). Interestingly, the number of B cells continues to rise as other myeloid lines decreased (Cain and Deepe 1998). The number of B cells in the spleen does not significantly change until the end of the second week of infection when all cell subsets have nearly doubled (Fojtasek, Sherman et al. 1993).

However, the role of humoral immunity in protection against *H. capsulatum* is uncertain as a beneficial function of B cells has not been established. Immune sera can increase phagocytosis of *H. capsulatum* by macrophages *in vitro* (De Sanchez and Carbonell 1975), but adoptive transfer experiments of serum from mice immunized with *H. capsulatum* ribosomes or live yeast cells is not protective (Tewari, Sharma et al. 1977). However, the mechanism of antibody action is extremely complex and studies using sera to investigate the usefulness of antibody may be inconclusive since there the concentration of protective antibodies may not be adequate, non-protective or blocking antibodies may inhibit protective effects, or antigens that illicit protective antibodies may not be immunogenic (Casadevall and Scharff 1995). Hence, a negative result does not preclude a potentially protective humoral response. For example, blocking antibodies in polyclonal responses to *Candida albicans* abolished the protective power of immune sera (Bromuro, Torosantucci et al. 2002). Survival of B cell deficient mice is not significantly different from wild-type animals (Allendorfer, Brunner et al. 1999), but mice lacking B cells would not be expected to respond differently from wild-type mice if the mice with B cells made an ineffective antibody response to infection.

Recently, antibody has been shown to affect histoplasmosis. A monoclonal antibody to *H. capsulatum* histone H2B expressed on the fungal cell surface increased the survival of lethally infected mice (Nosanchuk, Steenbergen et al. 2003). The antibody increased phagocytosis of *H. capsulatum* yeast cells by macrophage and inhibited cellular replication. The protective effect of the antibody was appreciably improved by the administration of sub-inhibitory concentrations of amphotericin B, which could have resulted from the activation of Toll-like receptors by amphotericin B (Sau, Mambula et al. 2003).

8. VACCINE DEVELOPMENT

For *H. capsulatum*, the prevailing dogma is that $CD4^{+}$ and $CD8^{+}$ T cells are necessary for optimal vaccine efficacy. To date, immunization with either *H. capsulatum* antigens or attenuated organisms requires the presence of one or both subsets of T cells to express functional activity. Immunization of mice with recombinant heat shock protein 60 from *H. capsulatum* confers a vigorous protective immune response (Gomez, Allendoerfer et al. 1995). However, the efficacy of vaccination of mice with heat shock protein 60 from *H. capsulatm* is abolished in the absence

of CD4$^+$ cells (Deepe and Gibbons 2002). On the other hand, vaccination of CD4$^+$-deficient mice via the subcutaneous route with *H. capsulatum* yeast confer protection in mice subsequently challenged intranasally (Wuthrich, Filutowicz et al. 2003). Thus, the results indicate that CD8$^+$ T cells can confer protection in the absence of CD4$^+$ cells, and stimulation of CD8$^+$ T cells can be efficiently achieved by dendritic cells (Lin, Yang et al. 2005). The findings are significant since they indicate that it may be possible to immunize effectively those with profoundly altered immunity.

Immunization with heat shock protein 60 in immunologically intact mice results in the expansion of Vβ8.1/8.2$^+$ T cells, which are essential for vaccine efficacy (Scheckelhoff and Deepe 2002). In addition to the requirement for specific CD4$^+$ T cells, effective cytokine responses are necessary for protection following immunization with heat shock protein 60 (Deepe and Gibbons 2002). Specifically, IL-10, IL-12, and IFN-γ are essential, since the activity of heat shock protein 60 is abolished if endogenous IL-10, IL-12, or IFN-γ is neutralized. Interestingly, immunization with a second heat shock protein of 70 kDa does not result protective cellular responses (Allendoerfer, Maresca et al. 1996) and it fails to significantly induce the production of IL-10 or IL-12 (Deepe and Gibbons 2002). The protective activity of heat shock protein 60 has been mapped to amino acids from 171–443. Only a small portion of Vβ 8.1/8.2$^+$ T cells recognize this fragment. Hence, a small population of cells confers protection.

Although heat shock protein 60 has been the major focus of vaccine development for *H. capsulatum*, additional targets have been studied. In 1971, immunization with a ribosomal-protein complex and an ethylenediamine extract of the fungus has been shown to be protective (Garcia and Howard 1971). Recently, newer techniques have been applied to generate extracts containing *H. capsulatum* cell-free antigens that are immunogenic and protect against lethal infection (Sa-Nunes, Medeiros et al. 2005). Protection can also be achieved with immunization with recombinant H antigen, a secreted beta-glucosidase (Deepe and Gibbons 2001). Finally, immunity can be achieved by passive antibody treatment with a monoclonal antibody to *H. capsulatum*, which results in prolongation of survival particularly in conjunction with the administration of antifungal drug (Nosanchuk, Steenbergen et al. 2003).

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SECTION 5

FUNGAL IMMUNE EVASION MECHANISMS

CHAPTER 19

ESCAPE MECHANISMS FROM THE IMMUNE RESPONSE

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Abstract: Fungal pathogenesis is by definition a situation in which a fungal species is successfully surviving in host tissues and causing local destruction or toxicity. The immune system is exquisitely equipped to ward off infection, and the strategies employed by fungi to survive immune defences during pathogenesis are diverse. This review examines the diversity of mechanisms used by fungi to escape immune responses, and specifically explores the observation that these mechanisms range from clear virulence traits to more opportunistic consequences of fungal environmental growth and survival strategies

1. INTRODUCTION

Our bodies are persistently exposed to countless microorganisms that have the potential to cause serious disease, but the organisms are recognized as foreign and promptly eliminated. In order to establish infection, pathogenic organisms must evade or subvert first innate and then acquired immune responses. In this chapter, I will discuss various mechanisms by which fungal pathogens avoid effective mammalian immune defenses. Previous chapters in this book have specifically explored the pathogenicity of specific fungi and touched on the immunology of defense against them. In this chapter I will discuss the various strategies that microbes can use to evade immune responses and pull examples of these evasion mechanisms from a variety of fungal pathogens. With this approach, I hope the reader will come away with an appreciation of the diversity of immune evasion strategies used, and how individual organisms must use multiple strategies to be successful.

2. MECHANISMS OF EVASION

Innate immune defenses are our first protection against infection. Innate immunity, by definition, is inherant in our physical make up and development. Innate immune mechansims include simple physical barriers to infection such as the skin,

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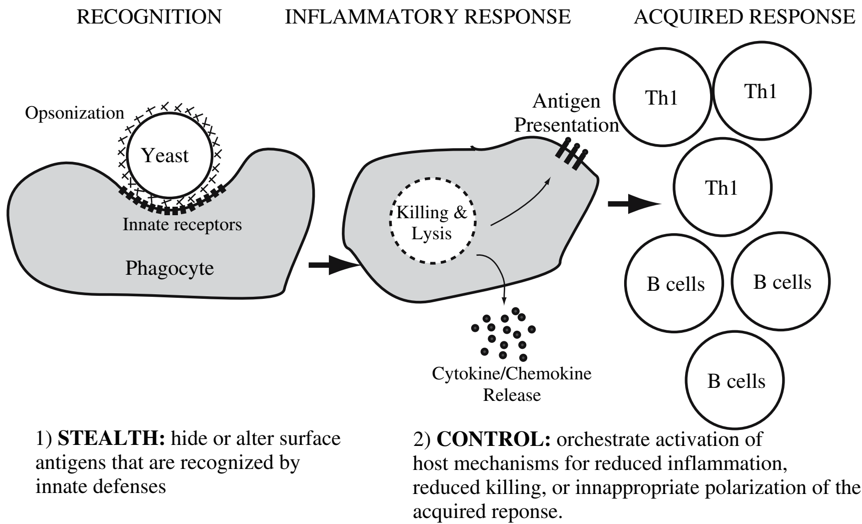
sequestration of vital nutrients such as iron, and production of anti-microbial peptides and complement. A host of specific receptors including Toll-like receptors, lectins, and scavenger receptors have arisen that mediate direct recognition of microbes (Janeway and Medzhitov 2002; Taylor et al. 2005). A variety of myeloid phagocytes such as macrophages and neutrophils are specialized for innate recognition of microbes leading to internalization (phagocytosis) and killing (Underhill and Ozinsky 2002). The unifying feature of these mechanisms is that they are immediate and require no training from previous exposure. Innate recognition of microbes leads to local and systemic inflammatory responses that activate further innate defenses and direct the development of effective acquired immune responses. Acquired immunity includes activation of antigen-specific T cells and production of specific antibodies by B cells. Acquired immune responses include long-lived memory components that permit enhanced responses upon later re-exposure to potential pathogens. The power of acquired immunity is that it is enormously variable, arising from combinatorial mutation of DNA encoding T cell receptors and B cell receptors/antibodies. The vast repertoire of antigen-specific acquired responses evolves very quickly (days to weeks) and is thus very difficult to subvert directly. As a consequence, most microbial evasion mechanisms focus on thwarting the innate immune system which is "hard-wired" in the host genome and evolves enormously slowly.

In order to survive in the face of these formidable immune defenses, successful pathogens utilize a variety of approaches. *First*, organisms may shield or camouflage themselves so that they are largely overlooked by immune surveillance systems (Figure 1, "Stealth"). This is a simple tactic that only requires that the organism find or produce a surface coating that is not recognized by the immune system or that is recognized but interpreted as "self". *Second*, organisms may display on their surface or secrete molecules that specifically activate host mechanisms for regulating immune responses (Figure 1, "Control"). In this case, the pathogen may direct either inhibition of immune responses or elaboration of types of immune responses that are ultimately not effective against the organisms. *Third*, organisms may express on their surface or secrete molecules that directly harm or counter specific host immune defenses (Figure 1, "Attack"). Secretion of toxins or proteases falls into this category.

Before moving on, it is useful to note that immune evasion mechanisms may be either "intentional" or "opportunistic". An "intentional" mechanism is one whose sole purpose is to thwart the infected host. For example, a microbe producing a secreted toxin that targets immune cells makes it only for that purpose. Deletion of the toxin does not render the microbe less viable, but does render it less pathogenic. An "opportunistic" mechanism is one that presumably developed to provide some growth advantage in the environment but that also happens to facilitate evasion of host defenses during infection. For example, proteases that are secreted to degrade wood and release nutrients to support fungal growth may also break down host tissues and permit pathogenic invasion. In this case, deletion of the proteases would reduce pathogenicity as well as environmental fitness. This creates a gray area when discussing virulence factors and mechanisms. A traditional virulence factor



3) **ATTACK:** kill or actively counter host defense mechansims

Figure 1. Mechanisms of Immune Evasion. Successful pathogens must thwart host immune defenses. The types of immune evasion mechanisms used have been divided into three categories for discussion in this chapter. 1) Stealth. Successful pathogens may effectively hide themselves from detection by specific immune cells or specific immune recognition molecules. 2) Control. Successful pathogens may specifically activate host immune inhibitory mechanisms or actively steer immune responses towards types that are not especially effective against the microbe. 3) Attack. Successful pathogens may produce molecules that specifically destroy or disable host immune defenses

is "intentional" in that it is produced only for infection/pathogenicity. Being indiscriminate with the discussion of "opportunistic" virulence mechanisms could lead one to characterize nearly any enzyme required for cellular growth and replication as a virulence factor. Many such genes turn up in screens for microbial mutants that are less pathogenic than wild type organisms and are reported to be virulence factors. In pulling examples for this chapter, I will restrict my definition of "opportunistic" virulence mechanisms to ones that seem to counter direct pressures put on the organism by the innate or acquired immune system of the host.

3. STEALTH

One of the simplest ways to avoid being killed by host immune defenses is to hide or disguise oneself so as not to be recognized. The best example of this in fungal pathogenesis is the thick polyssharide capsule of *Cryptococcus neoformans. C. neoformans* is an important human pathogen that can cause fatal meningoencephalitis in immunosuppressed individuals (Buchanan and Murphy 1998). *C. neoformans* is usually inhaled as a small, dehydrated basidiospore that

converts to an encapsulated budding yeast form that spreads into the nervous system. The thick polysaccharide capsule, which is required for pathogenicity, is made primarily of glucuronoxylomannan and has been demonstrated to inhibit phagocytosis of the yeast (Buchanan and Murphy 1998). Acapsular mutant strains of *C. neoformans* are ingested readily by macrophages, and both mannose and β-glucan receptors have been implicated in this recognition (Cross and Bancroft 1995). Although the capsule protects the organism from recognition by phagocytic receptors (and it thus "stealthy"), it is not entirely transparent to the innate immune system. The capsule is recognized by Toll-like receptors (TLRs) and triggers an inflammatory response. This inflammatory response is important for restricting the growth of the pathogen during infection since mice deficient in TLR2 are significantly more susceptible to *C. neoformans* infection (Yauch et al. 2004).

Another example of stealth comes from the ability of *Candida albicans* to switch between yeast and filamentous growth. *C. albicans* grows primarily as a budding yeast, but specific environmental cues such as serum and increased temperature can trigger growth in long filaments that can be true hyphae (sharing cytoplasm between cells) or pseudohyphae (having cell wall barriers between attached cells) (Figure 2). The ability to grow as a filament is critical for pathogenicity (Gale et al. 1998; Lo et al. 1997). The β-glucan receptor, Dectin-1, which has been specifically discussed earlier in this book, recognizes small patches of β-glucan called bud scars and birth scars that are exposed on the surface of the yeast as a consequence of budding growth (Gantner et al. 2005). During filamentous growth, these patches of β-glucan are not exposed, and Dectin-1 cannot recognize the organism (Figure 2). Dectin-1 is important for phagocytosis of yeast by myeloid cells and for signaling activation of the NADPH phagocyte oxidase which produces antimicrobial reactive oxygen species (ROS). ROS production is important for killing *C. albicans*, and humans and mice deficient in the NADPH phagocyte oxidase are more susceptible to *C. albicans* infection (Aratani et al. 2002a; Aratani et al. 2002b; Foster et al. 1998; Lehrer and Cline 1969). *C. albicans* is an opportunistic pathogen, and it is likely that there are growth advantages for the yeast and filamentous forms in the environment, and that the ability to switch between the two forms arose independent of any advantages conferred by growth in infected mammals. *Saccharomyces cerevisiae*, that is rarely a pathogen, also grows in yeast and filamentous forms, and Dectin-1 recognizes only the yeast form [(Gantner et al. 2005), and personal observation]. Thus although filamentous growth permits evasion of Dectin-1 mediated host defenses, this is likely an "opportunistic" evasion mechanism of *C. albicans* and not one that evolved exclusively as a pathogenic trait.

Another feature of *C. albicans* growth that has advantages in the environment, but also during infection, is the ability to form biofilms. Biofilms are microbial communities that are attached to surfaces and held together by a matrix of extracellular polymers. By growing en masse, organisms in a biofilm acquire increased resistance to environmental stresses such as dehydration and nutrient deprivation (Douglas 2003), but they are also typically more resistant to antimicrobial peptides

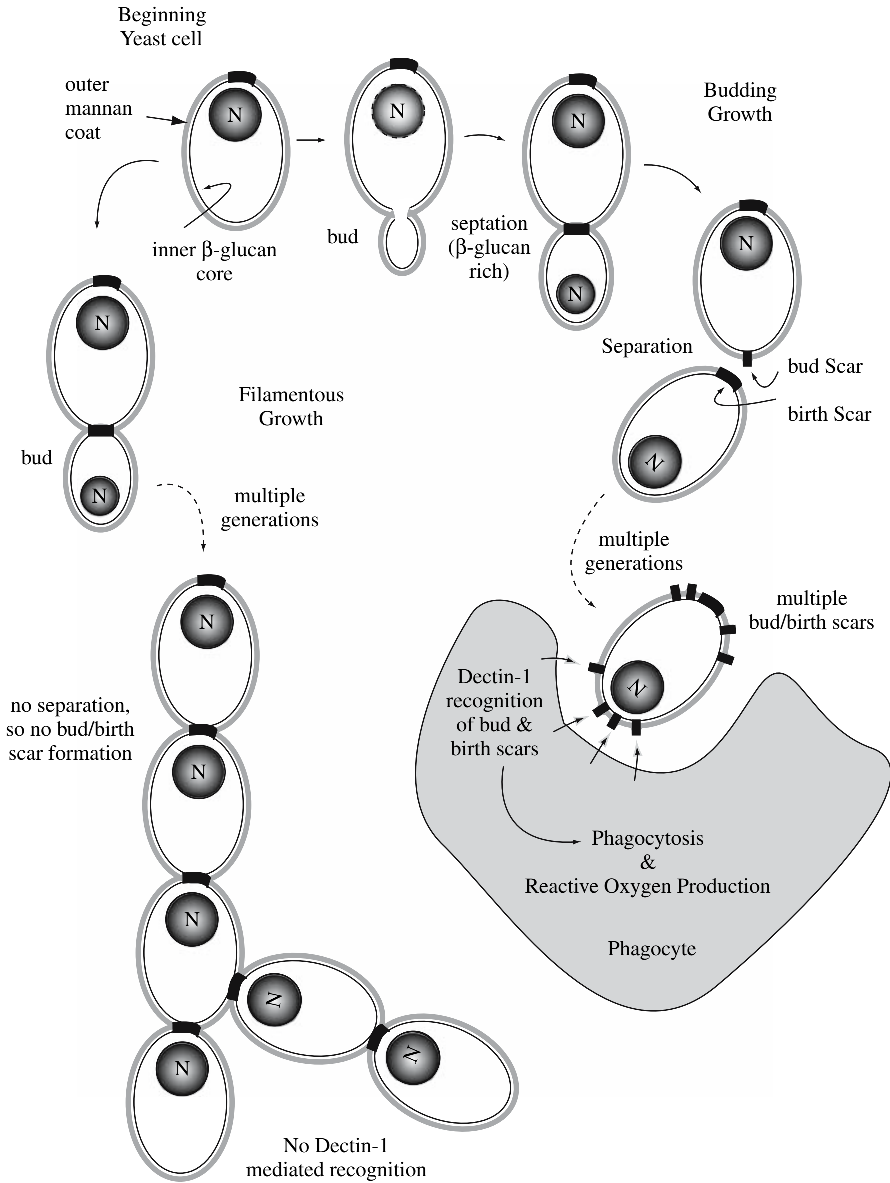


Figure 2. C. albicans filamentous growth protects from Dectin-1 recognition. *C. albicans* is a dimorphic fungus and can grow in both yeast and filamentous forms. *C. albicans* cell walls consist of an inner β-glucan structural core surrounded by a mannan-rich coat. During budding yeast growth, permanent deformities in the cell wall are formed during separation of mother and daughter cells. These bud and birth scars expose β-glucan from the cell wall to recognition by Dectin-1, a phagocyte receptor that can trigger antimicrobial responses. During filamentous growth, mother and daughter cells do not separate, and cell wall β-glucan is not exposed. As a consequence, Dectin-1 cannot recognize filaments

and antibiotics. *C. albicans* biofilms are typically 5–8 times more resistant to commonly used anti-fungal drugs compared to dispersed cultures (Hawser and Douglas 1995). *C. albicans* yeast cells embedded deep within a biofilm under a canopy of filaments and matrix are also effectively hidden from attack by phagocytes. *C. albicans* biofilms frequently grow on medically implanted devises such as catheters, endotracheal tubes, and joint replacements, and the organism is a major cause of nosocomial infections (Wenzel 1995).

Blastomyces dermatitidis is a dimorphic pathogenic fungus that is endemic in the central United States. In the environment it grows as a mold that produces airborne spores that are inhaled. The increased temperature, together with other environmental cues in the lung, induces growth as a budding yeast (Brandhorst et al. 2002). Blastomycosis, a systemic *Blastomyces* infection, can be life-threatening even in immunocompetent individuals. The cell wall of the mold form contains both β- and, unusually, α-glucan. Conversion to the yeast form is accompanied by greater production of the α-(1,3)-glucan; mature yeast glucan can be as high as 95% α-(1,3)-glucan (Kanetsuna and Carbonell 1971). Similar production of α-(1,3)-glucan has been reported in other pathogenic dimorphic fungi including *Paracoccidiodes brasiliensis* (Borges-Walmsley et al. 2002) and *Histoplasma capsulatum* (Eissenberg et al. 1997; Eissenberg et al. 1996). In all three fungi, loss of cell wall β-glucan in different strains has been correlated with reduced virulence leading to the suggestion that production of α-glucans may be a stealthy immune evasion mechanism (Klein 2000).

4. CONTROL

Stealth is not always possible, and generally an infectious organism is going to be recognized by the host in some ways. We've already seen this in the *Cryptococcus* capsule that blocks phagocytosis but still triggers inflammatory responses. Successful pathogens often find ways to take advantage of host recognition systems and control them for their own means. One example of this is the ability of *C. albicans* to bind to inhibitors of the complement pathway. The serum complement pathway for microbe opsonization and lysis is a central component of innate immune defense. Complement deposition on microbial surfaces can be activated by three pathways: the classical pathway, the lectin pathway, and the alternative pathway (Figure 3). The classical and lectin pathways activate complement deposition through formation of a C3/C5 convertase made up of protein fragments of the C4 and C2 complement proteins (C4b,C2b). Complement component C4b binding protein (C4BP) is a natural serum component inhibitor of this enzyme; the protein binds to and promotes degradation of the C4 complement protein, thus inactivating the C3 convertase. C4BP has also been demonstrated to have activity against the alternative pathway C3/C5 convertase (Blom et al. 2003; Fujita and Nussenzweig 1979). The alternate pathway activates complement deposition through formation of a C3 converatase made up of protein fragments of C3 and Factor B (C3b, Bb). Factor H is a major serum component inhibitor of the alternate pathway.

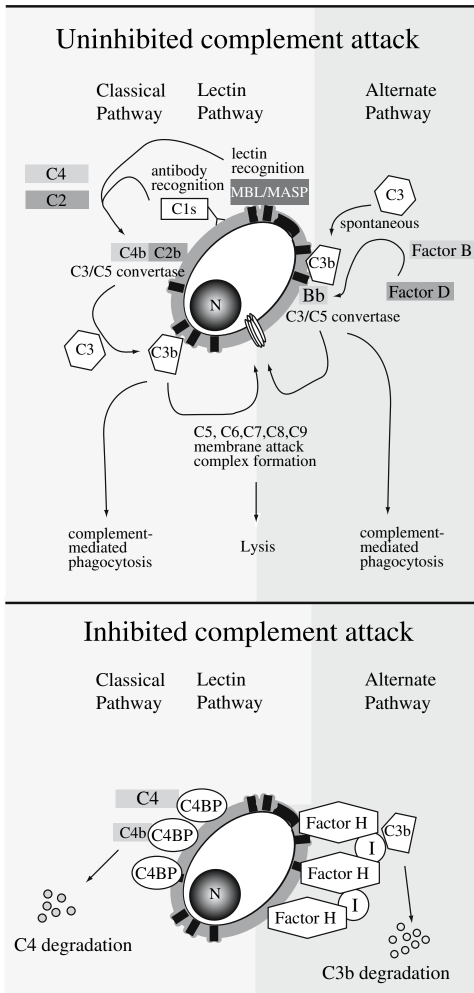


Figure 3. Evasion of complement. The serum complement cascade is an important method for antimicrobial defense. Complement deposition on fungal cell walls can be initiated through 3 pathways: The classical pathway, the lectin pathway, and the alternate pathway. Fungal recognition by the classical pathway occurs through antibodies. Fungal recognition through the lectin pathway occurs through soluble lectins such as the mannose binding lectin (MBL). Activation of the classical and lectin pathways stimulates the proteases C1s and MASP respectively to catalyze formation of the C4b/C2b active C3/C5 convertase. This enzyme catalyzes opsonization with C3b, and initiates formation of the membrane attack complex. In the alternate pathway, the C3/C5 convertase (C3b/Factor Bb) is formed through spontaneous C3b deposition and Factor D catalyzed membrane deposition of Factor Bb. *C. albicans* has been noted to bind two host protein inhibitors of the complement pathway. C4 binding protein (C4BP) binds to C4 and catalyzes its degradation, blocking activation of the classical and lectin pathways. Factor H binds to C3b and, using Factor I as a cofactor, catalyzes C3b degradation, blocking activation of the alternate pathway

Factor H binds to C3b preventing formation of the active convertase, and Factor H acts a cofactor for the plasma serine protease factor I in the degradation of C3b (Pangburn et al. 1977; Weiler et al. 1976). *C. albicans* cell walls can activate all three complement deposition pathways (Kozel 1996). Mannan on the cell surface is recognized by the mannan binding protein which activates the lectin pathway and by common anti-mannan antibodies that activate the classical pathway, and the wall is non-specifically targeted by C3b of the alternate pathway.

In order to evade killing by the complement pathway, *C. albicans* binds to C4BP and Factor H (Meri et al. 2004; Meri et al. 2002). Both complement inhibitors bind strongly and specifically to *C. albicans* yeast and filament cell walls, and the bound proteins retain their ability to inhibit the three complement pathways (Figure 3). The in vivo consequence of the ability to bind to complement inhibitory components has yet to be defined through identification of mutants that cannot bind to these proteins, but it is presumed that the binding provides significant protection from complement-mediated lysis and complement opsonization for phagocytosis. Thus *C. albicans* takes control of host mechanisms for complement regulation.

B. dermatitidis takes control of complement receptor signaling in phagocytes for evasion of innate immune defenses. The pathogen expresses blastomyces adhesin 1 (BAD1) on the cell wall, and this protein binds to CD14 and complement receptor 3 (CR3, MAC1) on the surface of macrophages (Newman et al. 1995). Why would a pathogen specifically target binding to a phagocyte? Macrophages produce important pro-inflammatory cytokines such as TNF-α when activated, and CR3 signaling has been implicated in suppression some of these inflammatory responses (Morelli et al. 2003). This suppression is thought to be mediated by production of the anti-inflammatory cytokine TGF-β, and normally probably plays a role in ensuring that macrophages do not become activated while performing routine housekeeping functions such as clearing apoptotic cells. Through binding to CR3, BAD1 specifically inhibits macrophage activation (Brandhorst et al. 2004). *B. dermatitidis* mutants deficient in surface BAD1 expression are much less pathogenic than wild type organisms (Brandhorst et al. 1999).

5. ATTACK

If host defense mechanisms cannot be avoided completely or controlled sufficiently, the last resort for a pathogen is simply to survive or destroy the defenses. To the extent that fungi are simply robust and hardened against their environments, this is not formally "immune evasion" so much as it is simple survival. However, there are many examples of cases in which fungal pathogens actively destroy or counter specific immune defenses.

ROS production by macrophages and neutrophils is a primary mechanism for killing internalized microbes. The reactive chemical species destroy microbial enzymes and attack cell walls. Macrophages and neutrophils protect themselves

from destruction by the ROS they produce by synthesizing catalases and superoxide dismutases that mop up any ROS that leaks out of the phagosome. A number of fungal pathogens have taken the same approach to protect themselves from ROS. *Aspergillus fumigatus* is a common soil fungus that is a frequent human pathogen, especially in immunocompromised patients. It is typically delivered into the lung as a spore-like conidia where it germinates and produces an extensively branched mycelium that invades lung tissue (Latge 2001). *A. fumigatus* produces at least three catalases with which to "attack" phagocyte ROS, one that is produced only in spores and two that are produced by mycelium (Rementeria et al. 2005). A double mutant lacking the two mycelial catalases demonstrated a reduced rate of infection compared to wild type *Aspergillus* (Paris et al. 2003). *Aspergillus* also produces at least two superoxide dismutases, although their specific contributions to virulence have not been determined (Rementeria et al. 2005). In addition to these mechanisms for defense against ROS, *Aspergillus* spores produce melanin that is thought to mop up ROS and protect from killing by alveolar macrophages (Latge 1999) Catalases and superoxide dismutases are also produced by *C. albicans, C. neoformans*, and *Histoplasma capsulatum*, among others.

Another example of fungal attack on innate immune defenses is the secretion of proteases that degrade tissue barriers and promote fungal invasiveness. As noted earlier, tissue barriers such as skin are the most basic innate defenses to infection. Most tissue barriers have an innate ability to distinguish between microbes and self; although they are impermeable to foreign microorganisms, they permit entry of host cells during growth, remodeling, and as part of normal inflammatory responses. *A. fumigatus* secretes dozens of proteases that are likely important for releasing nutrients from the environment, but that are also thought to be involved in successful pathogenesis and penetration of the lung epithelium (Rementeria et al. 2005). Given the cocktail of proteases secreted, it is difficult to attribute a specific advantage to a specific protease, since they likely work as a highly redundant group to disrupt tissue layers. Generally studies looking at pathogenicity of *A. fumigatus* strains deficient in single specific proteases have not demonstrated reduced virulence although secretion of proteases in general correlates strongly with pathogenicity (Blanco et al. 2002; Kogan et al. 2004; Latge 1999). The *C. albicans* secreted aspartyl proteinase (SAP) family of ten proteases is a more tightly defined set of secreted enzymes and more comprehensive studies on knockout strains have been performed. Triple knockout *C. albicans* strains deficient in SAPs 1–3 or 4–6 are significantly less virulent than wild type strains, although they grow normally in rich media (Hube et al. 1997; Sanglard et al. 1997). However, it is difficult to determine how much of the virulence contribution of the SAPs is due to tissue destruction since both strains grow poorly in media in which proteins provide the sole nitrogen source. Also, one group has demonstrated that SAP 4–6 knockout yeast are killed more efficiently by macrophages than wild type yeast, suggesting that the proteases may play a protective role within the phagosome by "attacking" or disabling antimicrobial components of the compartment (Borg-von Zepelin et al. 1998).

Many fungi produce toxins, which are generally noxious and may protect the fungi from other organisms in the environment as well as cause damage to mammalian host tissues during infection. For some of these toxins, specific roles in targeting immune cells and immune responses have been proposed. For example, *A. fumigatus* produces an alkaloid metabolite called fumigaclavine C which is an inhibitor of DNA synthesis (Rementeria et al. 2005). The toxin seems to have some specificity for T cells, and use of the purified toxin has been explored in mouse models of T cell induced disease. In a model of T cell induced liver damage, fumigaclavine C inhibited tissue damage in a dose dependent manner and reduced production of IL-2 (Zhao et al. 2004). In a mouse model of colitis, fumigaclavine C administration protected mice from disease and suppressed production of cytokines including IL-2 and IFN-γ (Wu et al. 2005). Another *Aspergillus* toxin of 14 kDa, still to be fully characterized, is produced by conidia and potently inhibits macrophage phagocytosis and ROS production (Bertout et al. 2002; Mitchell et al. 1997).

Competition for iron is another case in which microbes must actively thwart the host. Iron is required for aerobic life, but biologically available iron is scarce. In mammals it is mopped up by transferrin, which has a high affinity for ferric iron (10^{-20} M) and greedily sequestered away in cells, mainly in ferritin complexes (Conrad et al. 1999). To compete with this, pathogenic microbes must be able to “steal” this iron (Barasch and Mori 2004). Bacteria produce siderophores, soluble iron-capturing molecules, to harvest iron from the environment and to compete with other bacteria and the host for precious iron (Braun and Killmann 1999). Enterochelin, a siderophore produced by *E. coli* and related enteric bacteria, has an astonishingly high affinity for iron of 10^{-49} M (Loomis and Raymond 1991). This high affinity permits the siderophore to “steal” iron from iron acquisition systems such as transferrin. The bacteria express transport systems for internalization of the iron-siderophore complexes. The importance of this competition to the immune response is highlighted by the production of Lipocalin-2 by immune cells in response to Toll-like receptor activation. Lipcalin-2 (NGAL) is a host protein that strongly binds to enterochelin and blocks its uptake by bacteria (Goetz et al. 2002). Mice deficient in lipocalin-2 are highly susceptible to *E. coli* infection (Flo et al. 2004). Surprisingly, *Aspergillus* also expresses a transport system for internalizing iron-bound enterochelin, even though it does not produce the siderophore (Haas et al. 2003). The fungus also expresses several other siderophore transport systems for internalizing fungal-produced siderophores.

C. albicans expresses a single siderophore transport system encoded by the *Sit1* and *Arn1* genes (Ardon et al. 2001). This iron acquisition system is required for invasion of epithelial cell layers, but not for systemic infection (Heymann et al. 2002; Hu et al. 2002). These data suggest that siderophore-based acquisition of iron may be especially important within specific tissues, while other mechanisms of iron acquisition may suffice at other sites. Intracellular compartments are especially iron-starved environments, and the facultative intracellular fungus, *Histoplasma capsulatum*, produces a variety of siderophores that are thought to be required

for survival within this environment (Howard et al. 2000). The fungus is ingested by macrophages where is grows and replicates intracellularly, thus siderophore production is one mechanism for evasion of macrophage-mediated host defense.

6. CONCLUSION

Despite the efficiency, complexity, and scope of mammalian immunity, microbial pathogens have acquired strategies to subvert these defenses. Some mechanisms of defense are difficult to think of as "immune evasion" mechanisms. For example, although having a cell wall instead of an unprotected plasma membrane is certainly an advantage to fungal pathogens compared to some other eukaryotic organisms, it is not useful to think of this as an "immune evasion" mechanism since there is no specific immune response targeted. However, as several examples above have illustrated, the specific composition and structure of cell walls varies between organisms and morphotypes and this variation can be demonstrated to have specific utility in immune evasion. Other immune evasion mechanisms appear to be more classical "virulence" factors in that they are produced exclusively to suppress host immunity. The strategies used to evade host immune responses are varied, and no single strategy seems sufficient to convert a non-pathogen into a pathogen. Instead, successful pathogenic strains of fungi make use of multiple strategies simultaneously to replicate and disseminate within the host.

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SECTION 6

IMMUNE-BASED THERAPEUTIC STRATEGIES

CHAPTER 20

CYTOKINE TREATMENT OF FUNGAL INFECTIONS

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Abstract: Resolution of invasive fungal infections is often dependent on recovery from an immunocompromised state, indicating that host defense mechanisms are extremely important in the clearance of fungal pathogens. Immunotherapy aimed at enhancing host defense mechanisms may improve clinical outcome of invasive mycoses. Recombinant cytokines and growth factors have been applied in vitro and in animal models to augment host defense mechanisms that are involved in invasive fungal infections. In patients with invasive mycoses, prospective phase 2 studies with recombinant G-CSF and IFNγ have been performed. Recombinant IFNγ is a candidate for phase 3 trials on adjunctive immunotherapy for cryptococcal meningitis, invasive aspergillosis and candidemia

1. INTRODUCTION

1.1. Rationale

The development of a wide variety of potent antimicrobial agents has been very successful in reducing the morbidity and mortality associated with many infections. However, severe nosocomial bacterial and fungal infections remain a serious problem. Invasive fungal infections are an increasingly important complication among hospitalized and immunocompromised patients (Edmond et al. 1999; Wisplinghoff et al. 2004). The use of broad spectrum antibacterials, aggressive instrumentation of patients, including the use of chronic indwelling intravenous catheters, the ability to prolong patient survival through life-sustaining procedures, such as mechanical ventilation and haemodialysis, are some of the reasons hospitalized patients are at risk for nosocomial fungal infections, especially invasive candidiasis (Wisplinghoff et al. 2004). In addition, invasive fungal infections are a threat for patients with iatrogenic immunosuppression, such as chemotherapy for patients with solid organ and haematologic malignancies, therapy to manage graft

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versus host disease among allogeneic haematopoietic stem cell transplant recipients, and chronic immunosuppressive therapy for solid organ transplant recipients. There are a growing number of patients with underlying conditions such as rheumatoid arthritis, systemic lupus erythematosis, and Crohn's disease, requiring chronic immunosuppressive therapy with glucocorticosteroids, cytotoxic agents, and agents such as infliximab and etanercept, in whom serious invasive fungal infections may occur (Weisman 2002). While the risk of specific invasive fungal infection varies with the underlying condition, collectively these patients are at risk for a wide range of fungal infections, most frequently invasive aspergillosis or disseminated candidiasis. Less frequent but often life-threatening infections are caused by other moulds, such as zygomycosis and phaeohyphomycosis, or yeasts, such as cryptococcosis and histoplasmosis or other endemic (geographically restricted) fungal infections.

In response to this emerging problem, new classes of antifungal drugs have been introduced at an unprecedented pace, that bear promise for achieving cure from infection and a lower incidence of adverse effects (Herbrecht et al. 2002; Mora-Duarte et al. 2002; Kullberg et al. 2005). For patients with invasive aspergillosis, therapy with voriconazole, a new triazole antifungal, has significantly increased the survival rate, and other new antifungal agents may have similar benefits (Herbrecht et al. 2002). Despite these developments, treatment failure still is a significant problem, amounting 20–30% of patients with the most common opportunistic fungal infections (Anaissie et al. 1998; Herbrecht et al. 2002; Rex et al. 2003). In specific groups of patients, such as those with persistent neutropenia, failure rates are even substantially higher (Denning 1996). This paradox underscores the need for an intact immune system to prevent invasive fungal infections and to successfully control infection once established. During antifungal therapy, resolution of invasive fungal infections is often dependent on recovery from granulocytopenia or restoration of cellular immunity, indicating that host defence mechanisms are extremely important in the clearance of these pathogens. Therefore, immunotherapy aimed at enhancing host defence mechanisms may prove extremely useful. The design of trials of immunotherapy against fungal pathogens brings about several specific difficulties, and requires profound knowledge of the host defence mechanisms that are involved in invasive fungal infections.

1.2. Host Defence Mechanisms as Targets for Immunotherapy

Infection can be envisaged as a disequilibrium between the invading micro-organism and the host. The recent discovery of new classes of molecules capable of stimulating and regulating the haematopoietic and immune systems has now opened the door to the design of antifungal immunotherapy. Several arms of the immune system are amenable to modulation through immunotherapeutic approaches.

1.2.1. Adhesion and recognition of invading fungi

The first step in the host defence comprises the adhesion of the pathogen to the body surface of the host, and recognition by the pattern recognition receptors.

The recent identification of lectins (such as the mannose receptor, dectin-1, and DC-SIGN), and Toll-like receptors (TLR, in particular TLR2 and TLR4) as essential pattern recognition receptors for fungal pathogens suggest that modulation of these receptor signalling pathways may be explored as immune intervention (Netea et al. 2004b). For blood-borne fungal pathogens, adhere to and invasion of endothelial cells is the subsequent step of invasion, and endothelial cells have an active metabolic role in this process (Cannom et al. 2002). Interference with adhesion to endothelial cells or their subsequent activation may provide additional opportunities for immunotherapy. The identification of members of the *C. albicans* Als protein family (Als1p and Als5p) currently appears to provide the most promising opportunity for interfering with this process, most likely through development of a vaccine preventing fungal adhesion and subsequent invasion (Sheppard et al. 2004).

1.2.2. Innate host defence

At present, the downstream pathways of the fungal pattern recognition receptors governing the cellular pathways of the innate immune system are the most promising targets for immunotherapy. Phagocytes, i.e., PMN and mononuclear phagocytes, are essential components of the innate host resistance. Both epidemiological data on patients receiving anti-cancer chemotherapy and experimental animal studies have demonstrated that the contribution of PMN and mononuclear phagocytes to innate resistance against invasive candidiasis is crucial. The recent discovery of new classes of molecules capable of recruiting, stimulating and regulating these cells has now enabled the application of antifungal immunotherapy in clinical practice. A wide range of molecules – including the cytokines (interleukins, interferons) haematopoietic growth factors, and monoclonal antibodies – is currently becoming available for clinical use, and their use will be discussed in paragraph 2.

1.2.3. Granulocyte transfusions

Since the recognition of serious bacterial infections among patients with neutropenia as a consequence of cancer chemotherapy in the 1960s, granulocyte infusions have been considered as options to restore the host defence. However, enthusiasm waned largely because data supporting the efficacy of granulocyte infusions was sparse and the absolute increase in granulocyte numbers following infusion was relatively minor. Recently, there has been renewed interest in granulocyte infusions in persistently neutropenic patients with fungal infections as an adjunct to antifungal therapy, with the advent of haematopoietic factors to stimulate neutrophil production in the granulocyte donors. These data will be discussed in paragraph 2.4.1.

1.2.4. Humoral immunity and vaccination

Whereas circulating antibodies can be detected against major invading pathogens, such as *Candida, Aspergillus*, of *Cryptococcus* species, it is generally agreed that humoral immunity does not play a major role in the host defence against the invasive manifestations of these pathogens. Nevertheless, passive immunotherapy has been advocated as a potentially important adjunct to conventional antifungal

therapy in the management of invasive mycoses, especially in patients with cryptococcosis (Casadevall and Pirofski 2001) (further discussed in Chapter 21). The interest in specific "serum therapy" for cryptococcosis is not new, and the use of "hyperimmune" equine serum was advocated in the 1930s as part of the recommended therapy of cryptococcosis (Taber 1937). The potential for augmentation of host response to invasive candidiasis infection has been suggested in experimental animals, where mice treated with human intravenous immunoglobulin (IVIG) together with amphotericin B demonstrated modest prolongation of survival from *Candida* infection (Neely and Holder 1992), but this benefit has never been demonstrated in humans. Studies of IVIG prophylaxis in higher risk patients have yielded conflicting results. Whereas a reduction of the incidence of fungal infections initially was suggested in liver transplant recipients given IVIG and acyclovir prophylaxis (Stratta et al. 1992), a subsequent study did not show a beneficial effect of IVIG in preventing serious fungal infections in bone marrow transplant recipients compared to placebo (Klaesson et al. 1995).

2. CYTOKINE-BASED STRATEGIES TO AUGMENT INNATE HOST DEFENCE

Cytokines represent an essential class of mediators in physiology and pathology. Almost all cytokines known have clearly beneficial as well as deleterious effects for the organism. The latter effects are mainly seen when cells are exposed to relatively high concentrations of cytokines, whereas the beneficial effects generally occur at lower concentrations. From an evolutionary standpoint, these mediators are undoubtedly meant to be beneficial, but nevertheless, harmful effects mediated by cytokines do occur in pathology. Cytokine-mediated damage of cells, such as endothelial cells, at crucial sites of the body will be deleterious to the host. Not only the pro-inflammatory cytokines can be hazardous under circumstances; the anti-inflammatory cytokines may blunt the immune response to such an extent that a state of immunodeficiency ensues. It is however impossible to make a simple dichotomy between the good and the bad effects of these cytokines. Under certain circumstances a particular cytokine effect may be beneficial, whereas under other circumstances a similar effect may harm the host.

It is clear from these considerations that modulating the cytokine response during disease may lead to improvement of the clinical status, and these therapeutic approaches have become a great challenge in medicine. However, such interventions may have a substantial impact on the delicate and complicated cytokine balance and may lead to disturbances that adversely affect the status of the host.

In the attempts to modulate the cytokine response there are four options:

1) enforce the proinflammatory cytokine response to enhance host defence;
2) decrease the anti-inflammatory response to enhance the effects of innate proinflammatory cytokines on host defence.
3) decrease the proinflammatory cytokine response to limit inflammatory damage;
4) enforce the anti-inflammatory response to limit inflammatory damage.

Enforcement of the proinflammatory cytokine response and inhibition of the anti-inflammatory response (options 1 and 2) are aimed at treatment of invasive fungal infection. Inhibition of the pro-inflammatory response and enforcement of the anti-inflammatory response (options 3 and 4) are especially applicable during overwhelming inflammation, such as the systemic inflammatory response syndrome occurring during severe sepsis, and in serious non-infectious inflammatory diseases, e.g., rheumatoid arthritis and Crohn's disease.

2.1. Anti-inflammatory Strategies

In recent years, most of the clinical studies on immunotherapy of infection and inflammation have dealt with the options 3 and 4, the inhibition of the pro-inflammatory response and enforcement of the anti-inflammatory response, in particular through strategies aimed at inhibition of the effects of tumour necrosis factor alpha (TNFα) and interleukin-1 (IL-1). The failure of immunotherapeutic interventions attempted in sepsis trials to date (Bone 1996) underscores the need for more profound knowledge of the immunoregulatory mechanisms during infection, and highlights the delicateness of the balance between pro- and inflammatory profiles during the course of infection (Netea et al. 2003a; Netea et al. 2003b). Nevertheless, immunomodulation towards an anti-inflammatory profile has become common practice in *Pneumocystis jiroveci* pneumonia in HIV-positive patients through administration of corticosteroids (MacFadden et al. 1987). Also during other life-threatening fungal infections, such as fulminant candidaemia, acute invasive pulmonary aspergillosis, cryptococcal meningitis, or cerebral coccidioidomycosis, the host's inflammatory response may be largely responsible for tissue damage and inflammatory sequelae. However, the timing of an anti-inflammatory intervention and its potential deleterious impact on the innate host defence and the clearance of the micro-organisms is crucial and beyond clinical feasibility. In general, augmentation of the pro-inflammatory response, especially during the early phase of infection, appears to have a beneficial net effect, in spite of the potential tissue damage by the host's inflammatory response (Netea et al. 2003b). Indeed, preliminary data from clinical immunotherapy trials in patients with candidaemia or cryptococcal meningitis have confirmed this notion, as discussed below (Kullberg et al. 1998b; Pappas et al. 2004).

2.2. Pro-inflammatory Strategies: The Interferons

Despite the availability of recombinant interferon-γ (IFNγ) for almost two decades, clinical trials on immunotherapy for infectious diseases using IFNγ are extremely sparse. Interestingly, the only published major trial on prophylactic administration of recombinant IFNγ has been aimed at preventing invasive fungal infections, and was performed in patients with chronic granulomatous disease (CGD) (Gallin et al. 1991), a group of rare disorders in which the cytochrome oxidase in the membrane

of phagocytic cells is defective. This leads to insufficient generation of reactive oxygen intermediates, which are required to kill bacteria and fungi.

Prophylactic administration with IFNγ was shown to reduce the frequency of infections in these patients without severe side effects (Gallin et al. 1991; Weening et al. 1995). In a large, prospective, randomized, placebo-controlled trial, the administration of IFNγ proved beneficial in terms of prevention of bacterial and fungal infections in these patients, by a mechanism which has not been fully elucidated. In a second, open study, the incidence of invasive fungal infections, predominantly aspergillosis, in CGD patients who received rIFNγ was significantly reduced from 24% to 4% in 2 year (Gallin et al. 1991). Although it has been shown that IFNγ is able to stimulate the phagocytes to respond with oxygen-independent antimicrobial mechanisms (Murray et al. 1985), and improvement of superoxide production by CGD monocytes has been suggested (Jendrossek et al. 1993), the exact protective mechanisms of the cytokine in CGD patients has not been clearly identified (Ezekowitz et al. 1988; Rex et al. 1991).

IFNγ has been shown to be an essential cytokine for activating fungicidal activities of phagocytes in vitro, as was shown for a wide variety of fungal species, including *C. albicans, Aspergillus fumigatus, Cryptococcus neoformans*, and *Histoplasma capsulatum* (Djeu et al. 1986; Roilides et al. 1993; Nakamura et al. 1994), and adjunctive treatment of established fungal infections would be a conceivable next step. However, there are few data on the treatment of human mycoses with IFNγ, and most experience has been limited to anecdotes, and include patients with refractory cryptococcosis, hepatosplenic candidiasis, invasive aspergillosis, and zygomycosis (Phillips et al. 1991; Williamson et al. 1992; Poynton et al. 1998).

2.2.1. Candida

Administration of a single dose of recombinant IFNγ to mice with disseminated candidiasis significantly reduced the outgrowth of *C. albicans* in their organs, even when the infection had already been established for several days (Kullberg et al. 1993). Of note, a single injection of rIFNγ resulted in a beneficial effect that persisted for at least one week. These data indicate that it might be feasible to study combination therapy of a conventional antifungal agent and recombinant IFNγ in treating disseminated candidiasis. In severely neutropenic mice, treatment with IFNγ did not alter the course of disseminated candidiasis, suggesting that the effect of IFNγ is mediated through the activation of PMN (Kullberg et al. 1993). This is in agreement with the observation that neutrophils isolated from IFNγ-treated mice show enhanced intracellular killing of *Candida albicans* (Kullberg et al. 1993). Clinical data on immunotherapy with IFNγ in patients with candidiasis are lacking. In a single case report, Poynton and colleagues reported two patients with progressive chronic disseminated candidiasis, who responded to six weeks of rIFNγ therapy, which had initially been combined with rGM-CSF (Poynton et al. 1998). It is likely that particularly the rIFNγ was responsible for the observed effect, rather than the rGM-CSF. Based on the current insights on host defence mechanisms,

it appears that rIFNγ may be a major candidate for further studies on adjunctive immunotherapy of disseminated and invasive candidiasis in non-neutropenic hosts.

2.2.2. *Aspergillus*

In vitro, IFNγ augments oxygen radical production and neutrophil-mediated damage of *A. fumigatus* hyphae (Roilides et al. 1993). IFNγ is able to ameliorate the immunosuppressive effects of corticosteroids on the fungicidal activity of human mononuclear cells against *A. fumigatus*, as evidenced by restoration of the capacity for hyphal damage and of oxidative burst in response to hyphae (Roilides et al. 1996). The combination of IFNγ and G-CSF exerts an additive effect on neutrophil-mediated hyphal damage against *A. fumigatus* (Roilides et al. 1993; Roilides et al. 1994).

In experimental infection models, the development of Th1-type protective immunity is correlated with resistance to a lethal *Aspergillus* infection, and neutralization of endogenous IFNγ has deleterious effects on the course of fungal infections (Romani 2004). Conversely, production of the Th2 cytokines IL-4 and IL-10 is associated with disease progression in experimental models (Cenci et al. 1998; Romani 2004). Indeed, clinical observations have confirmed that high concentrations of IL-10 or low IFNγ/IL-10 production ratios are associated with a poor outcome in patients with aspergillosis (Roilides et al. 2001; Hebart et al. 2002). The importance of Th1/Th2 dysregulation in the course of invasive aspergillosis in humans was further supported by analysis of lymphocyte responses in patients with active infection. Stem cell transplant recipients, in particular those receiving steroids, have a Th2-biased cytokine response, rendering them susceptible to invasive aspergillosis (Hebart et al. 2002). On exposure to *Aspergillus* antigen, lymphoproliferative T-cell responses in uninfected patients and in patients with invasive aspergillosis who were responding to antifungal therapy demonstrated strong Th1 lymphoproliferative responses, with increased IFNγ/IL-10 ratios (Hebart et al. 2002). Patients with progressive invasive aspergillosis despite antifungal therapy, however, showed low IFNγ/IL-10 ratios consistent with a Th2-predominant response (Hebart et al. 2002). Together, clinical and experimental observations suggest that a Th1/Th2 dysregulation with suppression of host Th1-type lymphocyte response and a switch to a Th2-type immune response may contribute to the development of an unfavourable outcome of invasive aspergillosis (Hebart et al. 2002; Romani 2004). Immunotherapeutic strategies therefore should target at modulating the host response towards a favourable Th1/Th2 balance, leading to enhanced activity of cytokines such as IL-1, TNFα, IL-18, and IFNγ, which are required for effector cell activation against fungal pathogens (Kullberg and Anaissie 1998). Recently, the NIH/NIAID Bacteriology and Mycology Study Group initiated a placebo-controlled trial comparing the use of IFNγ 100 μg three times weekly versus placebo in conjunction with conventional therapy with voriconazole for patients with invasive aspergillosis. However, this study was permaturely discontinued for logistical reasons, so that there are presently no insights into the use of IFNγ as an adjunctive therapy for immunocompromised patients with invasive aspergillosis.

2.2.3. Cryptococcus

IFNγ is crucial for the stimulation of anti-cryptococcal mechanisms in macrophages and neutrophils, and in-vitro studies have demonstrated that killing of *C. neoformans* by macrophages can be enhanced with recombinant IFNγ (Flesch et al. 1989). Subsequently, experimental animal studies have demonstrated improved survival, decreased tissue burden, and enhanced anticryptococcal activity in animals experimentally infected with *C. neoformans* that have been treated with IFNγ alone or in conjunction with systemic antifungal therapy (Joly et al. 1994; Kawakami et al. 1999; Lutz et al. 2000; Clemons et al. 2001).

Patients with HIV and CD4+ lymphopenia, who constitute the major risk group for acquiring invasive cryptococcosis, have a decreased IFNγ production upon cryptococcal stimulation (Murray et al. 1984). Recently, it was demonstrated that HIV-negative patients with idiopathic CD4+ lymphopenia and cryptococcal meningitis also have a defective production of the protective proinflammatory cytokines TNFα and IFNγ, whereas the release of the anti-inflammatory cytokine IL-10 is normal (Netea et al. 2004a). The defect in Th1-type cytokine production in these patients could be restored by injection of IFNγ, whereas IL-10 production decreased, further contributing to a favourable Th1 profile (Netea et al. 2004a). Netea et al. have described a patient with idiopathic CD4+ lymphopenia and treatment-refractory cryptococcosis, in whom the Th1/Th2 balance was restored by administration of recombinant IFNγ, ultimately leading to clearance of cryptococci from the CSF (Netea et al. 2004a). After 4 years of combined therapy with recombinant IFNγ and fluconazole, the IFNγ could be discontinued in this patient without subsequent relapse, despite the persistent severe idiopathic CD4+ lymphopenia. These findings, indicating the ability of recombinant IFNγ to create a sustained increase of pro-inflammatory response and a suppression of IL-10 in patients with cryptococcosis, provide the rationale for immunotherapy in CD4+ deficient patients. The recently-published randomised trial involving HIV-positive subjects with acute cryptococcal meningitis is the only controlled human study of IFNγ in invasive mycoses to date (Pappas et al. 2004). In this randomized, double-blind, placebo-control trial, 75 patients with acute cryptococcal meningitis were enrolled. Patients were randomized to receive 100 μg of recombinant IFNγ (27 subjects), 200 μg of recombinant IFNγ (25 subjects), or placebo (23 subjects) subcutaneously three times weekly for 10 weeks in conjunction with standard therapy with intravenous amphotericin B followed by fluconazole. The results of this trial indicated an important trend towards more rapid clearance of *C. neoformans* from cerebrospinal fluid in IFNγ recipients compared to placebo recipients ($P = 0.064$). At 2 weeks, conversion of positive cerebrospinal fluid (CSF) fungal culture to negative has occurred in 38% of rIFNγ recipients compared to 18% of patients receiving antifungal therapy only. At the end of the study, serum cryptococcal antigen titres had decreased by 50 to 70-fold in the IFNγ treatment group, versus 24-fold in the placebo group. Although at week 10, the culture conversion rate was similar across treatment groups, 62% of subjects showed improved combined mycological clinical outcome in the active treatment arm compared with 48% on placebo ($P > .05$) (Pappas et al. 2004).

However, there was no obvious clinical benefit to IFNγ recipients compared to placebo recipients in terms of improved survival rates, and there were no important clinical or mycological trends noted among patients who received higher versus lower dose IFNγ. Overall, IFNγ was well tolerated, however, there were more side effects including fever, myalgias, headache and nausea in the higher dose group.

Whereas this pilot study provides no definite proof of the efficacy of antifungal immunotherapy in clinical practice, it has provided valuable information on the safety and applicability of immunomodulators in patients with invasive mycoses, opening ways to do subsequent phase 3 studies.

Indeed, based on the results of this study, the NIH/NIAID Bacteriology and Mycology Study Group has designed a phase-III randomized trial comparing placebo to 100 μg IFNγ three times weekly as adjunctive therapy with conventional antifungal therapy. Both this study and a study of recombinant IFNγ as adjuvant therapy against invasive aspergillosis have been prematurely abandonned, due to lack of commercial interest from the pharmaceutical industry and logistic issues.

Nevertheless, these data collectively lend support to the concept that IFNγ may be most useful as adjunctive therapy among patients with a variety of invasive fungal infections. To further develop clinical immunotherapy for these infections, which remains highly rational in view of the immune defects in this patient group, it will be critically important that marketing issues and industry funding policies do not impede the conduct of randomised clinical trials in this area.

In such future studies on IFNγ immunotherapy, it will be crucial to demonstrate not only surrogate endpoints, such as improved clearance of *C. neoformans* from CSF with IFNγ compared to placebo, but also to examine and compare the impact on clinical outcome and survival among IFNγ recipients.

2.3. Pro-inflammatory Strategies: The Cytokines

2.3.1. Stimulation of pro-inflammatory cytokines

The first reports on augmenting host resistance to experimental fungal infection using recombinant cytokines were on the role of interleukin-1 (IL-1). A single injection of either recombinant IL-1α or IL-1ß was shown to protect neutropenic mice against lethal disseminated candidiasis (Van ‘t Wout et al. 1988; Kullberg et al. 1990). Treatment also significantly decreased the numbers of *C. albicans* in the organs of infected normal mice and of mice rendered immunocompromised by cyclophosphamide, hydrocortisone acetate, or total body irradiation (Kullberg et al. 1990). A similar protective effect of IL-1 has been demonstrated in models of bacterial infection caused by a variety of species (reviewed in Vogels and Van der Meer 1992). Although the mechanism of IL-1-induced protection has not been fully elucidated, modulation of cytokine receptors and induction of acute phase proteins

and other humoral factors contribute to its effect (Vogels and Van der Meer 1992), which is independent of the presence or activation of phagocytic cells (Kullberg et al. 1990).

Interleukin-12 (IL-12) is produced very early during the infective process and antigen presentation by macrophages, and it enhances both the production of IFNγ and T-cell differentiation. In a preclinical study, recombinant IL-12 was administered to rats with disseminated cryptococcosis (Clemons et al. 1994). After 2 weeks of infection, treatment with either IL-12 or fluconazole reduced the outgrowth of organisms in the brain, and the combination of IL-12 and fluconazole had an additive effect. In the liver, fluconazole failed completely to contain the infection, whereas treatment with IL-12 was effective in reducing the outgrowth of *C. neoformans* (Clemons et al. 1994).

IL-18 is cytokine that serves as a costimulus for IFNγ production in the context of microbial stimulation, and its role in the induction of IFNγ by *C. albicans* has recently been demonstrated (Netea et al. 2002; Stuyt et al. 2002). Recombinant IL-18, either given as prophylaxis or after onset of disseminated candidiasis, leads to enhanced clearance of C. *albicans* in a mouse model, associated with increased production of IFNγ (Stuyt et al. 2004).

The feasibility of clinical application of these pro-inflammatory strategies is uncertain. Indeed, recombinant IL-1 has been administered to patients in small trials (Nemunaitis et al. 1994; Weisdorf et al. 1994), but this cytokine has considerable side effects, such as fever, chills, and hypotension. Whereas it is unlikely that recombinant IL-1, IL-12, or IL-18 will be applicable as immunotherapeutic drugs against infections, the experimental data underscore the requirement for a pro-inflammatory or Th1-type profile to promote the clearance of invasive fungal infections. Indirect ways to enhance endogenous pro-inflammatory responses, e.g., by vaccination, gene therapy, or receptor stimulation, may prove useful immunotherapeutic interventions in the future (Kullberg et al. 2004).

2.3.2. Inhibition of anti-inflammatory cytokines

The release of IFNγ and the activation of macrophages are both inhibited by anti-inflammatory cytokines, such as IL-4 and IL-10. Failure to clear a subacute or chronic infection with *C. albicans* corresponds with sustained production of IL-4 and IL-10 in susceptible mouse strains, whereas so-called constitutively resistant mouse strains produce significantly less IL-4 and IL-10 (Romani et al. 1994). Likewise, preliminary data suggest that that high concentrations of IL-10 or low IFNγ/IL-10 production ratios are associated with a poor outcome in patients with invasive aspergillosis (Roilides et al. 2001; Hebart et al. 2002). In animal models, neutralization of endogenous IL-4 or IL-10 by specific monoclonal antibodies augments host resistance, leads to increased survival of mice infected with *Candida* or *Aspergillus* species, and enhances the ability of their macrophages to kill *C. albicans* in vitro (Romani et al. 1992; Romani et al. 1994; Cenci et al. 1998; Romani 2004). In a different approach, recombinant soluble receptors to IL-4 (sIL-4R) have been cloned (Puccetti et al. 1994). The sIL-4R circulate in the bloodstream and are

able to bind and neutralize circulating IL-4. Treatment of mice with recombinant sIL-4R was able to cure potentially lethal subacute disseminated infection caused by *C. albicans* (Puccetti et al. 1994). Despite initial enthusiasm to develop anti-IL-10 or sIL-4R for clinical application in immunotherapy for invasive fungal infections, this path seems not to have been pursued any further.

2.4. Pro-inflammatory Strategies: The Haematopoietic Growth Factors

The colony stimulating factors (CSF) are able to augment the numbers of circulating phagocytes and their precursors in the bone marrow (reviewed in Herbrecht and Cordonnier 1995). In addition to its effect on cell numbers, the CSFs have been shown to enhance a variety of functional properties of phagocytic cells, such as chemotaxis, expression of cellular adhesion molecules, and superoxide production. In cancer patients, these factors have been used both to shorten the duration of chemotherapy-induced granulocytopenia (Antman et al. 1988; Crawford et al. 1991) and as adjunctive therapy in patients with febrile neutropenia (Maher et al. 1994). Although most of these studies failed to show an effect of CSF therapy on recovery from infection or survival, the potential beneficial role of rGM-CSF has been demonstrated in a prospective, randomized, placebo-controlled study of patients with acute myelogenous leukaemia (Rowe et al. 1995; Rowe et al. 1996). rGM-CSF was associated with a higher rate of complete haematological response than placebo, and longer overall survival. Furthermore, the fungal infection-related mortality rate was only 2% for those randomized to receive rGM-CSF compared with 19% for those receiving placebo ($P = 0.006$). Among rGM-CSF recipients, three of four with aspergillosis and all three patients with candidiasis survived compared with two of seven and one of four in the placebo group, respectively.

Various animal studies suggest a beneficial effect of rG-CSF on experimental infection with *C. albicans* (Uchida et al. 1992), *A. fumigatus* (Uchida et al. 1992) or *C. neoformans* (Uchida et al. 1992) in neutropenic animals, and neutrophil recovery rather than specific antifungal activation of phagocytes is thought to be responsible for these effects. In non-neutropenic animals, administration of a single dose of murine rG-CSF reduced the mortality and significantly decreased the outgrowth of *C. albicans* in the organs of the animals (Kullberg et al. 1998a). Treatment with rM-CSF has been associated with a favourable outcome in models of subacute or chronic disseminated candidiasis (Cenci et al.; Vitt et al. 1994), but others found that administration of rM-CSF aggravated the course of experimental candidaemia in mice (Hume and Denkins).

Several case reports and case series have been published describing successful therapy with antifungal agents in combination with rG-CSF, rM-CSF or rGM-CSF, in patients with invasive mucormycosis, fusariosis, acute or chronic disseminated candidiasis or invasive aspergillosis (Nemunaitis et al. 1993; Spielberger et al. 1993; Hennequin et al. 1994; Gonzalez et al. 1997; Maertens et al. 1997; Poynton et al. 1998). Generally, the value of such case reports or uncontrolled case series is very limited, in the absence of controlled clinical trials. Of note, several reports have

described serious side effects of rGM-CSF, such as severe capillary leak syndrome (Bodey et al. 1993; Maertens et al. 1997).

The first randomized placebo-controlled study addressing adjunctive immunotherapy for invasive mycoses has compared fluconazole alone versus rG-CSF with fluconazole in non-neutropenic patients with disseminated candidiasis (Kullberg et al. 1998b). The results of this Phase 2 pilot study indicate that adjunctive therapy with rG-CSF is safe and may be given in patients with invasive candidiasis or candidaemia. Although this study was not planned to detect a statistically significant effect of rG-CSF on outcome of infection, a trend towards faster resolution of infection was found, the median time to resolution of infection being 21 days in placebo-treated patients, versus 14 days in rG-CSF-treated patients (hazard ratio (HR) 1.88; 95%CI, 0.90, 3.92). Hazard ratios suggest that patients were approximately twice as likely to resolve their infection on rG-CSF compared to placebo, and a similar beneficial trend was seen in mortality (HR, 0.49; 95%CI, 0.15, 1.63) (Kullberg et al. 1998b). Additionally, this study suggests a previously unreported correlation between increasing leukocyte numbers during therapy and outcome of disseminated candidiasis, in non-neutropenic hosts. Although the relation between outcome of candidaemia and numbers of leukocytes has been well known for neutropenic patients, such influence has not been described for patients with normal leukocyte counts at onset of infection. In the non-neutropenic patients studied, a leukocyte increase with $>15 \times 10^9$/L was associated with the most rapid time to resolution of candidaemia (Kullberg et al. 1998b). These results would argue for further, sufficiently powered, studies on the role of colony-stimulating factors in treatment of candidiasis.

2.4.1. Cytokine-elicited white blood cell transfusions

In the past, several clinical trials have demonstrated a beneficial effect of white blood cell transfusions in patients with refractory neutropenia-related infections. In these studies, most patients had refractory bacterial infections, and they were randomized to receive treatment with white blood cell transfusions plus antibiotics versus antibiotics alone. Five of the studies showed that white blood cell transfusions could be life-saving among those patients with prolonged neutropenia (Freireich 1964; Graw et al. 1972; Alavi et al. 1977; Herzig et al. 1977; Vogler and Winton 1977), or could improve overall survival (Higby et al. 1975), whereas two showed no benefit of white blood cell transfusion therapy (Fortuny et al. 1975; Winston et al. 1982). Unfortunately, none of these trials evaluated the role of this modality in patients with invasive fungal infections. In addition, this modality was abandoned because of the costs and toxicity to recipients including, fever, chills, hypotension, pulmonary infiltrates, respiratory distress (Dana et al. 1981; Wright et al. 1981; Karp et al. 1982; Chanock and Gorlin 1996; Price et al. 2000; Hubel et al. 2001), transmission of cytomegalovirus, graft-versus-host disease, alloimmunization, and haemolytic reactions. Some authors also reported severe pulmonary toxicity with the simultaneous administration of amphotericin B and white blood cell transfusions

(Wright et al. 1981; Chanock and Gorlin 1996; Hubel et al. 2001). Subsequently, this modality was abandoned because of the toxicity to recipients.

Recently, the possibility was raised that administration of rG-CSF to white blood cell donors would increase their neutrophil to levels that would lead to a higher yield of better quality cells. Donors achieved a 4- to 10-fold increase of their neutrophil count, and the 24-hour post-transfusion counts in recipients were favourable (Bensinger et al. 1993). In an other study, rG-CSF-elicited white blood cell transfusions were given to 15 adult cancer patients with neutropenia and documented and refractory fungal infections (Dignani et al. 1997). All patients had haematological malignancies and 7 were bone marrow transplant recipients. Infections included aspergillosis, fusariosis, candidiasis and trichosporonosis. Appropriate antifungal therapy had failed to produce a response in these patients. At the end of white blood cell transfusion therapy, 11 of 15 patients had a favourable response (Dignani et al. 1997). Whereas this small pilot study demonstrated that rG-CSF-enhanced white blood cell transfusions may be both safe and life-saving for patients with refractory neutropenia-related fungal infections, it is important to note that there have been no further randomised follow-on studies. Thus, the role of granulocyte transfusions and the management of neutropenic patients with invasive proven or suspected invasive fungal infection remains to be determined (Pappas 2004).

3. IMMUNOTHERAPY TRIAL DESIGN ISSUES AND CONCLUSIONS

3.1. Design of Immunotherapy Trials

Clinical trials of potential adjunctive immunotherapeutic agents do serve two goals: first, to demonstrate clinical effectiveness and safety, and, second, to generate data that support registration of new agents for the treatment of these potential lethal conditions. The gold standard for achieving these goals is a prospective, randomized, controlled trial. However, comparative trials of antifungal therapies bring about a number of hurdles (Kullberg et al. 2004). Invasive mycoses usually occur in patients with complex and divergent underlying conditions, making the interpretation of the outcome parameters and safety issues extremely complex. In addition, in view of the diagnostic difficulties surrounding invasive fungal infections, unequivocally proven infections are relatively rare, especially in case of aspergillosis. Studies of invasive fungal infections have traditionally accrued subjects very slowly, and the feasibility of completing any study in a reasonable period of time is a major concern. Thus, the large sample size required for randomised, controlled clinical trials is difficult to attain. This requirement for large sample sizes is even greater, since trials on combination therapy should aim at showing superiority of the combination, rather than equivalence. Indeed, the study arm combining an antifungal with an immunomodulatory drug should be shown to be clearly superior to the arm with antifungal therapy only, to warrant addition of the immunomodulator in clinical practice (Kullberg et al. 2004). Additionally, as any immunotherapeutic agent is

likely to be highly expensive, the treatment benefit should be not only statistically significant, but also clinically relevant, in terms of resolution of infection or reduction of mortality. It is highly unlikely that a statistically significant, but clinically negligible effect on mortality would lead to clinical use of an expensive immunomodulatory drug (Kullberg et al. 2004).

Studies involving cryptococcosis or other HIV-associated invasive fungal infections must necessarily involve developing countries, where opportunistic infections are more prevalent. The conduct of studies involving costly therapies in very resource-limited environments is impractical and may be considered unethical (Edwards and Edwards 1998; Pappas 2004).

Finally, the market for patients with invasive fungal infections, even in the developed world, is relatively small compared to areas of cardiovascular diseases and more-prevalent bacterial infections, and the reluctance of pharmaceutical and biotechnology companies to invest in these markets hinders the funding of large, sufficiently powered trials in the complex area of invasive mycoses.

Despite these hurdles, these challenges can be met with creative study design, adequate industry and federal funding, and the collaborative efforts of committed clinical investigators through international multicentre trials (Pappas 2004).

3.2. Concluding Remarks

Both innate and cell-mediated immunity are important for host defence against invasive fungal infections. Despite the creation of new antifungal drugs, treatment failure remains a significant problem if these host defence mechanisms fail. There is considerable promise in the development of adjunctive immunotherapy for prevention and treatment of invasive fungal infections. However, most knowledge to date is based on the results of in-vitro and animal studies. Data concerning the clinical efficacy of these innovative interventions in patients are still lacking. The conduct of randomised clinical trials is hampered by significant logistical and clinical obstacles. Nonetheless, such trials are urgently needed. Based on the current knowledge of host defence mechanisms, as well as promising results in initial studies, adjunctive immunotherapies with recombinant IFNγ or G-CSF may yield new options for future immunomodulatory strategies to improve outcome of invasive fungal infections.

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CHAPTER 21

FUNGAL VACCINES AND VACCINATION: PROBLEMS AND PERSPECTIVES

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Abstract: Vaccines against human pathogenic fungi, a rather neglected medical need until few years ago, are now gaining steps in the public health priority scale. The awareness of the rising medical threat represented by the opportunistic fungal infections among the health care-associated infections, the advances in the knowledge of fungal pathogenicity and immune response and the extraordinary progress of biotechnology have generated enthusiasm and critical new tools for active and passive anti-fungal vaccination. The discovery that antibodies play a critical role for protection against fungal infection has greatly contributed to the advancements in this field, in recognition that almost all useful vaccines against viral and bacterial pathogens owe their protective efficacy to neutralizing, opsonizing or otherwise effective antibodies. Overall, there is more hope now than few years ago about the chances of generating and having approved by the regulatory authorities one or more antifungal vaccines, be active or passive, for use in humans in the next few years. In particular, the possibility of protecting against multiple opportunistic mycoses in immuno-depressed subjects with a single, well-defined glucan-conjugate vaccine eliciting directly anti-fungal antibodies may be an important step to achieve this public health goal

1. INTRODUCTION

The vaccines represent the most useful immunological application for human health. They are the only medical tools that, when used prophylactically, allow disease elimination or even eradication of the causative agent, as has happened with smallpox and is hopefully close to occur with poliomyelitis. When disease elimination is, for various reasons, bound to the habitat of the infecting organism and the natural history of infection, impossible or unlikely, yet the availability of a protective vaccine and effective vaccination procedures results into an effective control of the disease as witnessed for diseases such hepatitis B, measles and pertussis, just to cite only a few. To reach these goals in terms of public health, ranking

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from disease eradication to elimination and control, the availability of a safe and efficacious vaccine is obviously essential. However, critical factors are also the definition of the medical need of any given vaccine in terms of cost/benefit, the prospected advantage in relation to the existing preventive or therapeutic measures, the correct identification of the target population to vaccinate. The capability of the public health system to implement an effective vaccination policy must also be carefully considered. This includes, among others, the capacity to persuade healthy subjects or their parents or guardians in case of pediatric vaccines, of the personal and societal advantages of being vaccinated, weighting the minimal though actual risk of an adverse event against all future benefits of avoiding the diseases and its transmission to neighbours. Finally, of utmost importance is also establishing whether the disease could be contrasted not only with a preventive vaccination but also with the use of a therapeutic vaccine, i.e. a vaccine formulation that could be used in the ill subject. This is an exciting, very attractive approach which deserves particular attention in the field of fungal diseases. However, no therapeutic vaccine successfully used to fight infection has been so far provided.

All the elements above are balanced in the decision of undertaking vaccine manufacturing and establishing vaccination priority. This becomes particularly cogent in the case of diseases for which either the true incidence and impact on public health are unknown or the identification of the target population is problematic. Fungal diseases of humans are exactly a case in point. Besides few severe but geographically-limited and relatively low-incidence deep-seated infections such as, for instance, coccidiomycosis and blastomycosis, most other incident worldwide infections such as aspergillosis, cryptococcosis and candidiasis (in this last case, with the possible exception of the vaginal candidiasis) typically occur in the immunocompromised host, a clinical setting which raises remarkable obstacles to the rationale itself of the immunopreventive or even therapeutic vaccination. This is mostly due to difficulties in achieving an efficient and long-lasting immune response, in identifying who could really benefit of the vaccine, and establishing how and when exactly to vaccinate. Thus, it doesn't make a surprise that fungal vaccines have been unfairly placed in a rather remote room in the public health building, as witnessed by the practical absence of information on antifungal vaccines in one renowned, most famous textbook on Vaccines (Plotkin, and Orenstein, 1999), or in special editorial overviews,with only few lines of description even in the Jordan Report (2004) the most detailed state of art document annually released by the National Health Institutes.

This being said, there are several reasons and evidence gathered in the last years commanding the scale up of fungal vaccines for humans on a rather high position in the priority scale. Strong advocacy is the recent "call to arms of the immune system" launched by Stevens (2004) claiming for a vaccine against aspergillosis, a fungal disease which nobody was seriously thinking about a vaccine against until few years ago, as well as the upsurge of publications in the field of Candida vaccine (see Mochon and Cutler, 2005). It is my intention here, first, to discuss the evidence for the above command and, second, to illustrate some recent advances in the field

of fungal vaccines which makes it rather optimistic the achievement of at least a couple of effective vaccine in the next few years. I will finally focus on some novelties and conceptual advances coming from the area of fungal vaccines which could be applicable to vaccination against other human pathogens, as represented by the killer-toxin mimicking idiotypic vaccine and the ß-glucan conjugate vaccine.

Conversely, no attempt will be made here to cover all previously published findings in this area, and no details about the many different vaccine candidates and adjuvant proposed until now against the various fungal diseases will be given. Nonetheless, the most relevant information on proposed vaccine antigens and the respective references to the principal agents of fungal infections in humans are given in Table 1, while Table 2 offers indications about the most promising vaccine candidates against Candida, a rapidly growing field not only for the number of these antigens but also for the discovery of protective antibodies and their mechanism of protection (Mochon and Cutler, 2005). Moreover, mentions will be made throughout of some of the most recently proposed protective fungal antigens when their activity may account for a proposal about the immunology of protection. Finally,

Table 1. Major antigen components suggested as candidate vaccine against fungal infections (for Candidiasis, see Table 2)

Fungal disesases	Antigen	Some References
Cryptococcosis	Capsular GXM Mannoproteins Other antigens	Casadevall and Pirofski,2006; Maitta et al. 2004; Oscarsson et al. 2005
Pneumocystosis	Major surface glycopotein (gPa) p55 antigen Kexin	Smulian et al. 2000;Zheng et al. 2005
Histoplasmosis	Ribosomal proteins, Cell wall proteins, HSP60	Deepe 2004; Deepe et al. 2005;
Parococcidioidomycosis	43 Dal glycoprotein (gP43) and related multimeric peptides	Taborda et al. 2004
Blastomycosis	Blastomyces adhesin (BAD-1/or W 1–1)	Deepe et al. 2005; Wutric et al. 2005
Coccidioidomycosis	Whole cells (attenuaded or inactivated), Spherule and spherule outer wall extracts 27k-antigen Ag2/PRA Urease, HSP60	Cox and Magee, 2004; Tarcha et al. 2006.
Aspergillosis	Whole inactivated cells and crude extracts, Live attenuated conidia, Asp f 16 protease, KT-neutralizing antibody	Feldmesser, 2005; Stevens, 2004; Bozza et al. 2004

Table 2. Proposed Candida vaccines

Vaccine	Protection Mucosal; Systemic	Nature of protective immunity
Attenuated Candida strain (CA2)	−; ++	CMI, Th1
Beta −1-2 mannan	++; ++	Opsonic Abs
Ribosomal vaccine	?; +	Undefined
KT-mimicking, IdAb	++, ++	Fungicidal Abs
Inactivated whole cells (IWC)	?; +	Undefined, CMI?
Mannan-deprived IWC	?; ++	IgM Abs
Sap2	++; +?	IgG/IgA
HSP90	?; ++	Abs
P43 B cell mitogen	?; +	Abs
Ag-loaded Dendritic Cells	?; +	Th1 CMI

?: doubtful or not tested
−: virulent in the mucosal model
+: moderate protection; needs confirmation
++: strong protection
For References, see text.

no veterinary vaccines against dermatophytes will be dealt with here. For those who wish to go into more details in all the above aspects, I advise to read a number of excellent reviews and expert opinions published by leaders in this area (just to quote a few: Deepe, 1997 and 2004; Deepe et al. 2005; Casadevall et al., 2002; Cox and Magee, 2004; Feldmesser, 2005).

2. WHY FUNGAL VACCINES

2.1. Incidence of Fungal Diseases

The first reason supporting the need of generating a fungal vaccine is that, overall, the prevalence and incidence of fungal infections has markedly increased worldwide, with special concern for opportunistic agents of infection. With restriction to two most frequent among the latter such as Aspergillus and Candida spp reasonable estimates suggest that at least 5% of all hospital infections are caused by these two fungi, with a specifically attributable mortality between 30 and 40% of all cases. In Italy, the estimate incidence of nosocomial infections varies from 350,000 to 400,000 new cases per year. This means that, as a minimum, between 6'000 and 8,000 subjects each year die of invasive fungal infection, a figure much larger than the actual death rate for AIDS in the same country (Urciuoli et al. 2004). These figures are rather similar in all other industrialized countries where organ transplant, invasive surgery and the plethora of all other medical conditions predisposing to fungal infection are similar. In a recent survey, Australian investigators have reported an extra 15,000 cases of aspergillosis and candidiasis per year occurring in hospitalized patients in their country (Slavin et al. 2004). In some particular at risk subjects such as the bone marrow transplanted subjects, the mortality associate with

aspergillosis may reach a figure as high as greater than 50% (Sheppard and Edwards, 2004). A recent report from the Institute of Medicine in US has emphasized that nosocomial infections are a big concern- in that they are associated with thousands of deaths each year, leading to much longer hospital stays and of course tremendous cost increase. i.e. billions of dollars in additional hospital cost each year. For aspergillosis, Stevens (2004) has reported that nearly 2'000 deaths occurred in 1996 in US, with associated 176,300 hospital days and 633 millions USD in cost. Estimates for Candida incidence mostly derive from those diseases which can be more easily diagnosed such as bloodstream and mucosal infections, particularly candidal vaginitis. Overall, this fungus has become the fourth most common agent of bloodstream infections in carefully monitored surveillance programs, with close to 10% rate respect to all other agents of bloodstream infections, and rising incidence on non-albicans species, which are intrinsically refractory to one or more antifungals (Sims et al. 2005). This fungal agent has also become a very common urinary isolate, although in this case its etiological role is much less clear. On the other side, mucosal infections by Candida have long been known as being very common, not only in immunocompromized subjects but also in apparently normal ones. A specific case here is the vaginal thrush which, in its recurrent, chronic forms, affects a substantial percentage (3 to 5%) of all women who have suffered an initial acute attack of the disease (Fidel and Sobel, 1998). Most of these infections may irreversibly affect the quality of life since they require chronic, hardly compliable antimycotic treatment and with enhanced risk of acquiring antibiotic resistance.

The focus on the most common agents of opportunistic fungal infections worldwide should not be considered a disregard of other agents of systemic fungal infections which are endemic in one or other parts of the different continents. Examples here are blastomycosis, coccidioidomycosis and paracoccidioidomycosis for which the interest in developing a vaccine remains quite high, particularly for a vaccine against Coccidioides species, despite their geographic limitation and the limited number of subjects who get sick following infection by soil spores. The questions here are, on one side, an imperfect knowledge as to whether their incidence is rising or declining and, on the other side, the difficulties to establish treatments capable of eliminating any focus of dormant cells, potentially constituting a lifelong reactivation threat.

2.2. How Fungal Infections are Actually Treated

In contrast to most bacterial infections which can be effectively diagnosed and, despite the strong concerns about the spread of antibiotic resistance, may still be largely cured with antibiotics, fungal infections enjoy relatively few effective treatments and, for some of them, remarkable diagnostic difficulties. Some of the drugs used for combating these infections are also endowed with rather serious side-effects. The reasons for the historical paucity of efficacious antifungal treatments basically reside in the difficulties of finding suitable targets for selective drug toxicity in a microrganism which is eukaryotic as its host, coupled with some delay

by the pharmaceutical industries in investing for drugs with a limited market, as compared to bacterial and viral infections. While this gap has been partially filled in the last ten years, particularly with the novel class of echinocandins inhibiting glucan synthesis (Polak, 2003), it has also become apparent that antifungal drug resistance is not such a sporadic or episodic phenomenon as suspected until few years ago, and may rise in parallel with more widespread use of new drugs (Sanglard, 2002). The whole scenario wants either the availability of one or very few drugs, as for instance those active against some endemic mycoses, or the existence of multiple potential treatments but with the inevitable association of antibiotic resistance, as for instance in chronic mucosal candidiasis.

As mentioned above, opportunistic invasive infections by fungi also suffer from remarkable diagnostic difficulties. For instance, invasive candidiasis is diagnosed ante-mortem in less than 50% of the patients, a diagnostic insufficiency which makes the therapeutic treatment with antifungals both delayed and often inappropriate. All this truly constitutes a sound rationale for the development of immunological preventive or therapeutic approaches.

2.3. Vaccine Target

For endemic fungal infections which occur in otherwise healthy subjects and for which either the therapeutic options are limited or a trend toward antifungal drug resistance emerges, possibly associated with difficulties for a prompt, specific and sensitive diagnosis, the question of the vaccine target is simply answered: potentially all population groups in a defined geographic area with given characteristics, both genetic or occupational, placing them at risk of disease, in relation to the cost of any other non-specific intervention (Cox and Magee, 2004). This may also apply to other subjects which are at risk of developing fungal infections because of predisposing underlying conditions such as diabetes or invasive surgery or other local factors, but are not systemically immune-depressed to such an extent as to make vaccination unlikely to raise the correct protective immune response. Here the cost-benefit ratio is of the paramount importance as is the accurate information about the prevalence of the disease, both factors being unfortunately in most cases unknown.

Much more complex is the identification of the target population in subjects with deep immunodepression either of the natural immunity, as for instance, the neutropenic subjects undergoing conditional chemotherapy for and after bone marrow transplantation, or of the adaptive immunity, as for primary or acquired T cell deficiency as in AIDS. Clearly these subjects are unlikely to respond protectively to vaccination owing to the partial or total lack of immune-competence, rather, they may suffer from aggravation of the immunological disorder following the immunostimulation by vaccine antigens and adjuvants. Here a crucial question is the definition of risk criteria which are associated to the likelihood of becoming ill and identify exactly the population with higher risks which could justify the cost and the possible side-effects of a vaccination before becoming immunosuppressed. While the discussion is very active in this area, there are several indications

that a rather high number of subjects at risk could indeed benefit of a advance active immunization against Candida and Aspergillus These may also include, for instance, patients candidate to transplant and those affected by tumours the therapy of which predisposes to fungal infection (Stevens 2004; Sheppard et al. 2004). Moreover, opportunistic agents of diseases have low-penetrance virulence traits and the immune responses which contribute to their control are usually redundant and impinging on both natural and adaptive immunity. Thus, a vaccine which simply potentiates a residual setting of the immunity may nonetheless be beneficial. A particular case in point here is the observation that CD8 cell activation can replace CD4 in the induction of protection against histoplasmosis in a model of CD4 cell deficient mouse, as well as the report about the direct anticandidal and anticryptococcal activity of cytotoxic CD8 T cells (Deepe, 2004; Levitz et al. 1995). Clearly, the issue is here the knowledge of the type of immune responses which help the host to rid a transmissible agent or to control a commensal fungus. This is probably the most critical aspect in the definition of a priority for an antifungal vaccine aimed to combat opportunistic fungal infections.

2.4. Cell-Mediated and Antibody Responses: Which to Rely Upon for Vaccine-Induced Protection?

There has been a considerable debate on whether cell-mediated or antibody response is the protective arm of the antifungal immunity. Put in these terms, it is a false question, since there is no doubt that induction and fine regulation of CMI, particularly T-helper type 1 response, is a core factor in antifungal, and in general, antimicrobial response. Since an effective vaccine formulation requires induction and persistence of a protective memory response, CMI elicitation is a non-dispensable prerequisite for a valid vaccine. The question could rather be: are the cellular or the antibody effectors of immunity mostly involved in antifungal protection? These question remains of relevance for vaccination since the approach for generating a vaccine which must induce persistently activated cellular effectors, thus sustaining elevated activity of the pro-inflammatory Il-12-IFN-γ Th1 axis, may be quite different from the one which relies on B memory cells and antibody immunity, a fact which becomes still more important in partially or totally immunodepressed subjects. Thus, nature of immunizing antigen and its immunodominant epitopes, interaction with antigen presenting cells (mostly the dendritic cells) and processing through MHC class II or MHC class I pathway, and type of adjuvant, all determine the nature of elicited immunity and its outcome in terms of protection.

Most of the support for CMI effectors being the main arm of protection comes from clinical observations and well-defined animal models showing, for instance, that abolition of $CD4^{+}$ T cells or genetic knock-out of the Th1 cytokine pattern greatly enhances susceptibility to experimental infection by several fungi, both the dimorphic, endemic pathogens and the opportunistic ones. In addition, adoptive transfer of T cells from immunized animals has been shown to confer protection to naive counterpart in both mucosal and systemic models of infection (Romani,

2004; Santoni et al. 2002). In *C.albicans*, recombinant IFN-gamma is protective and expectedly, knock-out IFN-gamma animals are highly susceptible to infection (Romani, 2004). In humans, the situation varies with the nature of the specific infecting fungus. For instance, CMI defects, innate or acquired, predispose to severe forms of mucocutaneous but not systemic candidiasis or aspergillosis, which rather recognize neutropenia as the main predisposing condition. In contrast, cryptococcal meningo-encephalitis, pneumonia by *Pneumocystis carinii* and deep-seated infections by dimorphic fungi such as hystoplasmosis and coccidiodomycosis are clearly favoured by the CMI defects typical of AIDS subjects. Clearly, fungicidal neutrophils and macrophages may better do their job when activated by Th1 cytokines such as Interferon-gamma or type 1 Interferon and TNF-alfa. We have also mentioned above the few cases where a direct anti-fungal activity by cytotoxic effector T cells has been detected in ex-vivo experiments, though the in vivo relevance of these observations remains to be established. It should not be forgotten that type 1 cytokine response is critically requested for the formation of some, highly protective antibodies against protein and most polysaccharide antigens, (e.g. IgG2a), not differently from other Th2- dependent antibodies such as IgG1 and others. Thus, the observation that CMI deletion or modification enhances diseases is not per se a definitive proof that cellular effectors of the protection are eventually involved. Moreover, various antibodies have been generated which are evidently protective in the same animal models in which cellular CMI effectors are elicited and advocated to be responsible for protection (Casadevall et al. 2002), suggesting that CMI induction eventually regulated, or was accompanied by, generation of protective antibodies. Even in one of the most evident case for a critical role of CMI effectors in controlling the infection such as the coccidioidomycosis (Cox and Magee, 2004), a recent report on a candidate vaccine antigen describes the generation of potentially protective antibodies (Tarcha et al. 2005).

DNA vaccines are believed to be the strongest immunization approach for CD4 and CD8 cytotoxic effector generation, owing to preferential antigen processing through MHC class I pathway. Nonetheless, a DNA vaccine using the Pneumocystis gene coding for kexin, a furin-like protease, and CD40 ligand as adjuvant has been recently shown to generate anti-*P.carinii* protective antibodies both in CD4-depleted and CD4 repleted mice (Zheng et al. 2005). In another approach to a vaccine against pneumocystosis with the use of the cell wall-associated, glutamic acid-repeat-rich protein of 414 amino acids (p55), Smulian et al (2000) demonstrated that partial protection from rat pneumonia was accompanied by both CMI and antibody responses. Finally, antibodies against the major surface glycoprotein (gpA) of the above fungus were manifestly protective independently on the presence of T cells (Harmsen et al. 1995). Remarkably here, pneumocystosis clinically is a major example of disease caused by CD4 T cell deficiency as in AIDS.

Three other considerations would speak against CMI effector cells being uniquely or predominantly exploited for vaccine protection: 1. Maintaining a persistent-activation of CMI effectors, that is usually acquired by whole cell or DNA vaccines, while positively controlling the infectious agent, may be nonetheless inducing strong

inflammation with potential untoward effects. Classical is the Koch phenomenon in the case of tuberculosis and this may more easily happen with some endemic dimorphic fungi, like Coccidioides which show reactivation disease (Cox and Magee, 2004). This is so true that the immune system has evolved potent means to regulate inflammation and hyper-activation of CMI and its cellular effectors through regulatory cells and counteracting cytokines. 2. Practically all bacterial and viral vaccines which have been successfully used so far owe their protective effects to antibodies, particularly toxin-neutralizing antibodies (Plotkin and Orenstein, 1999). Vaccines conceived to stimulate CMI responses with cytotoxic effectors and the accompanying array of cito- and chemokines, thus mimicking what is considered to be the protective immune response, as in the cases of the HIV and the new anti-TB vaccines, are proving extremely difficult to be achieved, despite strong efforts and investments. Recently Deepe et al. (2005), while contending that T cells, not antibodies, are the chief mediators of protective immunity against blastomycosis and histoplasmosis, also emphasized the remarkable difficulties in generating this type of vaccines; 3.Theoretically, memory-bound antibodies can be induced by vaccination in at-risk subjects before they become immunosuppressed. Because of the relative longevity of IgG and IgA, their persistence at a good protective titer in serum and mucosal secretions even during a relatively prolonged immunosuppression period is more than likely. There are several examples that this approach may work, one of the last being the protection achieved against pneumocystosis in cortisone-treated rats following vaccination with the p55 antigen or vaccination with kexin and CD40 ligand, in a DNA format, in $CD4^{+}$-depleted mice (Smulian et al. 2000; Zheng et al. 2005) This is clearly not achievable with vaccines merely eliciting antifungal T cells and activating macrophages or neutrophils.

2.5. Do Antibodies Contribute to the Antifungal Protection?

If the role of CMI does not rule out antibody participating in antifungal protection, what is the "positive" evidence that antibodies do indeed have a role in this? The clinical evidence that antibodies are protective against fungal infections is rather limited to few cases (Mathews et al. 2003; Mathews and Burnie, 2004) but, in recent years, a rather strong evidence has been accumulated about the protective role of some antibodies in experimental models, some of which are close to the human disease and directly related to the use of various vaccine formulation (Tables 1 and 2). Also, thanks to the pioneering studies by Arturo Casadevall and his colleagues at Albert Einstein College of Medicine, in New York, there is now some convincing explanation of the difficulties in obtaining clinical evidence for antibody role in protection. Both for *C.neoformans* and, more recently, for *C.albicans*, this appears to be attributable to the existence of inhibitory antibodies, rather than the absence of protective ones (Casadevall, 1995; Torres et al. 2005; Bromuro et al. 2002) This situation is likely to be present in other fungal infections, both in the human- commensal or the environmental ones, and may explain the variable and inconsistent results obtained with many fungal whole cell vaccines. In turn,

the existence of inhibitory antibodies makes a new hurdle for the generation of a subunit vaccine based on antibody–mediated protection, in that it requires a critical definition and discrimination of an antigen preparation which does exclusively stimulate the production of protective antibodies, not only for their specificity but also for their isotype (Casadevall, 1995).

Meanwhile, the demonstration of protective antifungal antibodies has opened the way to the use of them as immunoprophylactic or immunotherapeutic agents against some fungi, i.e. to the feasibility of passive vaccination, an intervention which has several prospective advantages, thus deserving great attention in the area of fungal infections. Use of T cell lines and primed dendritic cells for adoptive vaccination remains the counterpart on the side of CMI-inducing vaccines (Bozza et al., 2004; Feldmesser, 2005).

3. PASSIVE VERSUS ACTIVE VACCINATION

Historically, therapy of infection with immune sera preceded the use of both vaccines and antibiotics. Indeed, therapeutic or prophylactic antisera against diphtheria, meningitis and pneumonia, just to cite few of them, have been used soon after, or even preceding, the discovery of the causative agent of infection. The practice of passive immunization was largely abandoned not because the sera were non protective, (they were highly protective, indeed) but simply because they were either non available in a sufficient quantity or too toxic, or even caused the induction of a sometimes lethal hypersensitivity reaction to foreign proteins (serum sickness). The entry of antibiotics into the scene also contributed to push serum therapy into a corner. Nonetheless, passive vaccination has remained a viable medical approach by the use of standard or hyperimmune human immunoglobulin preparations for both pre- and post-exposure prophylaxis of diseases such as viral hepatitis, measles, varicella, tetanus and rabies. Limited specificity and limited supply are of course major disadvantages of these preparations, together with the risk of transmitting to the recipients unrecognized or undetected infectious agents (virus, prions).

It is quite clear that the present-day recombinant DNA technology is going to substantially replace the foreign sera and human immunoglobulin preparations with highly-specific humanized or human antibodies, in a variety of different formats (Traggiai et al. 2004). This has already occurred in the field of tumor and chronic, autoimmune diseases, and is taking place also in the field of infection, though to a slower rate than necessary (see for instance, Beninati et al. 2000 and Zhang et al. 2006). There are several examples of recombinant antibodies against fungal infection, and one of them is in the regulatory approval track (Mycograb, Mathews and Burnie, 2003) while others are ready to enter that path. Interestingly, a number of them are devoid of Fc component, suggesting that they can work efficiently even in the absence of phagocytic effectors cells or complement. Other monoclonal antibodies against Candida do indeed need the Fc component and a pattern of complement activation and deposition on cell surface for protection (Casadevall et al. 2002; Mochon and Cutler, 2005). So far no consistent evidence of a therapeutic

effect of passive vaccination has been provided for endemic dimorphic infections caused by Histoplasma, Coccidioides and Blastomyces, agents of typical diseases for which cellular effectors activated by the Th1 cytokines are advocated for protection. However, initial findings about protective antibodies are emerging (Deepe, 2004; Tarcha et al. 2006).

The fact that therapeutic antibodies can be generated in a human format not requiring the presence of the Fc component has particularly important implications for passive vaccination against opportunistic fungi. In theory, these antibodies can work without the cooperation of the immune system, also in such heavily immunocompromized patients, as the leukopenic ones, i.e. in the true setting of the majority of patients with deep-seated fungal infections. Because of the quantity and costs inherent in a pure antibody approach, it is more likely that antibody therapy will be used to synergize with effective antimycotics, as suggested by Mathews and Burnie in the case of Mycograb. Rather striking examples of this application have been recently provided, indirectly indicating previously undisclosed capacity of some proteins to work as protective antigens in both systemic and mucosal infection models (for instance, Sap2 and MP65 of *C.albicans*; De Bernardis et al. 2006).

4. VACCINES AND ANTIBODIES

Having recognized that antibodies may be relevant for the control of fungal infections, thus possibly correlating with protective vaccination, a reflection is needed on the most desirable function that must have the vaccination-induced antibodies. It has already been mentioned that opsonization and complement deposition are considered of utmost importance for an effective protection, both in active and passive vaccination models. However, these antibodies require the presence of cellular effectors to fully exert their activity, a fact which can ultimately be a strong limitation in their function in immune-suppressed patients. Other antibodies have been generated following vaccination which owe their activity to the inhibition of some form of toxicity or enzyme activity (De Bernardis, 2002). Of high relevance are also other antibodies which counteract their cognate adhesins, well recognized virulence traits of several fungi (Calderone and Fonzi, 2002; Latgè and Calderone, 2002). Possibly through this inhibition, biofilm formation is affected, being biofilms critical factors for fungal growth and disease induction. That this mechanisms could be relevant for a vaccine expected to generate protective anti-fungal antibodies has recently been shown in *Candida albicans* where dual-targeting anti-adhesin domain antibodies, i.e the smallest (MW around 12,000), genetically-engineered antibody fragments containing the three complementary-determining,antigen-binding regions, exerted high level of protection both in mucosal and systemic rodent candidiasis, (De Bernardis et al. 2006). An indirect demonstration of the above mechanism has also been provided for *C.neoformans* where selected protective, but not non-protective antibodies, decreased biofilm formation in vitro (Martinez, 2005). Both in Candida and in Aspergillus, antibodies which inhibit fungal growth and even kill growing fungal cells have been described. Some of these antibodies have been

generated through vaccination with either a monoclonal antibody neutralizing a wide-spectrum, antimicrobial killer toxin and generating internal images of the toxin (the so called “idiotypic vaccination”: see Polonelli et al. 1998; Cassone et al. 1997) or by immunization with stress mannoproteins, as shown by Moragues et al. 2003. More recently, a glycoconjugate vaccine composed of beta-glucan molecule, laminarin, and a diphtheria toxoid CRM197) already used as carrier protein in other bacterial vaccines, has been shown to generate fungus growth inhibitory antibodies (Torosantucci et al. 2005). This last approach warrants some specific description not only for its original immunological mechanisms of protection, i.e. direct antifungal effect not apparently relying upon host immune cooperation, but also for their potential to represent multi-target antifungal vaccines with a single preparation (cross-species immunization). If further studies will confirm the supposed identification of the killer toxin receptor in a ß-glucan molecule, the idiotypic vaccination quoted above may fall within the same kind of approach (Cassone et al. 1997).

5. MULTITARGET ANTIFUNGAL VACCINES AND CROSS-SPECIES IMMUNIZATION

Usually, vaccines prepared to fight a given disease are made by the whole attenuated or inactivated causative microbial agent, or one or more of its immunodominant antigenic components (the so-called subunit vaccines, see Tables 1 and 2). In the nowadays very popular mixed or combined pediatric vaccines, which are aimed to immunize simultaneously against multiple diseases such as, for instance, tetanus, diphtheria, polio, pertussis and hepatitis B, a mixture of antigens from each causative agent is used. In few cases, the vaccine is composed of related, antigenically cross-reactive strain, belonging to the same bacterial or viral species, such as, for instance, in the case of the antituberculous BCG and the smallpox vaccines. To our knowledge, there is no example in the literature that a single defined antigen could be used to protect against very different pathogens, belonging to quite distant families or orders, and we are not aware of any previous use of an antigen from a phylum organism to immunize against diseases caused by microrganisms from another phylum This is somewhat surprising in view of the existence of conserved, highly immunogenic proteins (for instance, the HSPs) in many different pathogens, or compounds such as the peptidoglycans or the lipopolisaccharydes so widely shared among bacteria. In a way, an immunological dogma asks that vaccine specificity may be acquired only by the use of highly specific antigens, in a sort of opposing counterpart to natural immunity where non-specific, widely cross-reactive recognition is the rule – Lipolysaccharides and various glycans are indeed major stimulators of natural immunity (the so-called pathogen-associated microbial patterns, PAMP) through their binding to the family of Toll-like receptors TLRs and other receptors, such as, for instance, the Dectin-1 for fungal glucan (Brown and Gordon, 2005).

In a series of past and recent investigations aimed to find novel approaches to passive and active vaccination against human opportunistic fungi, we realized that

the above dogmatic specificity concept, contrasting natural with adaptive immunity, could be reversed by using a compound from another phylum organism to immunize against fungi. Thus, we proposed a beta-glucan constituent (laminarin) from the alga *Laminaria digitata* as a candidate antigen for a single vaccine potentially protecting against various, different fungal infections (Torosantucci et al. 2005). Importantly, our investigations also revealed that the algal, glucan-based vaccine elicited antibodies with a direct inhibitory activity against these pathogens, thus adding a critical advantageous requisite for vaccination of immunocompromized subjects. A convergent approach in the same, if not wider purpose, has been the use of a monoclonal antibody neutralizing a wide-spectrum antimicrobial killer toxin as immunizing antigen to raise anti-idiotypic antibodies mimicking the activity of the killer toxin on fungi (Polonelli et al. 1997). The two approaches might have shared a common component if it will be definitely proven that the anti-idiotypic, killer toxin-mimicking antibodies raised by the immunization with the killer toxin neutralizing monoclonal antibody recognize ß-glucan constituent as cognate antigen.

5.1. Beta-Glucan Constituents of Fungal Cell Wall

Beta-glucans are structurally complex glucose homopolymers, found in the cell wall of fungi, algae and bacteria (Stone 1992, Masuoka 2004). Their basic molecular structure is relatively homogeneous, although type of bonding, molecular mass and overall molecular configuration may be variable depending on the different microbial source (Bohn 1995). Biologically, they are well-known for their immunomodulatory and anti-tumor properties (Cassone 1987; Brown 2003; Masuoka 2004) but, to our knowledge, have never been considered as vaccine antigens but rather as immunomodulators or, more recently, as PAMP.

In the opportunistic fungal pathogen *Candida albicans*, β-glucans are major structural components, accounting for approximately 50–60% of cell wall dry weight. Based on different solubility in alkali and acid, Candida β-glucan has been differentiated into an alkali-soluble polymer of a relatively low molecular weight and a branched, acid-soluble molecule, both predominantly composed of β-(1 → 6)-linked residues, and into an alkali-acid insoluble, highly branched complex, containing grossly equivalent amounts of β-(1 → 6) and β-(1 → 3) linkages in a complex with chitin providing form and structural integrity to the fungal wall (Chattaway et al. 1968; Cassone 1991).

It is generally accepted that in this fungus glucans preferentially enriched with β-(1 → 6)- or β(1 → 3) linkages (possibly a family of distinct molecules, widely interconnected to each other) are differentially located and play distinct structural roles in cell wall architecture. Recent models of cell wall structure suggest that β-(1 → 3)-linked glucan molecules form a three-dimentional matrix surrounding the fungal cell. At the inside, close to the plasma membrane, this skeletal framework is strengthened by chitin chains, whereas, at its outer edge, β-(1 → 6) glucan moieties link GPI-anchored cell wall mannoproteins to the skeletal framework (Klis 2001) (A scheme of cell wall organization in *C.albicans* is shown in Figure 1,

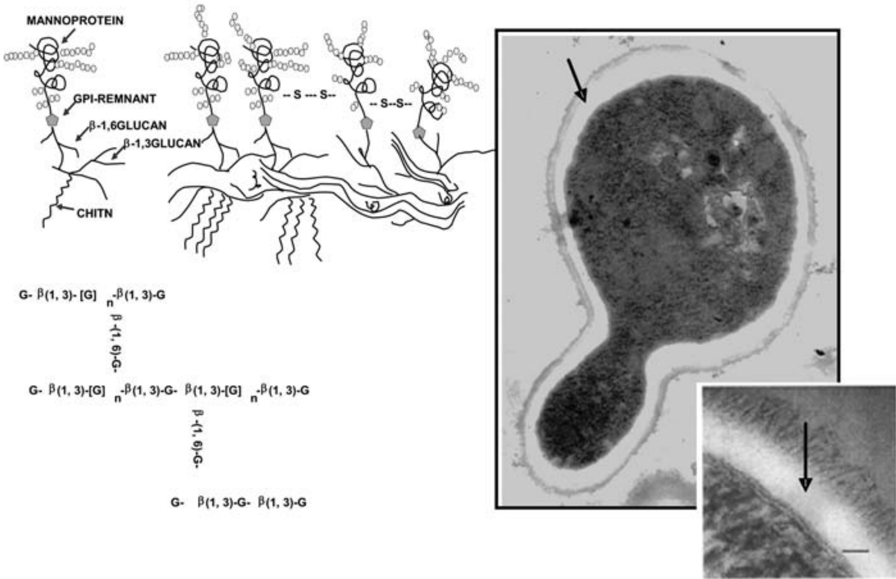

Figure 1. A shematic representation of the cell wall composition and structure in *C.albicans*. The arrows in the inset electron micrographs point to the inner layer of the cell wall where most of the glucan is present, some of it bound to chitin.(see however. Legend to Figure 2). The outermost fibrillar layer is considered to contain mostly mannoproteins. For references, see text

whereas Figure 2 shows the immuno-cytochemical detection of beta-glucan by the use of a specific murine monoclonal obtained by immunization with the Lam-CRM conjugate, see below).

While human pathogenic fungi contain both beta1–6 and beta 1–3 glucan, the expression and predominance of each of the two isomers is quite variable, depending on the fungus and its form of growth. In addition, beta-glucan can be replaced by alfa-glucan in some dimorphic fungi.

5.2. Protection against Candida and Aspergillus Conferred by a Glucan Vaccine

Glucans are per se very poor saccharide antigens, probably the "dullest" of all, as being constituted by a homopolymeric sequence of α-glucopyranosyl residues (Fig. 1). Very low, exclusively of IgM isotype, antibody levels in mice are achieved by even the most aggressive immunization schedules with pure, soluble or particulate glucans as antigens (Bromuro et al. 2000). However, as other polysaccharides, glucans may become strongly immunogenic when conjugated with a protein carrier. The findings of low-level,anti-β-glucan antibodies, in the serum of normal healthy human subjects probably is a consequence of natural exposure to glucan-protein

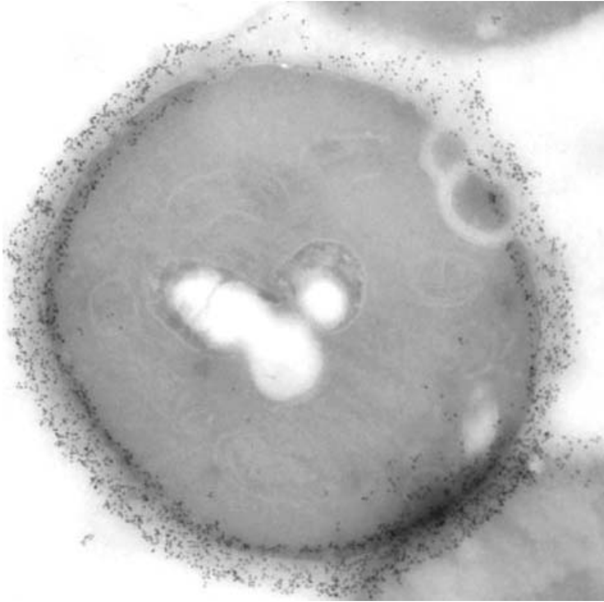

Figure 2. Immunogold labelling of cell wall glucan in *Canida albicans* through the use of mAb1E12 which detects, with different affinity, both ß1–3 and 1–6 glucan configuration. Note that glucan miolecules are also present on cell wall surface. For further details, see the text and Ref. Torosantucci et al. 2005

complexes as those found in the cell wall of many fungi (Chaffin et al. 1998). Previous evidence indicated to us that a vaccine composed by intact Candida or Saccharomyces cells treated to expose glucan rather mannan on cell surface conferred a substantial degree of protection, and that anti-ß-glucan antibodies could have been involved in the protection (Bromuro et al. 2000) For all said above, we considered that a vaccine based on a glycoconjugate between a ß-glucan molecule and a carrier protein could allow simultaneous immunization and protection against a variety of pathogenic fungi. To test the strength of this cross-immunization or even transphyletic vaccination we elected to use an algal, laminarin, rather than glucan extracted from Candida or other fungi, also to avoid possible contamination with other immunodominant fungal antigens, e.g. mannoproteins. As a carrier protein, CRM197, a genetically-detoxified diphtheria toxoid, already safely used in other current vaccines was selected. Shortly, this novel glycoconjugate met all expectations in terms of immunogenicity and protection from both mucosal and systemic candidiasis in rodents, as well as systemic aspergillosis in mice. The protection was clearly due to anti-ß-glucan antibodies which bound preferentially to the growing hyphal cell wall, both in *C.albicans* and in *Aspergillus fumigatus*. Moreover, a IgG2b monoclonal antibody, raised in vaccinated mice and recognizing at high affinity ß1–3 glucan configuration, mimicked the protective effect of the immune sera, and also bound to the hyphal cells.(Torosantucci et al., 2005). Figures 3 and 4 exemplify

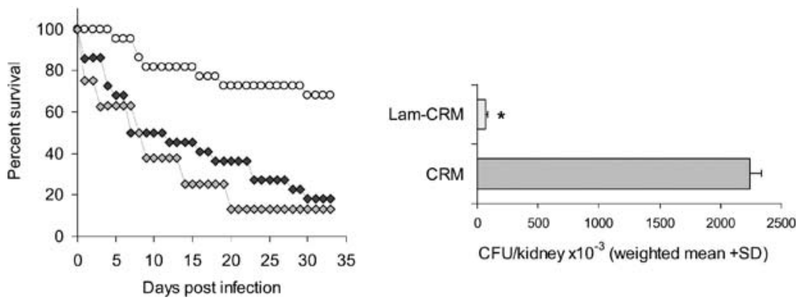

Figure 3. Protection induced by vaccination with the Lam-CRM conjugate as shown by Kaplan-Meyer survival curves (left panel) and Candida CFU counts in the mouse kidney (right panel). In the left panel,. the grey and blue dots refer to mice injected adjuvant or CRM protein, respectively, while in the right panel the grey color represent CFU of CRM-alone injected animals. In both panels, the yellow colours represent the values associated with Lam-CRM conjugate immuniozation. For further details, see Torosantucci et al. 2005

some of the immunogenic and protective activities induced by the vaccine. In particular, Figures 2 and 4 show that, in contrast with the common belief, ß-glucan molecules are present also on the cell surface of both Candida and Aspergillus, at least on the hyphal cells of these fungi, an observation in part matching the recent report of Dectin-1, a major glucan receptor, binding to cell surface of *C.albicans* (Gartner et al. 2005).

5.3. What anti- β-Glucan Antibodies do for Protection

β-glucan constituents are therefore present on fungal cell surfaces, particularly on growing hyphae, thus they are accessible to antibodies, which can opsonize the cells and facilitate complement deposition, a process whose importance for protection is quite obvious and has been highlighted in several studies (Casadevall et al. 2004). Antibodies to cell surface components of *C.albicans* have been shown to favour both intracellular and extracellular killing of the fungus and, quite recently anti-ß_-glucan antibodies have been shown to increase the candidacidal activity of macrophages in vitro. Antibodies binding the hyphal form of growth could also perturb adherence and tissue invasion, as has been demonstrated recently with a mAb directed against a stress-mannoprotein of *C.albicans* (Moragues et al. 2003).

While all above may be contributory factors, we believe that other properties of these antibodies may be truly relevant for the fungal cross-species protection conferred by the vaccine.

5.4. Hyphal Growth-Inhibitory Antibodies

In addition to the mechanisms already discussed, our data suggest that an additional mechanism for protection by anti- ß-glucan antibodies could operate in vivo. In

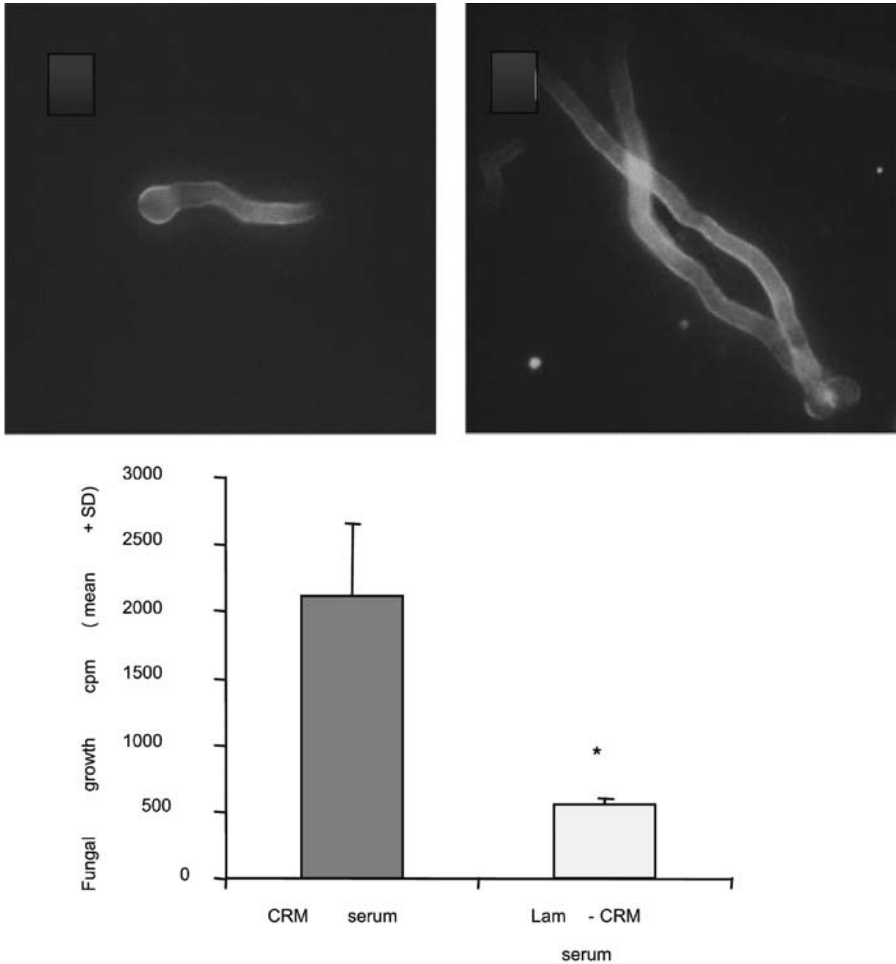

Figure 4. Binding (top panels) and in vitro hyphal growth inhibition (bottom panel) caused in *A.fumigatus* by incubation with immuneserum of mice vaccinated with the Lam-CRM conjugate. For further details, see Torosantucci et al. 2005

fact, the immune serum from vaccinated mice exerted a marked inhibition of Candida (and Aspergillus) hyphal growth in vitro, an effect that was also exerted by the affinity-purified IgG-rich fraction and that is in keeping with the preferential antibody binding to the hyphae. Together with previous data obtained with other yeast killer toxin–mimicking, anti-idiotypic antibodies our findings suggest that certain anti- ß-glucan antibodies may be endowed with direct inhibitory activity on fungi through some sort of interaction with such viability-critical molecules, which possibly include the oligosaccharide of nascent chains bound to the transg-lycosidases and glucan synthases.In accord with this hypothesis, recent data in our

laboratory have shown that the immune serum from Lam-Crm-vaccinated mice is able to inhibit certain stages of cell wall regeneration from protoplasts of *C.albicans*, coincident with beta-glucan deposition (unpublished data). The observation that these antibodies inhibit hyphal growth, possibly through inhibition of one critical component of cell wall machinery is of particular interest since, at least in *Candida albicans*, but probably also in Aspergillus (Stevens, 2004) hyphae appear to carry the main virulence traits, such as the adhesins and proteases, which contribute to fungus pathogenicity. In this context, these antibodies would work as a sort of cell wall inhibitory "antibiotics". In addition, the hyphae have been repeatedly shown to confer immunoevasion properties to the fungus, probably by activating the TLR2-mediated signaling pattern contrasting, through IL-10 production, the Th1 protective cytokine axis (see above). ß-glucan has been reported to activate this TLR2-dependent pattern through binding to dectin-1 receptor and, interestingly, in terms of immunoevasion, hyphae do not bind dectin-1 receptor (Gartner et al. 2005). Thus, in theory, anti-β-glucan antibodies may also exert protection by neutralizing the above signalling mechanism and shifting the cytokine profile toward the protective Th1 pattern, an observation already made, with other antibodies, in experimental cryptococcosis (Casadevall et al. 2004). Overall, anti-ß-glucan antibodies could be particularly protective by the expression altogether of their typical properties of immunomodulatory pro-defence components associated with some peculiar inhibitory properties featuring a sort of antibiotic action. Whatever the mechanism, this novel approach to a cross-immunizing vaccine may open the way to vaccination against at least some of the major opportunistic fungal agents of highly prevalent and incident diseases. This mostly whether it could be shown in future studies that the antibodies raised by this vaccine are still present in sufficient titer during immunosuppressive therapy and do not cause unbalance in the microbial flora and other untoward effects owing to their wide specificity. Our approach also provides for a novel vaccine which could be used to raise human or humanized antibodies for passive immunization, an approach which is now ongoing in our laboratories, and the outcome of which necessarily requires an epitope dissection and the precise identification of the cognate antigens within the beta1–3 and beta1.6 glucan molecules.

6. CONCLUDING REMARKS

Previously neglected vaccines such as the antifungal ones are gaining steps in the public health priority scale. The increased awareness of the medical threat represented by fungal infections, the advances in the knowledge of how fungi cause disease and which immune response may keep them at bay, together with improved biotechnological approaches to candidate vaccine antigens and engineered antibodies have offered critical new tools to anti-fungal vaccine generation. Fungal vaccines may also benefit of the clearly increased advocacy by the public and private sectors of the theory and practice of vaccination with its unrivalled risk- and cost-benefit ratios. All this has brought into the field increased enthusiasm and

commitment. The discovery that antibodies may play a critical role for protection is also giving strong impetus to the field of passive vaccination, which may eventually prove to be the first vaccine application against fungal infections, also helped by the spectacular advances in the generation of human and humanized monoclonal antibodies and various technological fragments of them. Research on active and passive vaccination against fungi is also offering novel ideas and some innovative approaches to the other fields of vaccine research (Casadevall and Pirofski, 2006). Clearly, there is more hope now than few years ago about the chances of generating and getting approved by the regulatory authorities one or more antifungal vaccines, be active or passive, for use in humans in the next few years.

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