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Crossroads Between Innate and Adaptive Immunity IV

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Crossroads Between Innate and Adaptive Immunity IV

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Preface

The reviews included in this volume are based on the work presented at the Aegean Conference "4th Crossroads between innate and adaptive immunity," which occurred between September 11 and September 16, 2011, at the Royal Myconian Conference Center in Elia, Mykonos, Greece. This conference was the fourth in the series and brought together an international group of experts whose research focuses on the mechanisms by which innate and adaptive immunity is mounted in the host and the cellular and signaling pathways that lead to effective immunity. The conference included sessions dedicated to host recognition of and response to pathogens, innate immune responses, antigen presentation, and adaptive immunity. The goal of this conference was to foster scientific exchange between these immunological fields, and promote discussion on the interactions between innate and adaptive immunity during host responses. The "4th Crossroads between Innate and Adaptive Immunity" Aegean Conference, with its highly interactive program, succeeded in bringing closely together scientists from around the world to discuss and establish collaborations in critical areas of innate and adaptive immunity.

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Vita-PAMPs: Signatures of Microbial Viability

1

Diego Mourao-Sa, Soumit Roy, and J. Magarian Blander

Abstract

Can the innate immune system detect and respond to microbial viability? Using bacteria as a model, we found that indeed the very essence of microbial infectivity, viability itself, can be detected, and notably, in the absence of the activity of virulence factors. The microbial molecule that serves as the signature of viability is bacterial messenger RNA (mRNA), common to all bacteria, and without which bacteria cannot survive. Prokaryotic mRNAs also differ from eukaryotic mRNAs in several ways, and as such, these features all fulfill the criteria, and more, for a pathogen-associated molecular pattern (PAMP) as originally proposed by Charles Janeway. Because these mRNAs are lost from dead bacteria, they belong to a special class of PAMPs, which we call vita-PAMPs. Here we discuss the possible receptors and pathways involved in the detection of bacterial mRNAs, and thus microbial viability. We also consider examples of vita-PAMPs other than bacterial mRNA.

Keywords

Pattern recognition • Vaccine • Bacterial messenger RNA • Virulence factors • Toll-like receptor • Inflammasome • Type I interferon

1.1 Introduction

As proposed by Charles A. Janeway Jr. in his seminal paper, which appeared in the Cold Spring Harbor Symposia on Quantitative Biology in 1989, activation of adaptive immunity is greatly dependent on the prior activation of innate immune antigen-presenting cells (APC) [1].

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Activation of APC would be achieved by engagement of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) present on pathogens and absent in the host [1]. Proof of this insightful conceptualization came with the identification of the first mammalian Toll-like receptor (TLR) which linked the innate and adaptive immune systems [2], a finding that paved the way for identification of several families of PRRs [3]. Subsequent detailed understanding of the mechanism of action of PRRs greatly progressed the field of immunology, and provided new ideas towards exploiting the innate immune system for vaccine development.

Standard vaccination follows the traditional principle of administering live attenuated microorganisms, dead microorganisms, or antigens administered in conjunction with adjuvants, in order to elicit immunity towards the desired pathogen. Adjuvants are necessary for the induction of adaptive immunity, and TLRs and other PRRs are often the principal targets of adjuvants. Live and dead vaccines are natural sources of antigens and adjuvants, and because of this there is little need for exogenous adjuvants in these vaccine formulations. An intriguing and long-known aspect of vaccination lies in the fact that live attenuated vaccines, such as polio, smallpox, tuberculosis, or typhoid vaccines, induce robust and long-lasting immunity compared to vaccine formulations that use dead pathogens or pathogen-extracted antigens together with adjuvants. Importantly, live attenuated vaccines have been very successful in eradicating smallpox and have greatly controlled human poliomyelitis, tuberculosis, and typhoid fever. This phenomenon suggests the existence of immune mechanisms for detecting pathogen viability and inducing a protective immune response. Here we discuss the effects of immunization with live and dead vaccines on development of immune responses, the different means by which microbes signal viability, and the manner that the immune system may recognize microbial viability.

1.2 Live Versus Dead Pathogens: Role of Virulence Factors

Accumulating evidence indicates that live pathogens elicit more robust adaptive immune responses compared to their killed counterparts [4–6], and these responses are both protective and durable. In light of our current knowledge of the immune system, these findings suggest that live and dead pathogens are fundamentally different in their ability to induce innate and subsequent adaptive immune responses. Nevertheless, the ability of live pathogens to proliferate inside hosts, therefore increasing the amount of PAMPs and antigens, could easily explain the increased immune response observed, transforming the differences in the immune activation to quantitative rather than qualitative differences. To solve this matter of pathogen proliferation and increased immune responses, Brockstedt and colleagues designed a metabolically active, but growth-impaired, attenuated form of *Listeria monocytogenes* (also called KBMA which stands for "killed but metabolically active") by treatment with psoralen and ultraviolet light [7, 8]. This mutant *L. monocytogenes* was deleted in one virulence factor, the ActA protein (*ΔActA*) that allows actin polymerization, thus cytosolic movement and cell-to-cell spread by the bacteria. Interestingly, KBMA *L. monocytogenes* induced stronger adaptive immune responses that were efficient and long lasting in a vaccination model [7]. These findings unambiguously demonstrated that the distinct ability of live and dead pathogens to induce immune responses lies in their differential capacity to activate the immune system, and not in the amount of signal that live pathogens provide. However, the activity of virulence factors in this case could not be excluded from accounting for the robust immune response. KBMA *ΔActA L. monocytogenes* still expressed a second virulence factor (LLO—listeriolysin O), which allowed their escape out of phagolysosome into the cytosol where they could engage cytosolic PRRs [7, 9]. However,

LLO-deficient *L. monocytogenes* can still generate protective immunity [10]. Thus, while one explanation for the differential immune responses induced by live and killed pathogens is the direct or indirect detection of virulence factors and pathogenicity [11], preservation of viability together with a complete absence of virulence factor activity may still be sufficient to induce a robust immune response. Careful investigations are still needed to address this question. A definitive answer would obviously have tremendous benefits for designing safe and effective vaccine formulations.

1.3 Microbial Viability Detection: Growth and Metabolism

As discussed above, the immune system responds more effectively and robustly to live microbes than to dead ones [4–7] in a manner that is largely considered to be due to the ability of live microbes to proliferate and express virulence factors [11, 12]. We reasoned that besides the ability of the immune system to detect microbial structures and virulence, it could additionally sense a more primordial signal, microbial viability itself. By studying immune responses to bacterial viability we found that bacterial mRNA is present only in live bacteria and can act as a distinct PAMP. We proposed the name of "vita-PAMPs" for a unique classes of PAMPs that are present only in live microbes and signal microbial viability to the immune system [13].

One method to detect microbial viability is to detect the ability of a microbe to grow. In this line, maybe the first microbial derived molecule that we could classify as a vita-PAMP is tracheal cytotoxin (TCT), a disaccharide-tetrapeptide monomer of peptidoglycan, present in the cell walls of all Gram-negative bacteria [14]. Despite the presence of TCT in the cell walls of these bacteria, it may serve to signal microbial viability as it is only released during active growth of *Bordetella pertussis* and *Neisseria gonorrhoeae*, and is destroyed during bacteriolysis [14]. TCT was shown to elicit innate responses, such as nitric oxide and IL-1α production by tracheal epithelial cells [15] and TNF-α, IL-6, KC, and IL-1β by macrophages [16]. Therefore, detection of microbial growth by the recognition of TCT induces innate responses that activate and prepare cells for future elimination of the microbe.

Another way that microbial viability can be detected is by sensing microbial metabolism. Several microbial components have been proposed such as bacterial pyrophosphates [17], quorum-sensing molecules [18], and bacterial second messengers such as cyclic-di-GMP [19, 20] and cyclic-di-AMP [21]. We will discuss these in a bit more detail. A common metabolic pathway in bacteria is the synthesis of isoprenoids, which are involved in a variety of functions such as electron transport and carbohydrate carrier function for peptidoglycan synthesis [22]. In bacteria, isoprenoid synthesis can be achieved either by the 2C-methyl-D-erythritol 4-phosphate (or MEP pathway) or by the classical mevalonate pathway, whilst animals exclusively use the classical mevalonate pathway [22]. Therefore, host detection of metabolites from the MEP pathway might signal the presence of metabolically active nonself microbes. Notably, metabolites of the MEP pathway could activate the immune system while metabolites derived from bacteria using the classical mevalonate pathway fail to do so (reviewed in ref. [23]). *Escherichia coli* utilize the MEP pathway, and the (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate metabolite, which is absent in species that use the classical mevalonate pathway, was described as a very potent activator of γδ T cells [17]. Considering the location of γδ T cells in epithelial surfaces, early detection of bacterial metabolic pathways might constitute an immune surveillance mechanism to prevent infection.

Quorum-sensing autoinducer molecules, such as homoserine lactones from *Pseudomonas aeruginosa*, play an important role in signaling to biofilm formation and virulence factor induction to neighboring bacteria [24]. The production of *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) by *P. aeruginosa* is sensed by the immune system and induces chemotaxis of human neutrophils [18]. The immune detection of this quorum-sensing metabolism of *P. aeruginosa*

might contribute to prevention of biofilm formation by early recruitment of neutrophils to the site.

Another way through which bacteria induce biofilm formation and virulence factor expression is by signaling via cyclic-di-nucleotides as second messenger. Interestingly, cyclic-di-nucleotides are used by most bacteria and not by eukaryotes or Archaea (reviewed in ref. [25]). Cyclic-di-GMP and cyclic-di-AMP were described as inducers of interferon (IFN)-β responses when delivered to the cytosol of macrophages [19–21]. Additionally, cyclic-di-GMP was recently shown to bind and activate STING, a signaling adaptor, to induce TBK1-IRF3 signaling for IFN-β induction [26].

1.4 Detection of Microbial Viability Independently of Microbial Growth

To determine the role of microbial viability in induction of immune responses without the compounding consequences of virulence factors and microbial growth, we studied the immune response to nonpathogenic thymidine auxotrophs of *E. coli* (*ThyA*$^-$ *E. coli*). Comparison of macrophage and dendritic cell (DC) responses to *ThyA*$^-$ *E. coli* indicated that live, but not killed, bacteria induced increased secretion of IFN-β and IL-1β, as well as the induction of pyroptosis (a caspase-1-dependent inflammatory cell death) [13]. Moreover, live bacteria in comparison to dead ones induced increased serum antibody titers in a vaccination model [13].

Comparison of "molecular patterns" in killed and live bacteria indicated that bacterial RNA is readily lost from bacteria after killing by different methods [13]. Analysis of RNA species indicated that the bacterial mRNA was required for the subsequent innate immune responses. The unique set of immune responses triggered by live bacteria (described above) was dependent on bacterial mRNA, as dead *E. coli* reconstituted with bacterial mRNA recapitulated the responses to live *E. coli* [13].

Detection of bacterial mRNA is a direct indication that the bacteria encountered are metabolically active, transcribing their genes and translating them into proteins, processes that are necessarily linked to viability. These observations prompted us to classify bacterial mRNA as a vita-PAMP.

1.5 Features of Bacterial mRNAs

Bacterial mRNA lacks a 7-methyl guanosine cap at the 5′ end and has exposed 5′ triphosphate groups (PPP), a feature resembling viral RNA which is sensed by the PRR RIG-I [27, 28]. Interestingly, features of the bacterial mRNA that elicit the immune responses described above did not appear to revolve around the 5′ end of the molecule because removal of the 5′-PPP moiety or capping with 7-methyl guanosine did not impair the elicited immune response [13].

More detailed characterization of the RNA was carried out by generation of in vitro transcribed RNA from the *E. coli* Gro operon, which encodes stress proteins induced once bacteria are phagocytosed [29]. Reconstitution of killed *E. coli* with single- or double-stranded in vitro transcribed RNA induced similar pyroptosis and IL-1β and IFN-β responses. Additionally, killed *E. coli* reconstituted with *E. coli* Era GTPase, DNA polymerase III, or Gro RNA transcripts equally stimulated IL-1β production and pyroptosis suggesting a degree of independence from a requirement for specific nucleotide sequences. Although single- and double-stranded RNA molecules showed similar effects, prediction analysis of single-stranded RNA structure revealed several areas of intramolecular base pairing and secondary structure formation that may influence RNA recognition and innate responses even by the so-called single-stranded RNA [13].

Notably, eukaryotic mRNA was not able to replace bacterial mRNA in the induction of innate immune responses, an observation that encouraged us to analyze further the immunostimulatory characteristics of the bacterial mRNA. Lack of poly(A) tails (polyadenylation at the 3′ end) in the *E. coli* mRNA was a feature required for induction of IFN-β, IL-1β, and pyroptosis by macrophages and DCs [13]. Bacterial mRNA could indeed serve as a vita-PAMP as such since the lack of a poly(A) tail is by and large not observed in eukaryotic mRNAs, with the exception of the

replication-dependent histone mRNAs H1, H2A, H2B, H3, and H4 (reviewed in ref. [30]).

1.6 Bacterial mRNA and Innate Immunity

Vita-PAMPs are only observed in live microbes, which share a plethora of other PAMPs with dead microbes. Therefore, identification of unique signaling outcomes is of great importance in pinpointing the action of the vita-PAMP itself. Investigation of signaling pathways indicated that bacterial mRNA did not appear to affect NF-κB activation, as live and dead *E. coli* similarly induced IκBα degradation, and transcription of IL-6 and IL-1β was similar [13]. Bacterial mRNA derived from live bacteria did, however, differentially activate IRF-3 and caspase-1. Activation of IRF-3 elicits increased IFN-β and is mediated by the kinase TBK1, which is a signaling molecule shared by distinct PRRs, such as STING, RIG-I, MDA5, TLR4, and TLR3 [3]. Other PRRs, such as TLR7 and TLR9, also induce increased type I IFN but use IRF-7 instead. Caspase-1 activation, on the other hand, is mediated by a protein complex called the inflammasome, which is in part controlled by members of the NOD-like receptor (NLR) family of PRRs. Inflammasome activation induces the posttranslational cleavage of the cytokines IL-1β and IL-18 allowing their secretion, in addition to the induction of pyroptosis [31]. Bacterial mRNA induced inflammasome activation via NLRP3, a NLR family member involved in responses against different stimuli that induce phagosomal leakage, ATP-mediated K^+ efflux, and generation of reactive oxygen species (ROS) [31]. Importantly, detection of microbial viability triggered inflammasome activation independently of ATP-mediated K^+ efflux, and comparable levels of ROS and phagosomal leakage were observed in response to dead and live *ThyA*$^-$ *E. coli* [13]. The lack of difference in the induction of pro-IL-1β between live and dead bacteria indicates NLPR3 inflammasome activation might occur by a yet unappreciated mechanism that orchestrates sensing of bacterial mRNA in the cytosol (more on this below).

Analysis of upstream signaling pathway components involved in the detection of microbial viability indicated that the TLR signaling adaptors, MyD88 and TRIF, play distinct and important roles. Signaling via MyD88 was non-redundantly required for NF-κB-dependent activation and induction of IL-1β mRNA; thus it was indispensable for the generation of pro-IL-1β which is one of the substrates of the inflammasome complex. Intriguingly, TRIF signaling was dispensable for induction of IL-1β mRNA, but played a central role in orchestrating the activation of IRF3 and the NLRP3 inflammasome. Therefore, TRIF was essential for the production of IFN-β, secretion of active IL-1β, and induction of pyroptosis [13]. The way that TRIF mediates NLRP3 inflammasome activation remains largely unknown; nevertheless, transcriptional events and/or post-translational events might play important roles in this process. Given the crucial role of TRIF in IRF3 activation, there is a possibility that this pathway may control transcription of a crucial gene required for inflammasome assembly and activation. Additionally, TRIF could possibly control post-translational modifications, such as phosphorylation, of inflammasome components that could subsequently regulate their activity (see Fig. 1.1).

1.7 Vita-PAMPs and Adaptive Immunity

As described above, live bacteria compared to killed ones induce increased innate immune responses, which also influence the quality of the ensuing humoral immune response. In a mouse vaccination model, live *ThyA*$^-$ *E. coli* or killed *ThyA*$^-$ *E. coli* reconstituted with bacterial RNA, when compared to killed *ThyA*$^-$ *E. coli*, induced increased serum antibody titers of IgG1, IgG2b, IgG2c, and IgG3, but not IgM [13]. This differential induction of IgG and not IgM is indicative of increased class-switch recombination in response to live bacteria. Furthermore, immunoglobulin class-switch is efficiently mediated by $CD4^+$ T cells, which further suggests that the increased immunity mediated by bacterial mRNA impacts different steps of the adaptive immune response [13].

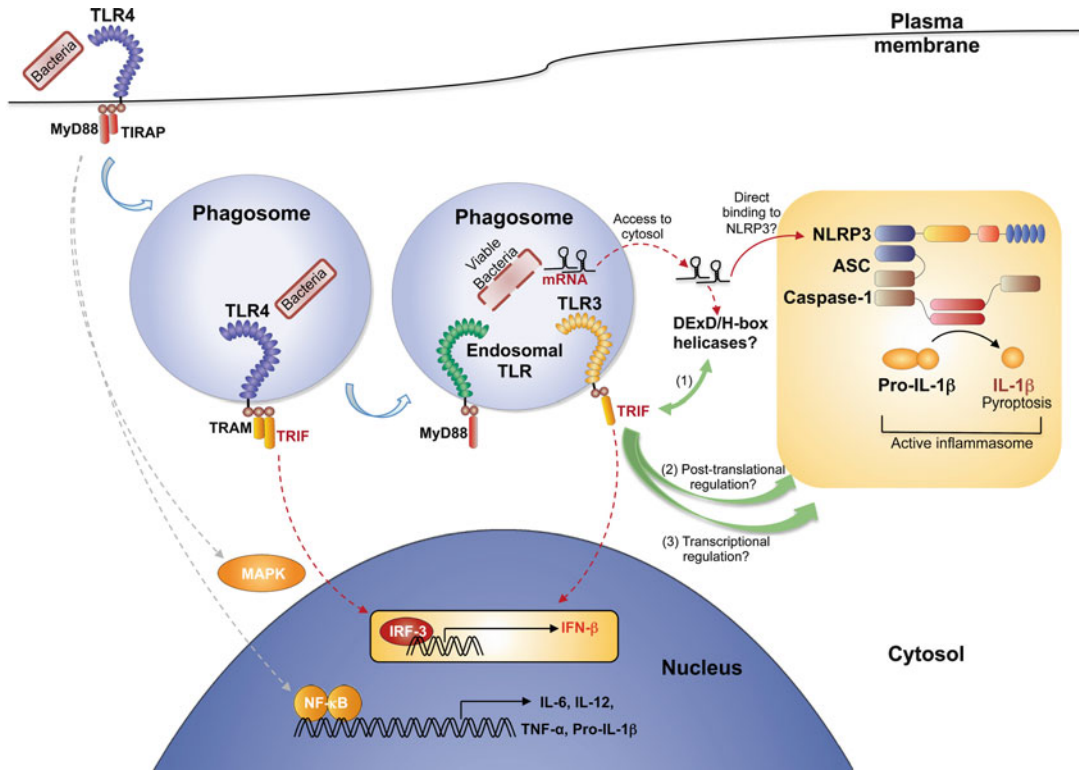

Fig. 1.1 Model of innate immune receptor engagement and signaling upon detection of viable and avirulent Gram-negative bacteria. Gram-negative bacterial cell wall components can engage surface TLR4 inducing TIRAP-MyD88 signaling that culminates in MAPK and NF-κB activation and expression of several inflammatory genes such as IL-6, IL-12, TNF-α, and pro-IL-1β. Upon phagocytosis of bacteria, trafficking of TLR4 to phagosomes allows signal transduction via TRAM-TRIF leading to IRF3 activation and type I interferon (IFN-β) production. Hydrolytic enzymes within the phagolysosome help degrade bacteria, further exposing bacterial components for immune detection, such as DNA, or in the case of viable bacteria, RNA (a vita-PAMP). This may allow additional TRIF signaling via TLR3 leading to augmented IRF3 activation. Phagosomal leakage, regardless of the viability of phagocytosed bacteria, allows a second phase of recognition in the cytosol. This cytosolic phase of detecting viable bacteria occurs by activation of the NLRP3 inflammasome, through direct or indirect recognition of bacterial mRNA, leading to the induction of pyroptosis and IL-1β secretion. Three additional TRIF-dependent events, indicated by *green arrows*, may also occur: (1) bacterial mRNA might engage DExD/H-box helicases (such as DDX1-DDX21-DHX36 complex) that couples to TRIF to further increase IRF3 activation, (2) post-translational modifications, which may regulate inflammasome assembly and activation, and (3) increased transcription of genes that encode critical components of the inflammasome, other than NLRP3. Innate responses unique to the detection of viable Gram-negative bacteria are indicated in *yellow boxes*

1.8 PRRs That Orchestrate the Response to Bacterial Viability

The ability of the immune system to detect microbial viability indicates the presence of cognate receptors, which sense vita-PAMPs and signal to augment the immune response. Analysis of innate signaling indicated cross-talk among different PRRs in the response to bacterial mRNA. As discussed above, MyD88 was required for the induction of pro-IL-1β, and TRIF was required for IFN-β induction and activation of the NLRP3 inflammasome, which was necessary for IL-1β secretion and pyroptosis [13]. Dependence on MyD88 is likely due to engagement of a surface TLR, such as TLR4,

which recognizes the abundance of LPS on the surface of both live and dead Gram-negative bacteria. This presumed TLR4 signaling would allow NF-κB activation and induction of pro-IL-1β, and could also engage TRIF signaling. However, we believe that the phagosomal presence of mRNA derived from live bacteria might also engage additional TRIF signaling downstream of endosomal TLR3, leading to the increased levels of IFN-β made uniquely in response to live *E. coli*. The leakage of phagosomal contents observed upon phagocytosis of both live and dead bacteria suggests that similarly, inflammasome activation may occur only when bacterial mRNA is present. This can indeed occur when bacterial RNA is directly delivered into the cytosol using lipofection, as previously described by Gabriel Nunez and colleagues [32] (see Fig. 1.1). Once in the cytosol, bacterial mRNA may trigger additional receptors other than NLRP3. Cytosolic delivery of a synthetic double-stranded RNA (poly I:C) was recently described to engage a DDX1-DDX21-DHX36 helicase complex that couples to TRIF signaling and induces IFN-β [33]. Accordingly, the cytosolic presence of bacterial mRNA might engage this or equivalent complexes in the cytosol, which would trigger TRIF signaling and IFN-β production (see Fig. 1.1).

1.9 Conclusions

Basic and applied immunology has greatly developed in the past 20 years in great part due to the understanding of the basics of adaptive immune response activation by the innate immune system. These studies, influenced and driven by Charles Janeway's original hypothesis [1], led to an understanding of how adjuvants could critically modulate the adaptive response. The finding that microbial viability, independent of microbial growth or virulence factor expression, plays important roles in the induction of unique immune responses, opens new avenues for the exploration of innate immunity. Defining new vita-PAMPs and delineating their mechanistic action in induction of long-lasting protective immune responses will help gain new insights into the signaling pathways involved. These studies would collectively hold the promise of designing new generations of vaccines that replicate the superior performance of live attenuated vaccines and without the associated safety concerns.

References

1. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology. 1989;54 Pt 1:1–13. Epub 1989/01/01.
2. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394–7. Epub 1997 Jul 24.
3. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805–20. Epub 2010 Mar 23.
4. Kawamura I, Yang JF, Takaesu Y, Fujita M, Nomoto K, Mitsuyama M. Antigen Provoking Gamma-Interferon Production in Response to Mycobacterium-Bovis Bcc and Functional Difference in T-Cell Responses to This Antigen between Viable and Killed Bcg-Immunized Mice. Infection and immunity. 1994;62(10):4396–403.
5. Orme I. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. Infection and immunity. 1988;56(12):3310–2.
6. von Koenig CH, Finger H, Hof H. Failure of killed Listeria monocytogenes vaccine to produce protective immunity. Nature. 1982;297(5863):233–4. Epub 1982 May 20.
7. Brockstedt DG, Bahjat KS, Giedlin MA, Liu W, Leong M, Luckett W, et al. Killed but metabolically active microbes: a new vaccine paradigm for eliciting effector T-cell responses and protective immunity. Nat Med. 2005;11(8):853–60. Epub 2005 Jul 26.
8. Jomaa H, Feurle J, Luhs K, Kunzmann V, Tony HP, Herderich M, et al. Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis. FEMS immunology and medical microbiology. 1999;25(4):371–8. Epub 1999 Aug 25.
9. Pamer EG. Immune responses to Listeria monocytogenes. Nat Rev Immunol. 2004;4(10):812–23. Epub 2004 Oct 02.
10. van de Loo AA, Arntz OJ, Bakker AC, van Lent PL, Jacobs MJ, van den Berg WB. Role of interleukin 1 in antigen-induced exacerbations of murine arthritis. Am J Pathol. 1995;146(1):239–49. Epub 1995 Jan 01.
11. Vance RE, Isberg RR, Portnoy DA. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell Host Microbe. 2009;6(1):10–21. Epub 2009 Jul 21.

12. Detmer A, Glenting J. Live bacterial vaccines–a review and identification of potential hazards. Microb Cell Fact. 2006;5:23. Epub 2006 Jun 27.
13. Sander LE, Davis MJ, Boekschoten MV, Amsen D, Dascher CC, Ryffel B, et al. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature. 2011;474(7351):385–9. Epub 2011 May 24.
14. Luker KE, Tyler AN, Marshall GR, Goldman WE. Tracheal cytotoxin structural requirements for respiratory epithelial damage in pertussis. Mol Microbiol. 1995;16(4):733–43. Epub 1995 May 01.
15. Flak TA, Heiss LN, Engle JT, Goldman WE. Synergistic epithelial responses to endotoxin and a naturally occurring muramyl peptide. Infect Immun. 2000;68(3):1235–42. Epub 2000 Feb 26.
16. Magalhaes JG, Philpott DJ, Nahori MA, Jehanno M, Fritz J, Le Bourhis L, et al. Murine Nod1 but not its human orthologue mediates innate immune detection of tracheal cytotoxin. EMBO Rep. 2005;6(12):1201–7. Epub 2005 Oct 08.
17. Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind R, Kollas A, et al. Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in Escherichia coli. FEBS letters. 2001;509(2):317–39.
18. Zimmermann S, Wagner C, Muller W, Brenner-Weiss G, Hug F, Prior B, et al. Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. Infect Immun. 2006;74(10):5687–92. Epub 2006 Sep 22.
19. Karaolis DKR, Means TK, Yang D, Takahashi M, Yoshimura T, Muraille E, et al. Bacterial c-di-GMP is an immunostimulatory molecule. Journal of Immunology. 2007;178(4):2171–81.
20. McWhirter SM, Barbalat R, Monroe KM, Fontana MF, Hyodo M, Joncker NT, et al. A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. J Exp Med. 2009;206(9):1899–911. Epub 2009 Aug 05.
21. Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science. 2010;328(5986):1703–5. Epub 2010 May 29.
22. Heuston S, Begley M, Gahan CG, Hill C. Isoprenoid biosynthesis in bacterial pathogens. Microbiology. 2012;158(Pt 6):1389–401. Epub 2012 Apr 03.
23. Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, Jomaa H. Microbial isoprenoid biosynthesis and human gammadelta T cell activation. FEBS Lett. 2003;544(1–3):4–10. Epub 2003 Jun 05.
24. Donabedian H. Quorum sensing and its relevance to infectious diseases. J Infect. 2003;46(4):207–14. Epub 2003 Jun 12.
25. Tamayo R, Pratt JT, Camilli A. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu Rev Microbiol. 2007;61:131–48. Epub 2007 May 08.
26. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, et al. STING is a direct innate immune sensor of cyclic di-GMP. Nature. 2011;478(7370):515–8. Epub 2011 Sep 29.
27. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, et al. 5′-Triphosphate RNA is the ligand for RIG-I. Science. 2006;314(5801):994–7. Epub 2006 Oct 14.
28. Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, et al. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science. 2006;314(5801):997–1001. Epub 2006 Oct 14.
29. Buchmeier NA, Heffron F. Induction of Salmonella Stress Proteins Upon Infection of Macrophages. Science. 1990;248(4956):730–2.
30. Davila Lopez M, Samuelsson T. Early evolution of histone mRNA 3′ end processing. RNA. 2008;14(1):1–10. Epub 2007 Nov 14.
31. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. Annu Rev Immunol. 2009;27:229–65. Epub 2009 Mar 24.
32. Kanneganti TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, et al. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature. 2006;440(7081):233–6. Epub 2006 Jan 13.
33. Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, et al. DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. Immunity. 2011;34(6):866–78. Epub 2011 Jun 28.

Innate Lymphoid Cells in Immunity and Disease

2

You Yi Hwang and Andrew N.J. McKenzie

Abstract

The family of innate lymphoid cells (ILCs) comprises of natural killer (NK) cells, Rorγt-dependent ILCs (lymphoid tissue inducer (LTi) cells, ILC22, and ILC17), and type 2 ILCs. Apart from a common requirement for inhibitor of DNA binding 2 (Id2) expression and common γ-chain (γ_c) signaling, the differentiation of ILC populations is regulated by distinct transcription factors. ILCs play fundamental roles in processes such as cytotoxicity, lymphoid organogenesis, intestinal homeostasis, immunity against infections, and wound healing. However, the dysregulation of ILCs has been implicated in autoimmune and inflammatory diseases. Here, we will review the recent advances in ILC development and their roles in immunity and disease, with a primary focus on type 2 ILCs.

Keywords

Type 2 • Innate lymphoid cells • ILC • Id2 • Rorγt • Rorα • LTi • ILC22 • ILC17 • Nuocyte • NHC • Ih2 • Immunity • Disease • Anti-helminth • Inflammatory bowel disease • IBD • Fibrosis • Allergy • Airway hyperreactivity • AHR

2.1 Introduction

The immune system has evolved as protection against a wide range of infectious agents ranging from simple pathogens such as viruses, bacteria, and fungi to multicellular parasites such as helminths. In vertebrates, the immune system can be broadly divided into two interdependent effector arms, the adaptive and innate immune responses. In the adaptive immune response, lymphocytes are activated to generate potent pathogen-specific

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Table 2.1 Common cytokines produced by innate and adaptive lymphoid cells

Innate lymphoid cell	Adaptive T helper cell	Common cytokine produced	References
NK cells	Th1	Interferon-gamma (IFN-γ)	[3–5]
LTi	Th17/Th22	Interleukin-17 (IL-17), IL-22	[6–8]
ILC22	Th22	IL-22	[8–10]
ILC17	Th17	IL-17	[11]
Type 2 ILC	Th2	IL-5, IL-13	[12–14]

responses (e.g., antibodies and cytotoxic T cells) via VDJ recombination and generation of memory T and B cells. The innate immune response is evolutionarily older, and pathogen recognition does not adapt to the infection. The innate immune system serves as the "first line of defense" in organisms by providing an immediate protective response against infection and helping to initiate the adaptive immune response.

The innate immune system comprises of leukocytes such as mast cells, eosinophils, basophils, macrophages, neutrophils, dendritic cells (DCs), and natural killer cells (NK cells). These leukocytes act together to combat infectious agents by secreting cytokines, chemokines, and antimicrobials. This leads to inflammation, phagocytosis of microorganisms and infected cells, antigen processing and presentation, and activation of the adaptive immune response. Up until the last decade, NK cells were unique in being the only identified innate cell derived from a lymphoid progenitor. Recent developments have now classified NK cells as the earliest identified member of a family of hematopoietic effector cells termed innate lymphoid cells (ILCs) that are dependent on the transcription factor Id2. Currently, ILCs can be broadly classified into three groups: (a) NK cells, (b) the retinoic acid receptor-related orphan receptor γ t (Rorγt)-dependent ILCs (lymphoid tissue inducer (LTi) cells, ILC17, ILC22), and (c) type 2 ILCs. These groups have recently been named ILC1, ILC3 and ILC2 respectively. These various ILCs have now been implicated in protection against infectious organisms, organogenesis of lymphoid tissue, tissue remodeling during wound healing and homeostasis in tissue stromal cells.

Because the key cytokines secreted by some ILCs mirror those of various T helper cell populations, it has been proposed that ILCs may represent the innate counterparts of T helper lymphocytes, at least in terms of cytokine production [1, 2] (Table 2.1). In this review we will provide a general overview of the ILC family, focusing on the recent advances with regard to type 2 ILCs in immunity.

2.2 Phenotype of ILCs

2.2.1 NK Cell Phenotype

NK cells were first described in 1975 and later defined as an innate effector lymphocyte [15]. They are mostly differentiated in the bone marrow and are widely distributed in many tissues such as the lungs, liver, spleen, and lymph nodes [16, 17]. In humans, a majority of NK cells (approximately 90%) are $CD56^{dim}$ and $CD16^{hi}$ and the minority (10%) are $CD56^{hi}$ and $CD16^{dim/-}$ [18]. A population of thymic derived NK cells has been described in mice that may be similar to human $CD56^{hi}CD16^{dim/-}$ NK cells. These thymic NK cells express CD127, high levels of Gata-3 and are Notch independent [3].

2.2.2 Rorγt-Dependent ILC Phenotype

In mice, LTi cells are characterized by a lack of T, B, and myeloid cell markers, but express integrin α4β7, CD45, CD4, lymphotoxin-α (LT-α), and LT-β, as well as multiple chemokine and cytokine receptors (CD127, CD117, c-Kit)[19]. Adult LTi cells differ from their fetal counterpart due to expression of the T cell costimulatory molecule ligand (OX40-L) and CD30L [20]. Human LTi

cells have been described in fetal mesentery and adult lymph node, spleen, gut, and tonsils [21]. They are similar to mouse LTi cells except that human LTi cells are all $CD4^-$ while a proportion of mouse LTi cells are $CD4^+$ [22].

ILC22 were first identified as an IL-22 producing NK cell subset [9, 10, 23, 24]. The reason for this classification was due to surface expression of NK cell markers such as CD56, NKp44 and low NKp46 expression (in humans), and NKp46 and some low NK1.1 expression (in mice). However, ILC22 differ from conventional NK cells because they lack cytolytic properties, lack killer inhibitory receptors (in humans), lack Ly49 (in mice) and do not produce IFN-γ. They also share similarities with LTi cells by expressing Rorγt and IL-22. In both human and mice, ILC22 are found mainly in the small intestine, colon, mesenteric lymph nodes and liver [9]. ILC22 (ILC3) have been termed NK22, NCR22, NKR^+LTi, LTi-like NK cells and $NKp46^+Ror\gamma t^+$ ILCs. We have adopted the nomenclature proposed by Spits and Di Santo and use ILC22 to describe this $Ror\gamma t^+IL\text{-}22^+NK\ receptor^+$ LTi-like ILC population [1].

IL-1β and IL-23 upregulate the production of IL-22 from ILC22 in both mice and humans [10], while IL-12 and IL-18 induce IL-22 production from mouse ILC22 [25]. It has also been shown that IL-25 produced by intestinal epithelial cells negatively regulates IL-22 production by $Ror\gamma t^+$ ILCs [26]. The common γ-chain (γ_c) cytokines (for example, IL-2, IL-7, and IL-15) can also activate proliferation and cytokine production of human ILC22 [27]. Depending on culture conditions, human ILC22 can be induced to secrete a spectrum of cytokines, including IL-2, IL-5, IL-8, IL-13, IL-17, TNF, IFN-γ, and B cell activation factor [27, 28]. Whether this is because human ILC22 possess cytokine plasticity, or because it is a heterogeneous population of cells, has yet to be determined.

Another mouse non-LTi population has been described, which is specialized to produce IL-17. These cells, termed ILC17, are Rorγt dependent and are $CD4^-CD117^-NKp46^-$, which separates them from both LTi and ILC22 [29]. In humans, an IL-17-producing ILC population has been described; however its expression of cell surface markers is different from mouse ILC17 [7]. Analysis of $lineage^-CD127^+CD117^+$ adult tonsil cells identified that a proportion of them produce IL-17, but not IL-22 [7, 30]. However, these $lineage^-CD127^+CD117^+$ cells appear to be heterogeneous because an $IL\text{-}17^+IL\text{-}22^+$ subpopulation was also identified [30].

2.2.3 Type 2 ILC Phenotype

The type 2 ILCs were independently discovered in 2010 by three separate groups, and were called nuocytes, natural helper cells (NHCs), and innate type 2 helper (Ih2) cells [13, 14, 31]. Using a combination of flow cytometry and microarray analyses, type 2 ILCs were shown to lack the expression of lineage defining surface markers for T cells, B cells, NKT cells, DCs, macrophages, neutrophils, eosinophils, mast cells, basophils, and LTi cells. Type 2 ILCs share a number of surface and functional similarities [32] (Table 2.2). Variability of surface expression markers may be attributed to the different tissues these type 2 ILCs were taken from, indicate a different activation state of the cell, or identify different type 2 ILC subsets. Certainly, activated nuocytes isolated from lung tissue showed a lower expression of Sca-1 and CCR9 compared to those isolated from the MLN [33]. Multiple other groups have since described type 2 ILC-like populations in the liver, bone marrow, lungs, and intestine [34–44]. All identified type 2 ILCs are lineage negative, respond to treatment with either IL-25 and/or IL-33, and can produce type 2 cytokines (IL-5 and/or IL-13). A report of particular interest by Mjösberg et al. characterized a possible human equivalent of mouse type 2 ILCs [36]. These human type 2 ILC cells share a similar phenotype and function with mouse type 2 ILCs and are found in the fetal and adult lung and gut tissues (Table 2.2). They also found these cells in the peripheral blood, but these cells express the chemokine receptor CCR6 and did not produce type 2 cytokines. This suggests that human type 2

Table 2.2 Phenotypic comparison of the type 2 ILCs

	Nuocytes	Natural helper cells	Ih2 cells	Human type2 ILCs
Lineage[a]	–[b]	–	–	–
IL-7Rα	+[b]	+	Not reported	+
IL-17BR (IL-25R)	+	+	+	+
ST2 (IL-33R)	+	+	+	+
c-Kit	+ (variable)	+	Low	+
Sca-1	+	+	–	Not reported
CD25	+	+	Not reported	+
Thy1	+	+	+	Not reported
CD44	+	+	+	Not reported
CD45	+	+	+	+
ICOS (CD278)	+	Not reported	+	Not reported
γc dependent	Yes	Yes	Yes	Responsive to IL-2
Other markers	CD43, MHC Class II, CCR9, ICAM-1, CD49d, Itgb7	CD27, CD38, GITR, CD69	CD122	CRTH2, CD161, AhR, CCR4, CCR6, CD7
Type 2 cytokines	IL-5, IL-6, IL-13 protein, IL-4 mRNA	IL-5, IL-6, IL-13 protein	IL-5, IL-13 protein, IL-4 mRNA	IL-13 protein, IL-5 mRNA (in cultured lines)
Maf	+	+	Not reported	Not reported
Gata-3	+	+	+	Not reported
Junb	+	+	Not reported	Not reported
Stat6	+	+	+	Not reported
Id2	+	+	+	Not reported
Rorγ	–	–	Not reported	Low (in gut) - (in polyps)
Rorα	+	+	Not reported	Not reported
Location	BM, lung, gut, MLN, spleen, blood (after induction)	FALC and lung	Systemic, especially in the MLN, spleen, and liver	Lung, intestines, and nasal polyps. Also blood (inactive)
Conditions for cytokine production	IL-7 + IL-33 or IL-2 + IL-7 + IL-25	IL-33 or IL-2 + IL-25	In vitro data not reported	IL-2 + IL25 or IL-33
Differentiation potential	No differentiation to T cells or myeloid cells	No T cell differentiation	Not reported	No NK cell, T cell, or myeloid cell differentiation
Method of induction/ expansion	IL-25, IL-33 treatment or helminth infection or OVA treatment	IL-25, IL-33 treatment or helminth infection or papain treatment	IL-25 or IL-33 treatment or helminth infection	Chronic rhinosinusitis
Anti-helminth properties	Yes	Yes	Yes	Not reported but found in the gut tissue
Airway allergy association	Yes	Yes	Not reported	Yes
References	[13]	[14]	[31]	[36]

[a]Cell surface markers for at least T cells and B cells
[b]–, Absence of expression or production; +, identified expression or production

ILCs may initially be released into the bloodstream in an inactivate form after which they home into the lung and gut tissue. There, they may mature, becoming activated in situ to start producing type 2 cytokines. Further research is required to determine if the cells identified by Mjösberg et al. are truly the human equivalent of type 2 ILCs.

Although multipotent progenitor type 2 (MPP^{type2}) cells also respond to IL-25 treatment, they can differentiate into myeloid cells following treatment with SCF and IL-3 [45]. This suggests that MPP^{type2} cells may represent a heterogeneous population which includes precursor cells which are not terminally differentiated. This differs from the other members of the type 2 ILC family and they are not included herein.

2.3 Development of ILCs

2.3.1 Inhibitor of DNA Binding 2 (Id2): An Early Common "Switch" for ILCs

The development of T cells, B cells, and DCs from progenitor cells is dependent on a group of basic helix-turn-helix (bHLH) proteins termed E proteins, which include the E2a isotypes (E12 and E47), E2-2, and human bHLH factor (HEB) [46]. Conversely, the E proteins inhibit the development of several ILC populations [47]. The transcription factor function of E proteins is neutralized by Id (inhibitor of DNA binding) proteins, by forming a heterodimer with each other [48]. Of the 4 Id protein members, Id2 has been shown to be important for the development of NK cells and LTi cells by blocking the transcriptional activity of E47 [47]. Id2 also promotes development of ILC22 and type 2 ILCs [14, 49]. It has been demonstrated in Rorγt$^+$ ILCs that Id2 is upregulated prior to Rorγt expression [50]. Taken together, the evidence suggests that the progenitors of different ILC populations share an early expression of Id2 protein, which acts as a developmental block against differentiation down the T cell and B cell pathway (Fig. 2.1).

2.3.2 NK Cells Development

NK cell development in the bone marrow is dependent on early IL-15 and Flt3 ligand signaling [51, 52]. Other transcription factors affecting NK cell differentiation and maturation include transcription factors such as Ets-1, Id2, Ikaros, PU.1, T-bet, Gata-3, NFIL3 (E4BP4), and Eomesodermin, as well as the Tox nuclear factor [51, 53]. The development of mouse thymic NK cells is dependent on IL-15, IL-7, and Gata-3 [4] (Fig. 2.1).

2.3.3 Rorγt-Dependent ILCs Development

As their name suggests, Rorγt-dependent ILCs express Rorγt, which is important for their development and function [2]. The retinoic acid receptor-related orphan receptors (Rorα, Rorβ, and Rorγ) are a family of DNA-binding transcription factors which are nuclear receptors. Cholesterol and its derivatives have been identified as natural ligands for Rorα while hydroxycholesterols have been proposed as a natural ligand for Rorγ [54, 55]. Rorγt is a short Rorγ isoform that is specifically expressed in cells of the immune system, and Rorγt-deficient mice lack lymph nodes and Peyer's patches [56]. All the Rorγt-dependent ILCs express the IL-7 receptor CD127, and IL-7 has been shown to be important for homeostasis of these ILCs [57, 58] (Fig. 2.1).

Fetal LTi cells differentiate from fetal liver CLPs by first upregulating Id2 which results in upregulation of α4β7, with the loss of B cell potential. This is followed by upregulation of the chemokine marker CXCR6, extinguishing their T cell potential, before final expression of Rorγt. An early pulse of Notch signaling has been reported to maximize the efficiency of LTi cell differentiation [50]. However the necessity of Notch signaling is still contentious as results show fetal liver CLPs can still generate LTi cells in the absence of Notch [59]. Postnatal LTi cells are derived from bone marrow CLPs, which enter the periphery as α4β7$^+$ cells to colonize

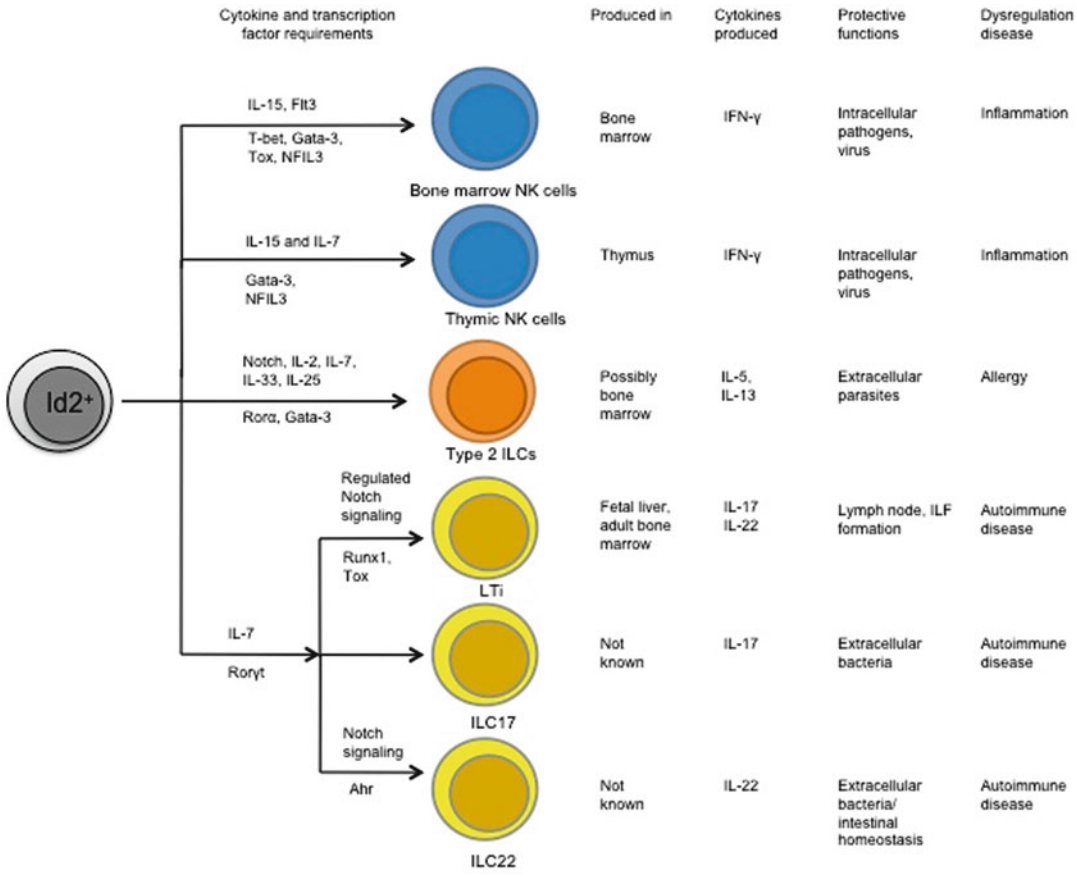

Fig. 2.1 An overview of known ILC developmental requirements. All ILC members are thought to derive from an Id2 expressing progenitor. Differentiation into NK cells, type 2 ILCs, or Rorγt-dependent ILCs is dictated by various cytokines, signals (*above arrow*), and transcription factors (*below arrow*). A brief summary of where these subsets are thought to differentiate as well as functions is included

the spleen and lamina propria before completing differentiation in situ into Rorγt$^+$ cells via a Notch-dependent pathway [59]. Additionally, LTi cell differentiation has also been shown to depend on the transcriptions factors Runx1 and Tox [60, 61].

Fate-mapping experiments and genome-wide microarray profiling have demonstrated that mouse ILC22 derive from Rorγt$^+$ precursors that are α4β7$^-$ [62, 63]. Although ILC22 development does not require the expression of α4β7, Id2-deficient mice do not possess ILC22 [49], suggesting that another function of Id2, apart from inducing α4β7 expression, is required for the differentiation of ILC22 [50]. ILC22 can develop from a CXCR6$^+$ CLP population in adult lamina propria but not in spleen, showing a compartmental specificity for the chemokine receptor [59]. These Rorγt$^+$ precursors in the small intestine, colon, and secondary lymphoid organs require Notch signaling to stably upregulate NK receptors in vivo and will express IL-22 as they mature into ILC22 [59]. ILC22 retain some degree of plasticity, for example, ILC22 in the small intestine remain Rorγt$^+$, while those in the colon and secondary lymphoid organs become RORγt$^-$IL-22$^-$ IFN-γ$^+$ cells, gaining NK cell markers but lacking cytolytic ability, thus differentiating them from true NK cells [63, 64]. In vitro, IL-7 has been implicated in maintaining

Rorγt expression and IL-22 production, while IL-2 and IL-15 promote the loss of Rorγt and the gain of IFN-γ expression [64]. Vornarboug et al. showed that the presence of commensal microflora also plays a role in maintaining Rorγt expression and subsequent induction of IL-22. However, the microflora is not essential for ILC22 development since they continue to develop in the small intestine of germfree mice [23, 24, 26, 62, 64].

Another factor implicated in Rorγt-dependent ILC development and function is the ligand-dependent transcription factor aryl hydrocarbon receptor (AhR) (Fig. 2.1) [65–67]. AhR is expressed by ILC22 in both mice and humans, and AhR-deficient mice have fewer ILC22 that have impaired IL-22 production [67, 68]. These mice also show defects in cryptopatch clusters and isolated lymphoid follicles (ILFs), suggesting that AhR is also important for postnatal LTi function [66]. The natural ligands for AhR are flavanoids and glucosinolates, which are dietary compounds commonly found in vegetables such as of the family *Brassicaceae* [66]. This suggests that balanced nutrition plays a part in priming the innate immune system in the gut, and maybe mother was right to make you eat your greens [65].

Some key questions about the Rorγt-dependent ILCs (LTi, ILC22, and ILC17) still remain to be answered. These include the developmental relationships between these subsets; should they be classified as distinct subsets or differently activated cells, and do they possess a degree of plasticity to change from one subset to another? Previous attempts to differentiate fetal mouse LTi cells into ILC22 have been unsuccessful, but human LTi cells from fetal lymph nodes and adult tonsils can become ILC22, suggesting that some degree of trans-differentiation is possible [62]. Future research will hopefully better define the development and function of the Rorγt-dependent ILCs in both humans and mice.

2.3.4 Type 2 ILCs Development

Type 2 ILCs are derived from the lymphoid lineage [69]. Collectively, they express a combination of hematopoietic markers such as CD45, c-Kit, and Sca-1, as well as lymphoid markers such as IL-7Rα, ICOS, Thy1.2, and CD44 [13, 14, 31, 36]. The development of NHCs and Ih2 cells is dependent on expression of the γc surface receptor, suggesting the developmental importance of γc-dependent cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21), some of which are central regulators of lymphocyte homeostasis [14, 31, 58]. Type 2 ILCs do not differentiate into other lineage cell types in a cytokine cocktail with SCF and IL-3, suggesting that they are terminally differentiated [13]. However, it is currently unknown if they can trans-differentiate under specific stimulus.

Yang et al. proposed that NHCs are derived from bone marrow lymphoid progenitor cells. They found that most NHCs expressed Rag1 at some point in their development and they differentiate in vivo from lymphoid progenitor cells [69]. Their findings were corroborated by Wong et al., wherein they showed that functional nuocytes differentiated from bone marrow CLP in both in vivo and in vitro models when treated with IL-7 and IL-33 [34]. Both studies agreed that NHCs and nuocytes required IL-7 receptor for in vivo development. In addition, Wong et al. demonstrated the requirement for Notch signaling for in vitro differentiation of nuocytes. Notch signaling is important for hematopoiesis, especially for T cell commitment of progenitor cells and T cell maturation in the thymus [70]. T cell precursors in the double negative stage 1 and stage 2 thymocytes also retain nuocyte differentiation potential when treated with IL-7 and IL-33 [34]. However, further differentiation down the T cell pathway has to be inhibited. The absence of NHCs in Id2-deficient mice suggests that Id2 is important for type 2 ILCs development, and could be responsible for the inhibition of T cell differentiation [14].

Significantly, Wong et al. also reported the requirement for Rorα for nuocyte differentiation [34]. Rorα is expressed in a wide variety of tissues, but is especially important for neuronal development [71, 72]. Mice deficient for Rorα exhibit ataxia, cerebellar atrophy, and a significantly diminished life span [73, 74]. Rorα had also been loosely linked to immunity because

Rorα-deficient mice were reported to have reduced T cell/B cell numbers in the spleen and thymus, as well as reduced OVA-induced airway hyperreactivity [71, 75]. Rorα has also been implicated in Th17 differentiation [76]. Microarray data for both nuocytes and NHCs show that they express Rorα mRNA and not Rorγt mRNA, further differentiating them from the Rorγt-dependent ILCs [13, 14]. A natural knockout of Rorα occurs in staggerer (*Rora*$^{sg/sg}$) mice [73]. Reconstituting lethally irradiated mice with staggerer mouse bone marrow followed by infection with *Nippostrongylus brasiliensis* showed that the nuocyte population did not expand in the reconstituted mice and these animals displayed impaired worm expulsion. The adaptive immune response remained normal based on normal T cell development and numbers [34]. An OVA-induced asthma model using staggerer mice demonstrated that these mice developed less airway inflammation, goblet cell hyperplasia, eosinophilia, and production of type 2 cytokines [75]. These results, together with those from a study by Halim et al. [160], support a model for Rorα as an important transcription factor for type 2 ILC development in the bone marrow. Interestingly, human type 2 ILCs express Rorγt, albeit at lower levels than the Rorγt-dependent ILCs [36]. It remains to be proved if Rorα is also important for the development of human type 2 ILCs.

Thus, type 2 ILCs differentiate from CLPs, and this is dependent on Notch and γc-dependent cytokine (IL-7) signaling [77]. A possible source of Notch ligands and IL-7 is the stromal cells in the bone marrow [78, 79], although this requires further investigation. Notch signaling encourages CLPs towards a T cell fate [70], but it appears that the expression of Id2 (by signals as yet unknown) inhibits the T cell commitment of these progenitor cells [2, 14]. Expression of Rorα correlates with the further differentiation of mouse type 2 ILCs [34] (Fig. 2.1), but the factors that regulate Rorα expression remain to be identified. Recently, Gata-3 expression has been demonstrated to license type 2 ILCs for IL-13 expression, and that Gata-3 and STAT6 both contribute to type 2 ILC development [80, 161].

2.4 ILC Roles in the Host Organism

2.4.1 NK Cells: Cytolytic Activity and Cytokine Production

The CD56^{dim} NK cell population is biased towards rapidly initiating a cytolytic response against virus-infected host organism cells or tumor cells without the need for pre-sensitization or activation via the major histocompatibility (MHC) molecules [81]. There are two mechanisms for this cytolytic activity. The first is granule-dependent cytotoxicity, where NK cells are activated to release perforin and granzymes in proximity to an infected cell to kill it. The other triggers the apoptosis pathway in target cells via NK cell-secreted tumor necrosis factor-α (TNF-α) that binds to the target cell, or via direct cell contact with NK cells leading to signaling through TNF-related apoptosis-inducing ligand (TRAIL) and/or Fas ligand (FasL) [16, 82].

CD56^{hi} NK cells and mouse thymic NK cells lack cytolytic activity and are primed towards producing cytokines such as IFN-γ, TNF-α, IL-10, and other growth factors [3, 81] (Fig. 2.1).

Apart from being effector cells, NK cells have a regulatory role during an immune response by affecting DCs, macrophages, and mast cells [83]. Recently, the notion that NK cells are truly innate cells has been called into question because specific subsets of mouse liver NK cells have been described to have the adaptive immunity property of lasting memory against specific viral antigens [84, 85].

2.4.2 LTi Cells and Organogenesis of Lymphoid Structures

LTi cells induce the formation of lymph tissues such as lymph nodes and Peyer's patches during embryogenesis in both humans and mice [86–88]. During mouse embryogenesis, fetal liver-derived LT-α_1LT-$\beta_2$$^+$LTi cells colonize developing lymph tissues and interact with mesenchymal-derived stromal organizer cells called lymphoid tissue organizer (LTo), which express vascular cell

adhesion molecule (VCAM-1) and LT-β receptor. Signaling through the LT-β receptor induces the upregulation of various cell adhesion molecules, production of IL-7 and TNF-related activation-induced cytokine (TRANCE), and secretion of lymphoid chemokines such as CXCL13, CCL19, and CCL21. These factors recruit additional LTi precursors as well as other hematopoietic cells, including B and T cells, and DCs, to the developing lymph node [89, 90].

Postnatal LTi cells have further developmental roles in secondary lymphoid tissues [7]. They are important for the formation of ILFs in the gut after recognition of pathogen-associated patterns (PAMPs) on commensal bacteria in order to maintain intestinal homeostasis [91, 92]. Additionally, it has been reported that postnatal LTi cells are involved in the repair of damaged lymph nodes after acute viral infections that destroy the T cell zone stromal cells [93]. They have also been shown to be involved in the segregation of B and T cell zones in spleen architecture, as well as in memory CD4 T cell generation [94, 95].

2.4.3 Rorγt-Dependent ILCs: IL-17 and IL-22 Producers for Intestinal Homeostasis

IL-17 is a pro-inflammatory cytokine that recruits neutrophils and promotes cytokine and antimicrobial peptide production from a variety of cells such as bronchial epithelial cells [96]. IL-17 has also been shown to have a role in the formation of germinal centers and neutrophilia in allergic asthma [97, 98]. IL-22 is a member of the IL-10 family and binds to its receptor, which is found exclusively on epithelial cells to induce the production of cytokines, microbial peptides, and mucins [99]. It can act as either a pro-inflammatory or an anti-inflammatory cytokine depending on the cellular and cytokine environment. It acts as a pro-inflammatory cytokine in diseases such as psoriasis and multiple sclerosis [100], but limits damage caused by the immune system in hepatitis and helps maintain mucosal immunity and integrity in eosinophilic airway inflammation and inflammatory bowel disease [101].

LTi cells are producers of the cytokines IL-17 and/or IL-22 after stimulation with IL-23 [21, 102]. Therefore, LTi cells may be involved with the early protection against microbial infections and maintaining the mucosal barrier in the host organism. ILC22 and ILC17 act as specialized producers of IL-17 and IL22, to support the protective responses in the gut during microbial infections.

Both ILC22 and ILC17 are recruited to the intestine under inflammatory conditions, and are involved in a protective role during intestinal infection and inflammation. IL-23 induces ILC22 and ILC17 to produce their respective cytokine [99]. ILC22 serve as a critical early source of IL-22 to protect against colitis-inducing *Citrobacter rodentium* infections [103], as well as other colitis models such as inflammatory bowel disease (IBD) and dextran sulfate sodium (DSS)-induced colitis [104]. Although ILC22 are not required to control a *Listeria monocytogenes* infection, the oral introduction of the pathogen still enhances IL-22 production from ILC22 [63]. It is unknown if these human-specialized IL-17 producers are present in the intestine during an infection. The different effector functions of ILC17 and ILC22 might explain the presence of specialized subsets of IL-22- and IL-17-producing ILCs, which would tailor the innate immune response to infections and maintain intestinal homeostasis.

2.4.4 Type 2 ILCs: Protective Response Against Helminths

Type 2 ILCs were initially described as important innate cells responsible for anti-helminth protection [105]. IL-25 and IL-33 have been shown to be important to activate type 2 ILCs to produce effector cytokines.

IL-25 (IL-17E) is a member of the IL-17 family that is associated with Th2-like inflammation and disease [106–108]. IL-25 mRNA transcripts are produced in Th2 cells and lung epithelial cells while the protein has been reported to be produced by alveolar macrophages, mast cells, eosinophils, and basophils [109–111]. IL-25

upregulates the production of type 2 cytokines by eosinophils, mast cells, type 2 ILCs, and Th2 cells [33, 112]. IL-25 signaling acts via the signaling molecule Act1 to increase expression of Gata-3 and subsequent production of type 2 cytokines [109, 113, 114].

IL-33 (IL-1F11) is a member of the IL-1 family that binds to the ST2 receptor (Il1lr1) in complex with IL1RAP [115]. ST2 is primarily expressed on mast cells, Th2 cells, and type 2 ILCs [33, 116, 117]. IL-33 mRNA is expressed in epithelial cells, endothelial cells, lung fibroblasts, DCs, and alveolar macrophages [118], and plays roles in disease symptoms such as fibrosis and airway hyperreactivity, as well as in autoimmune diseases such as arthritis [119–122]. The mechanism by which IL-33 is released by cells is unclear; it is thought that IL-33 acts as an "alarmin" during necrosis and initiates inflammatory signaling [123]. By contrast, if the cell undergoes programmed cell death, i.e., apoptosis, then caspase-1 cleaves the cytokine domain of IL-33 into a nonfunctional form that fails to initiate the inflammatory response [124].

Infection with helminths, such as *N. brasiliensis*, breaches and irritates the epithelial barrier of the lung and gut. TFF2 signaling and other unknown signals induce the production and release of IL-25 and IL-33 from epithelial cells and other cells such as alveolar macrophages [125]. Since IL-25-responsive epithelial cells are important for downstream IL-5 and IL-13 production in the lung, this suggests that epithelial cells could self-upregulate factors in a positive feedback loop that amplifies the downstream type 2 response [113, 114].

Type 2 ILCs activated by IL-25 or IL-33 are important for *N. brasiliensis* expulsion. Neill et al. demonstrated that mice lacking either one or both cytokine receptors have very few nuocytes and are unable to effectively clear helminth infections. However, the adoptive transfer of activated type 2 ILCs was able to rescue this defect. Furthermore, worm expulsion was dependent on IL-13 since transferring IL-13-deficient nuocytes into IL-13-deficient mice failed to mediate worm expulsion [13]. IL-13 is indispensible for the efficient expulsion of *N. brasiliensis* [126] because it induces a range of type 2 immune physiological responses such as smooth muscle contraction, goblet cell hyperplasia, and mucus hypersecretion, thus activating a "weep and sweep" mechanism which traps and expels the worms [127] (Fig. 2.2). IL-13 is partially involved during the infection with other parasite species such as *Trichuris muris* [128]. Therefore, type 2 ILCs are the early trigger of type 2 protective responses. An increased number of circulating Ih2 cells in the blood after IL-25 treatment suggests that additional type 2 ILCs could be recruited from the blood to enhance a local type 2 response and also suggests that a localized infection could initiate a systemic type 2 response via these circulating type 2 ILCs [31].

Apart from the innate immune system, the adaptive immune system is also activated following infection. Alarmins released by damaged epithelial cells, and parasite-derived antigens, promote Th2 cell differentiation via professional antigen-presenting cells [129]. This secondary wave of type 2 cytokines amplifies the effects of type 2 ILCs as well as initiating other physiological responses such as promoting Th2 cell differentiation, activating B cells to produce antibodies, inducing IgE class switching and upregulating mast cells [130–132].

Even though type 2 ILC-derived cytokines are sufficient to resolve a helminth infection, the presence of Th2 cells (even if IL-4 and IL13 deficient) is still essential for effective helminth expulsion [133]. This could be explained by the observation that Th2 cells are required to maintain type 2 ILCs numbers during an infection [13]. Nuocytes are present in Rag2 knockout mice (which lack B cells and T cells) and are responsive to IL-25 and IL-33, but their population numbers decrease soon after induction, and these mice are unable to expel the worm burden effectively [13]. This suggests that T cells play a role in nuocyte maintenance, and in addition, boost the type 2 immune response by producing more type 2 cytokines. An area for further investigation is the interrelationship of innate type 2 ILCs and adaptive Th2 cells.

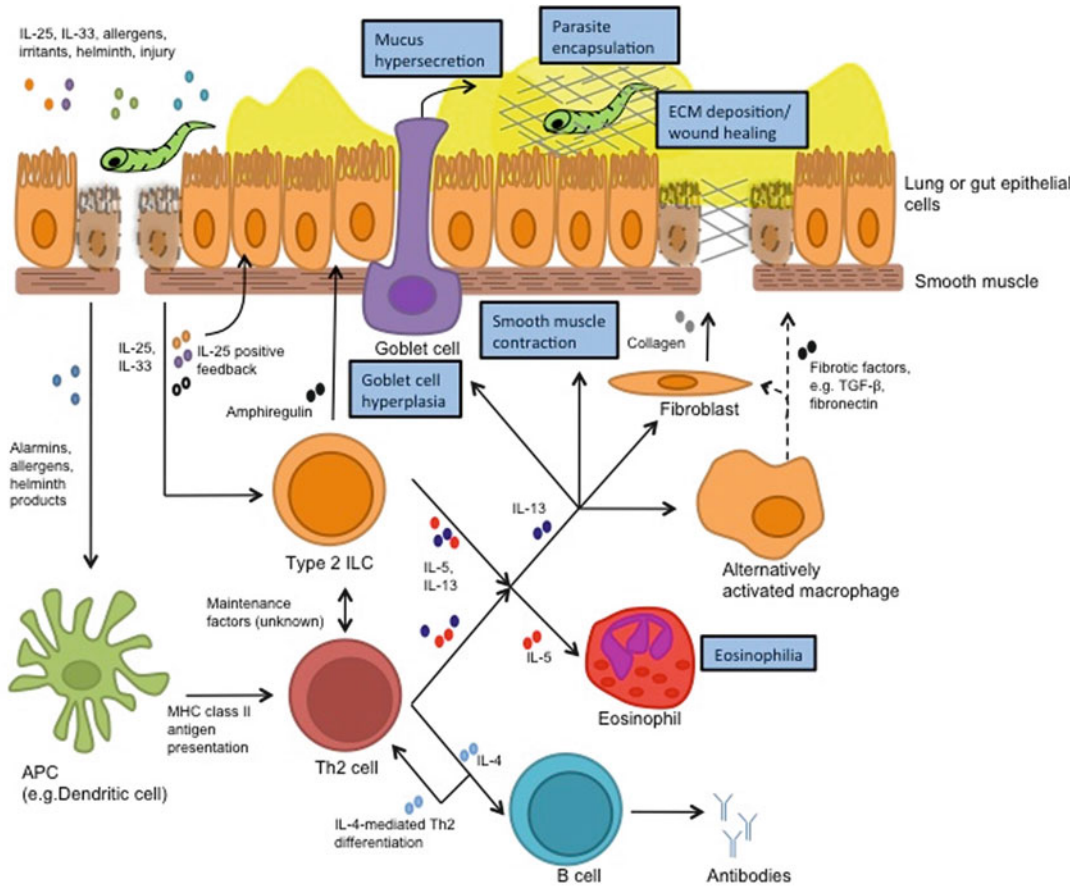

Fig. 2.2 Schematic of type 2 ILC function. Allergens, chemicals, irritants, or parasites induce lung or intestinal epithelial cells to release the type 2 ILC-activating cytokines, IL-25, and IL-33. IL-25 may act in a positive feedback loop on epithelial cells to amplify the activation of type 2 ILCs. Activated type 2 ILCs rapidly produce amphiregulin, IL-5, and IL-13. Amphiregulin promotes epithelial cell proliferation, while IL-5 promotes eosinophilia into the lung or gut tissues. IL-13 promotes smooth muscle contraction, goblet cell hyperplasia, and mucus hypersecretion. It also encourages deposition of extracellular matrix (ECM) by directly inducing collagen production from fibroblasts, and indirectly by inducing fibrotic factor production from alternatively activated macrophages (*dashed arrow*). Professional antigen-presenting cells (APC) activate adaptive Th2 cells in order to support the proliferation and function of type 2 ILCs. Th2 cells produce IL-5, IL-13, IL-9 (not shown), and IL-4, which perform functions specific to Th2 cells such as IL-4-driven class switching and antibody production

2.4.5 Wound Healing

Evidence suggests that type 2 ILCs are involved in wound healing and fibrotic processes. Type 2 ILCs directly produce amphiregulin, which promotes the proliferation of epithelial cells [38, 134]. Type 2 ILCs can also indirectly promote tissue remodeling via IL-13 and IL-5. In vitro studies show that IL-13 can directly induce the proliferation of myofibroblasts and collagen production from fibroblasts [135, 136]. IL-13 indirectly promotes fibrosis via the induction of fibrotic factors such as arginase, TFG-β, and fibronectin from fibroblasts and alternatively activated macrophages [137–140]. In vivo, IL-13 has been shown to mediate *Schistosoma mansoni*-induced liver fibrosis in a TGF-β-independent pathway [141–143]. IL-5 promotes

eosinophil recruitment and activation, which is thought to play a role in airway remodeling in chronic airway diseases [144].

Therefore, activation of type 2 ILCs may contribute to tissue repair following infection and injury to minimize the disease pathology (Fig. 2.2) [145].

Other ILC populations may also be involved in healing injuries sustained during infection. As mentioned previously, LTi cells can restore damaged lymph nodes after particularly severe viral infections. Other Rorγt-dependent ILCs may also promote healing via production of IL-22, which has been implicated in tissue repair after injury or alcohol-induced damage [146].

2.4.6 Dysregulation of ILCs: Autoimmunity, Allergy, and Fibrosis

The dysregulation of either IL-17 or IL-22 has been linked to autoimmune diseases such as psoriasis, rheumatoid arthritis, and IBD [147]. Therefore, if the activation of Rorγt-dependent ILCs (and production of IL-17 and IL-22) is not tightly regulated, they may contribute to these diseases. For example, Buonocore et al. have demonstrated that a Rorγt$^+$ILC population is stimulated by IL-23 in the colon to produce IL-17 and induces intestinal colitis [29].

Chronic activation of the type 2 response can cause allergic airway diseases (such as asthma), inflammatory gut diseases, as well as excessive fibrosis and tissue remodeling [148–150]. As potent type 2 cytokine producers, type 2 ILCs would be expected to play a part in these diseases. Research has shown that the activator (IL-25, IL-33) and effector (IL-5, IL-13) cytokines of type 2 ILCs are involved in allergic diseases.

IL-25 and IL-33 expression correlates with allergic airway diseases [118, 151], and IL-33 has been identified as an asthma-related gene based on a genome-wide study [152]. In asthmatic lung tissue, increased production of IL-25 and IL-33 bring about the same physiological changes in the lungs as during a helminth infection, such as a rapid type 2 response, increased production of IL-5 and IL-13, and increased mucus production, eosinophilia, and airway hyperreactivity [118, 151]. Blocking either IL-25 or IL-33 signaling in the airways can reduce eosinophilia and inflammation in an ovalbumin (OVA)-driven model of allergic airway disease [43, 151]. Overexpression or ablation of IL-13 within the lungs has underlined its role in inducing asthma-like phenotypes, such as nonspecific airway hyperreactivity and mucus hyperproduction [153, 154]. As mentioned above, IL-13 also contributes to tissue remodeling and fibrosis, and thus may contribute to fibrosis in diseases dominated by a type 2 immune response [155]. IL-5 promotes eosinophil infiltration into the lungs [156, 157].

Recently, multiple teams have identified type 2 ILCs in the lungs and their role in airway allergy has been investigated [37–44, 137]. They have shown that when challenged with IL-25, IL-33, papain, allergens (*Alternaria alternata*, OVA, house dust mite, glycolipid antigen), parasites, or viruses, type 2 ILCs proliferated and were activated to produce a rapid type 2 response characterized by increased production of IL-5 and IL-13, increased mucus production, eosinophilia, and airway hyperreactivity, reminiscent of the response during an allergic asthma.

When mice were treated with OVA (as per an OVA-induced asthma model), IL-25, or IL-33, nuocytes were induced in the lung tissue and bronchoalveolar lavage (BAL). These nuocytes represent a major source of IL-13 in the lung, explaining why IL-13 from T cells is partially dispensable for the allergic inflammation during an airway hyperreactivity response. Adoptive transfer of nuocytes into IL-13-deficient mice (which do not respond to IL-25 treatment) restores both AHR and eosinophilia, indicating that nuocytes have the capacity to upregulate asthma even in the absence of T cell-derived IL-13. However, infiltration of neutrophils into the lung during challenge with IL-25 was not restored, indicating that other cells and cytokines are responsible for other aspects of the allergic response [41]. Kim et al. also showed the importance of type 2 ILCs in

response to glycolipid antigens [40]. Halim et al. corroborated these earlier studies using the adoptive transfer of type 2 ILCs into Rag2 and γc double knockout mice, which restored the allergic phenotype [44]. Respiratory infections with rhinovirus or respiratory syncytial virus are known to promote type 2 responses, and exacerbate allergic asthma. Chang et al. demonstrated that influenza virus-induced asthma is not mediated by adaptive immunity, but by IL-33-dependent type 2 ILCs [39].

Significantly, Mjösberg et al. demonstrated that human type 2 ILCs are enriched in chronically inflamed airway tissues, such as the nasal polyps of patients suffering from chronic rhinosinusitis. These patients exhibited higher levels of IL-5 and IL-13 transcripts within the polyp tissue, which in turn contributes to eosinophil enrichment within the nasal polyps [36]. This may be attributable to the increased human type 2 ILC population.

The allergic response is not limited to only the lungs. As Camelo et al. demonstrate, activation of type 2 ILC leads to an overexpression of IL-13 in the gut, which then leads to chronic inflammation and ulcerative colitis [158]. It is also possible that type 2 ILCs represent a potent source of IL-13 in patients suffering from chronic asthma, which may contribute to the remodeling of lung tissue and lung fibrosis [159].

2.5 Conclusion

As we begin to understand the complexities of these newly identified ILC populations, it is apparent that the innate lymphoid cell compartment plays an important role for the host. It drives lymphoid tissue development, maintains tissue and barrier homeostasis, provides a rapid protective response against infectious agents, and promotes wound healing. In this way, they precede and also support the adaptive immune response.

Dysregulation of ILCs is also associated with disease. Rorγt-dependent cells are involved with colitis and IBD, while type 2 ILCs are associated with allergy in the gut and lungs. As we learn more about the innate lymphoid cells, they may come to represent viable therapeutic targets to combat such diseases.

References

1. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat Immunol. 2011;12:21–27.
2. Cherrier M, Ohnmacht C, Cording S, Eberl G. Development and function of intestinal innate lymphoid cells. Curr Opin Immunol. 2012.
3. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. Nat Immunol. 2006;7:1217–1224.
4. Ribeiro VS, Hasan M, Wilson A et al. Cutting edge: Thymic NK cells develop independently from T cell precursors. J Immunol. 2010;185:4993–4997.
5. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol. 2007;96:41–101.
6. Harrington LE, Hatton RD, Mangan PR et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005;6:1123–1132.
7. Cupedo T, Crellin NK, Papazian N et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. Nat Immunol. 2009;10:66–74.
8. Eyerich S, Eyerich K, Pennino D et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest. 2009;119:3573–3585.
9. Cella M, Fuchs A, Vermi W et al. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature. 2009;457:722–725.
10. Luci C, Reynders A, Ivanov II et al. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. Nat Immunol. 2009;10:75–82.
11. Takatori H, Kanno Y, Watford WT et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med. 2009;206:35–41.
12. Brinkmann V, Kristofic C. TCR-stimulated naive human CD4+ 45RO- T cells develop into effector cells that secrete IL-13, IL-5, and IFN-gamma, but no IL-4, and help efficient IgE production by B cells. J Immunol. 1995;154:3078–3087.
13. Neill DR, Wong SH, Bellosi A et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010;464:1367–1370.
14. Moro K, Yamada T, Tanabe M et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature. 2010;463:540–544.

15. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer. 1975;16:230–239.
16. Di Santo JP. Natural killer cell developmental pathways: a question of balance. Annu Rev Immunol. 2006;24:257–286.
17. van den Brink MR, Palomba ML, Basse PH, Hiserodt JC. In situ localization of 3.2.3+ natural killer cells in tissues from normal and tumor-bearing rats. Cancer Res. 1991;51:4931–4936.
18. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol. 1986;136:4480–4486.
19. Mebius RE, Rennert P, Weissman IL. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity. 1997;7:493–504.
20. Kim MY, Anderson G, White A et al. OX40 ligand and CD30 ligand are expressed on adult but not neonatal CD4+CD3- inducer cells: evidence that IL-7 signals regulate CD30 ligand but not OX40 ligand expression. J Immunol. 2005;174:6686–6691.
21. Kim MY, Kim KS, McConnell F, Lane P. Lymphoid tissue inducer cells: architects of CD4 immune responses in mice and men. Clin Exp Immunol. 2009;157:20–26.
22. Kim MY, Toellner KM, White A et al. Neonatal and adult CD4+ CD3- cells share similar gene expression profile, and neonatal cells up-regulate OX40 ligand in response to TL1A (TNFSF15). J Immunol. 2006;177:3074–3081.
23. Sanos SL, Bui VL, Mortha A et al. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. Nat Immunol. 2009;10:83–91.
24. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity. 2008;29:958–970.
25. Satoh-Takayama N, Dumoutier L, Lesjean-Pottier S et al. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. J Immunol. 2009;183:6579–6587.
26. Sawa S, Lochner M, Satoh-Takayama N et al. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. Nat Immunol. 2011;12:320–326.
27. Crellin NK, Trifari S, Kaplan CD, Satoh-Takayama N, Di Santo JP, Spits H. Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2. Immunity. 2010;33:752–764.
28. Cella M, Otero K, Colonna M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. Proc Natl Acad Sci USA. 2010;107:10961–10966.
29. Buonocore S, Ahern PP, Uhlig HH et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature. 2010;464:1371–1375.
30. Crellin NK, Trifari S, Kaplan CD, Cupedo T, Spits H. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. J Exp Med. 2010;207:281–290.
31. Price AE, Liang HE, Sullivan BM et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc Natl Acad Sci USA. 2010;107: 11489–11494.
32. Saenz SA, Noti M, Artis D. Innate immune cell populations function as initiators and effectors in Th2 cytokine responses. Trends Immunol. 2010;31: 407–413.
33. Neill DR, McKenzie AN. Nuocytes and beyond: new insights into helminth expulsion. Trends Parasitol. 2011;27:214–221.
34. Wong SH, Walker JA, Jolin HE et al. Transcription factor RORalpha is critical for nuocyte development. Nat Immunol. 2012;13:229–236.
35. Brickshawana A, Shapiro VS, Kita H, Pease LR. Lineage(-)Sca1+c-Kit(-)CD25+ cells are IL-33-responsive type 2 innate cells in the mouse bone marrow. J Immunol. 2011;187:5795–5804.
36. Mjosberg JM, Trifari S, Crellin NK et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol. 2011;12:1055–1062.
37. Yasuda K, Muto T, Kawagoe T et al. Contribution of IL-33-activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice. Proc Natl Acad Sci USA. 2012;109: 3451–3456.
38. Monticelli LA, Sonnenberg GF, Abt MC et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nat Immunol. 2011;12:1045–1054.
39. Chang YJ, Kim HY, Albacker LA et al. Innate lymphoid cells mediate influenza-induced airway hyperreactivity independently of adaptive immunity. Nat Immunol. 2011;12:631–638.
40. Kim HY, Chang YJ, Subramanian S et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. J Allergy Clin Immunol. 2012;129:216–27. e1–6.
41. Barlow JL, Bellosi A, Hardman CS et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. J Allergy Clin Immunol. 2012;129:191–8. e1–4.
42. Wolterink RG, Kleinjan A, van Nimwegen M et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. Eur J Immunol. 2012;42:1106–1116.

43. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage-CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. J Immunol. 2012;188:1503–1513.
44. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. Immunity. 2012;36:451–463.
45. Saenz SA, Siracusa MC, Perrigoue JG et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. Nature. 2010;464:1362–1366.
46. Kee BL. E and ID proteins branch out. Nat Rev Immunol. 2009;9:175–184.
47. Yokota Y, Mansouri A, Mori S et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature. 1999;397:702–706.
48. Engel I, Murre C. The function of E- and Id proteins in lymphocyte development. Nat Rev Immunol. 2001;1:193–199.
49. Satoh-Takayama N, Lesjean-Pottier S, Vieira P et al. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. J Exp Med. 2010;207:273–280.
50. Cherrier M, Sawa S, Eberl G. Notch, Id2, and RORgammat sequentially orchestrate the fetal development of lymphoid tissue inducer cells. J Exp Med. 2012;209:729–740.
51. Gascoyne DM, Long E, Veiga-Fernandes H et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat Immunol. 2009;10:1118–1124.
52. Nakamori Y, Liu B, Ohishi K et al. Human bone marrow stromal cells simultaneously support B and T/NK lineage development from human haematopoietic progenitors: a principal role for flt3 ligand in lymphopoiesis. Br J Haematol. 2012
53. Martin-Fontecha A, Lord GM, Brady HJ. Transcriptional control of natural killer cell differentiation and function. Cell Mol Life Sci. 2011;68:3495–3503.
54. Kallen J, Schlaeppi JM, Bitsch F, Delhon I, Fournier B. Crystal structure of the human RORalpha Ligand binding domain in complex with cholesterol sulfate at 2.2 A. J Biol Chem. 2004;279:14033–14038.
55. Jin L, Martynowski D, Zheng S, Wada T, Xie W, Li Y. Structural basis for hydroxycholesterols as natural ligands of orphan nuclear receptor RORgamma. Mol Endocrinol. 2010;24:923–929.
56. Sun Z, Unutmaz D, Zou YR et al. Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science. 2000;288:2369–2373.
57. Schmutz S, Bosco N, Chappaz S et al. Cutting edge: IL-7 regulates the peripheral pool of adult ROR gamma+ lymphoid tissue inducer cells. J Immunol. 2009;183:2217–2221.
58. Kang J, Coles M. IL-7: The global builder of the innate lymphoid network and beyond, one niche at a time. Semin Immunol. 2012
59. Possot C, Schmutz S, Chea S et al. Notch signaling is necessary for adult, but not fetal, development of RORgammat(+) innate lymphoid cells. Nat Immunol. 2011;12:949–958.
60. Tachibana M, Tenno M, Tezuka C, Sugiyama M, Yoshida H, Taniuchi I. Runx1/Cbfbeta2 complexes are required for lymphoid tissue inducer cell differentiation at two developmental stages. J Immunol. 2011;186:1450–1457.
61. Aliahmad P, de la Torre B, Kaye J. Shared dependence on the DNA-binding factor TOX for the development of lymphoid tissue-inducer cell and NK cell lineages. Nat Immunol. 2010;11:945–952.
62. Sawa S, Cherrier M, Lochner M et al. Lineage relationship analysis of RORgammat+ innate lymphoid cells. Science. 2010;330:665–669.
63. Reynders A, Yessaad N, Vu Manh TP et al. Identity, regulation and in vivo function of gut NKp46+RORgammat+ and NKp46+RORgammat-lymphoid cells. EMBO J. 2011;30:2934–2947.
64. Vonarbourg C, Mortha A, Bui VL et al. Regulated expression of nuclear receptor RORgammat confers distinct functional fates to NK cell receptor-expressing RORgammat(+) innate lymphocytes. Immunity. 2010;33:736–751.
65. Qiu J, Heller JJ, Guo X et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity. 2012;36:92–104.
66. Kiss EA, Vonarbourg C, Kopfmann S et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science. 2011;334:1561–1565.
67. Lee JS, Cella M, McDonald KG et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nat Immunol. 2012;13:144–151.
68. Veldhoen M, Hirota K, Westendorf AM et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature. 2008;453:106–109.
69. Yang Q, Saenz SA, Zlotoff DA, Artis D, Bhandoola A. Cutting edge: Natural helper cells derive from lymphoid progenitors. J Immunol. 2011;187:5505–5509.
70. Sandy AR, Jones M, Maillard I. Notch signaling and development of the hematopoietic system. Adv Exp Med Biol. 2012;727:71–88.
71. Dzhagalov I, Zhang N, He YW. The roles of orphan nuclear receptors in the development and function of the immune system. Cell Mol Immunol. 2004;1:401–407.
72. Jetten AM. Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. Nucl Recept Signal. 2009;7:e003.

73. SIDMAN RL, LANE PW, DICKIE MM. Staggerer, a new mutation in the mouse affecting the cerebellum. Science. 1962;137:610–612.
74. Dussault I, Fawcett D, Matthyssen A, Bader JA, Giguere V. Orphan nuclear receptor ROR alpha-deficient mice display the cerebellar defects of staggerer. Mech Dev. 1998;70:147–153.
75. Jaradat M, Stapleton C, Tilley SL et al. Modulatory role for retinoid-related orphan receptor alpha in allergen-induced lung inflammation. Am J Respir Crit Care Med. 2006;174:1299–1309.
76. Yang XO, Pappu BP, Nurieva R et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity. 2008;28:29–39.
77. Vonarbourg C, Diefenbach A. Multifaceted roles of interleukin-7 signaling for the development and function of innate lymphoid cells. Semin Immunol. 2012
78. Weber JM, Calvi LM. Notch signaling and the bone marrow hematopoietic stem cell niche. Bone. 2010;46:281–285.
79. Welch PA, Burrows PD, Namen A, Gillis S, Cooper MD. Bone marrow stromal cells and interleukin-7 induce coordinate expression of the BP-1/6C3 antigen and pre-B cell growth. Int Immunol. 1990;2:697–705.
80. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat Immunol. 2012;13:58–66.
81. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633–640.
82. Smyth MJ, Cretney E, Kelly JM et al. Activation of NK cell cytotoxicity. Mol Immunol. 2005;42:501–510.
83. Moretta A, Marcenaro E, Sivori S, Della Chiesa M, Vitale M, Moretta L. Early liaisons between cells of the innate immune system in inflamed peripheral tissues. Trends Immunol. 2005;26:668–675.
84. Gillard GO, Bivas-Benita M, Hovav AH et al. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. PLoS Pathog. 2011;7:e1002141.
85. Vivier E, Raulet DH, Moretta A et al. Innate or adaptive immunity? The example of natural killer cells. Science. 2011;331:44–49.
86. Kelly KA, Scollay R. Seeding of neonatal lymph nodes by T cells and identification of a novel population of CD3-CD4+ cells. Eur J Immunol. 1992;22:329–334.
87. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol. 2004;5:64–73.
88. Eberl G, Littman DR. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. Immunol Rev. 2003;195:81–90.
89. Eberl G, Lochner M. The development of intestinal lymphoid tissues at the interface of self and microbiota. Mucosal Immunol. 2009;2:478–485.
90. van de Pavert SA, Mebius RE. New insights into the development of lymphoid tissues. Nat Rev Immunol. 2010;10:664–674.
91. Hamada H, Hiroi T, Nishiyama Y et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J Immunol. 2002;168:57–64.
92. Bouskra D, Brezillon C, Berard M et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature. 2008;456:507–510.
93. Scandella E, Bolinger B, Lattmann E et al. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. Nat Immunol. 2008;9:667–675.
94. Kim MY, McConnell FM, Gaspal FM et al. Function of CD4+CD3- cells in relation to B- and T-zone stroma in spleen. Blood. 2007;109:1602–1610.
95. Lane P, Kim MY, Withers D et al. Lymphoid tissue inducer cells in adaptive CD4 T cell dependent responses. Semin Immunol. 2008;20:159–163.
96. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology. 2010;129:311–321.
97. Hsu HC, Yang P, Wang J et al. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. Nat Immunol. 2008;9: 166–175.
98. Mizutani N, Goshima H, Nabe T, Yoshino S. Complement C3a-Induced IL-17 Plays a Critical Role in an IgE-Mediated Late-Phase Asthmatic Response and Airway Hyperresponsiveness via Neutrophilic Inflammation in Mice. J Immunol. 2012.
99. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. Int Immunol. 2011;23:159–163.
100. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity. 2008;29:947–957.
101. Takahashi K, Hirose K, Kawashima S et al. IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation. J Allergy Clin Immunol. 2011;128: 1067–1076.
102. Kim S, Han S, Withers DR et al. CD117 CD3 CD56 OX40Lhigh cells express IL-22 and display an LTi phenotype in human secondary lymphoid tissues. Eur J Immunol. 2011;41:1563–1572.
103. Zheng Y, Valdez PA, Danilenko DM et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med. 2008;14:282–289.

104. Cox JH, Kljavin NM, Ota N et al. Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. Mucosal Immunol. 2012;5:99–109.
105. Fallon PG, Ballantyne SJ, Mangan NE et al. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. J Exp Med. 2006;203:1105–1116.
106. Fort MM, Cheung J, Yen D et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001;15:985–995.
107. Lee J, Ho WH, Maruoka M et al. IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. J Biol Chem. 2001;276: 1660–1664.
108. Saadoun D, Terrier B, Cacoub P. Interleukin-25: key regulator of inflammatory and autoimmune diseases. Curr Pharm Des. 2011;17:3781–3785.
109. Angkasekwinai P, Park H, Wang YH et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med. 2007;204:1509–1517.
110. Ikeda K, Nakajima H, Suzuki K et al. Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. Blood. 2003;101:3594–3596.
111. Kang CM, Jang AS, Ahn MH et al. Interleukin-25 and interleukin-13 production by alveolar macrophages in response to particles. Am J Respir Cell Mol Biol. 2005;33:290–296.
112. Corrigan CJ, Wang W, Meng Q et al. Allergen-induced expression of IL-25 and IL-25 receptor in atopic asthmatic airways and late-phase cutaneous responses. J Allergy Clin Immunol. 2011;128: 116–124.
113. Claudio E, Sonder SU, Saret S et al. The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation. J Immunol. 2009;182:1617–1630.
114. Swaidani S, Bulek K, Kang Z et al. The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation. J Immunol. 2009;182:1631–1640.
115. Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. J Exp Med. 2000;191:1069–1076.
116. Lohning M, Stroehmann A, Coyle AJ et al. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. Proc Natl Acad Sci USA. 1998;95:6930–6935.
117. Yanagisawa K, Naito Y, Kuroiwa K et al. The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. J Biochem. 1997;121:95–103.
118. Schmitz J, Owyang A, Oldham E et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005;23: 479–490.
119. Hong YS, Moon SJ, Joo YB et al. Measurement of interleukin-33 (IL-33) and IL-33 receptors (sST2 and ST2L) in patients with rheumatoid arthritis. J Korean Med Sci. 2011;26:1132–1139.
120. Kearley J, Buckland KF, Mathie SA, Lloyd CM. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. Am J Respir Crit Care Med. 2009;179:772–781.
121. Kondo Y, Yoshimoto T, Yasuda K et al. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. Int Immunol. 2008;20: 791–800.
122. Marvie P, Lisbonne M, L'helgoualc'h A et al. Interleukin-33 overexpression is associated with liver fibrosis in mice and humans. J Cell Mol Med. 2010;14:1726–1739.
123. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel "alarmin"? PLoS One. 2008;3:e3331.
124. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat Rev Immunol. 2010;10:103–110.
125. Wills-Karp M, Rani R, Dienger K et al. Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. J Exp Med. 2012;209:607–622.
126. McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. Curr Biol. 1998;8: 339–342.
127. Anthony RM, Rutitzky LI, Urban JFJ, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. Nat Rev Immunol. 2007;7:975–987.
128. Bancroft AJ, McKenzie AN, Grencis RK. A critical role for IL-13 in resistance to intestinal nematode infection. J Immunol. 1998;160:3453–3461.
129. Oliphant CJ, Barlow JL, McKenzie AN. Insights into the initiation of type 2 immune responses. Immunology. 2011;134:378–385.
130. Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, Sarfati M. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. J Exp Med. 1992;176:1319–1326.
131. Goswami R, Kaplan MH. A brief history of IL-9. J Immunol. 2011;186:3283–3288.
132. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res. 2006;16:3–10.
133. Voehringer D, Reese TA, Huang X, Shinkai K, Locksley RM. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. J Exp Med. 2006;203:1435–1446.
134. Enomoto Y, Orihara K, Takamasu T et al. Tissue remodeling induced by hypersecreted epidermal

growth factor and amphiregulin in the airway after an acute asthma attack. J Allergy Clin Immunol. 2009;124:913–920. e1–7.
135. Ingram JL, Rice A, Geisenhoffer K, Madtes DK, Bonner JC. Interleukin-13 stimulates the proliferation of lung myofibroblasts via a signal transducer and activator of transcription-6-dependent mechanism: a possible mechanism for the development of airway fibrosis in asthma. Chest. 2003;123:422S–424S.
136. Oriente A, Fedarko NS, Pacocha SE, Huang SK, Lichtenstein LM, Essayan DM. Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. J Pharmacol Exp Ther. 2000;292: 988–994.
137. Monticelli LA, Sonnenberg GF, Artis D. Innate lymphoid cells: critical regulators of allergic inflammation and tissue repair in the lung. Curr Opin Immunol. 2012
138. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med. 2006;12:99–106.
139. Lee CG, Homer RJ, Zhu Z et al. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). J Exp Med. 2001;194:809–821.
140. Lindemann D, Racke K. Glucocorticoid inhibition of interleukin-4 (IL-4) and interleukin-13 (IL-13) induced up-regulation of arginase in rat airway fibroblasts. Naunyn Schmiedebergs Arch Pharmacol. 2003;368:546–550.
141. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J Clin Invest. 1999;104:777–785.
142. Chiaramonte MG, Cheever AW, Malley JD, Donaldson DD, Wynn TA. Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. Hepatology. 2001;34:273–282.
143. Kaviratne M, Hesse M, Leusink M et al. IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. J Immunol. 2004;173:4020–4029.
144. Ohnishi T, Sur S, Collins DS, Fish JE, Gleich GJ, Peters SP. Eosinophil survival activity identified as interleukin-5 is associated with eosinophil recruitment and degranulation and lung injury twenty-four hours after segmental antigen lung challenge. J Allergy Clin Immunol. 1993;92:607–615.
145. Chen F, Liu Z, Wu W et al. An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. Nat Med. 2012;18:260–266.
146. Xing WW, Zou MJ, Liu S, Xu T, Wang JX, Xu DG. Interleukin-22 protects against acute alcohol-induced hepatotoxicity in mice. Biosci Biotechnol Biochem. 2011;75:1290–1294.
147. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity. 2008;28:454–467.
148. Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just T(H)2 cells. Nat Rev Immunol. 2010;10:838–848.
149. Shale M, Ghosh S. Beyond TNF, Th1 and Th2 in inflammatory bowel disease. Gut. 2008;57:1349–1351.
150. O'Reilly S, Hugle T, van Laar JM. T cells in systemic sclerosis: a reappraisal. Rheumatology (Oxford). 2012.
151. Ballantyne SJ, Barlow JL, Jolin HE et al. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. J Allergy Clin Immunol. 2007;120: 1324–1331.
152. Moffatt MF, Gut IG, Demenais F et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med. 2010;363:1211–1221.
153. Wills-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev. 2004;202:175–190.
154. Wynn TA. IL-13 effector functions. Annu Rev Immunol. 2003;21:425–456.
155. Fattouh R, Jordana M. TGF-beta, eosinophils and IL-13 in allergic airway remodeling: a critical appraisal with therapeutic considerations. Inflamm Allergy Drug Targets. 2008;7:224–236.
156. Warringa RA, Schweizer RC, Maikoe T, Kuijper PH, Bruijnzeel PL, Koendermann L. Modulation of eosinophil chemotaxis by interleukin-5. Am J Respir Cell Mol Biol. 1992;7:631–636.
157. Shi HZ, Xiao CQ, Zhong D et al. Effect of inhaled interleukin-5 on airway hyperreactivity and eosinophilia in asthmatics. Am J Respir Crit Care Med. 1998;157:204–209.
158. Camelo A, Barlow JL, Drynan LF et al. Blocking IL-25 signalling protects against gut inflammation in a type-2 model of colitis by suppressing nuocyte and NKT derived IL-13. J Gastroenterol. 2012.
159. Olman MA. Epithelial cell modulation of airway fibrosis in asthma. Am J Respir Cell Mol Biol. 2003;28:125–128.
160. Halim TY, MacLaren A, Romanish MT, Gold MJ, McNagny KM, Takei F. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. Immunity. 2012;37:463-474.
161. Hoyler T, Klose CS, Souabni A et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. Immunity. 2012;37:634-648.

Tuning Cross-Presentation of Apoptotic T Cells in Immunopathology

3

Vincenzo Barnaba

Abstract

Cross-presentation of several long-lived antigens associated with apoptotic T cells requires caspase-dependent cleavage to efficiently deliver antigenic fragments to the processing machinery of antigen-presenting cells. The resulting emergence of a large population of autoreactive $CD8^+$ T effector cells specific for apoptotic T cell-associated self-epitopes plays a key role in improving immunopathology in several infections and autoimmune diseases. Importantly, they endow mixed polyfunctional type-1, type-2, and type-17 responses and correlate with the chronic progression of various pathological conditions. This evolution is related to the selection of autoreactive $CD8^+$ T cells with higher T cell receptor avidity, whereas those with lower avidity undergo prompt contraction in patients undergoing disease resolution. The development of mixed responses with divergent differentiation requirements is consistent with distinct sites or kinetics of $CD8^+$ T cell priming in vivo. Therefore, we propose a strict link among cross-presentation of apoptotic T cells, the generation of high frequencies of mixed autoreactive $CD8^+$ T cells producing a broad array of cytokines (IFN-γ, IL-17, IL-4, IL-2, etc.), and the progression towards chronic inflammatory diseases.

Keywords

Cross-presentation • Apoptotic T cells • Apoptotic epitope-specific $CD8^+$ T cells

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3.1 Introduction

The capacity of conventional (or myeloid) dendritic cells (cDCs) to present exogenous antigens associated with other cells (usually necrotic or apoptotic cells) or soluble antigens on major histocompatibility complex (MHC) class I molecules

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is defined as cross-presentation [1–4]. In humans, cDCs are subdivided in two populations, on the basis of expression of CD1c (BDCA-1) or CD141 (BDCA-3). CD141$^+$ cDCs seem to represent the human counterpart of mouse lymphoid CD8$^+$ DCs, because both these subsets perform cross-presentation mechanism with high efficiency. This phenomenon seems crucial for inducing either cross-priming or cross-tolerance of CD8$^+$ T cells, based on the presence or absence of various infectious or danger signals influencing the switch from tolerogenic immature cDCs to mature cDCs with high stimulatory and migratory capacities [1–5]. Under steady state conditions, cDCs phagocytose self-antigens associated with dying (e.g., apoptotic) tissue cells (derived from the physiological cell turnover), process them, present the resulting peptides on MHC class II or class I molecules, and migrate into the draining lymph nodes with very low efficiency, where they can induce tolerance or cross-tolerance of autoreactive CD4$^+$ T cells or CD8$^+$ T cells, respectively (peripheral tolerance). By contrast, in an inflammatory context, cDCs are activated (for instance, through engagement by pathogen-associated molecular patterns [PAMPs] or necrotic cell products), increase for a short time the ability to internalize and to process both tissue-derived microbial and self-antigens as well as the expression of stimulatory, costimulatory, and pro-migratory molecules (i.e., the lymph node-specific chemokine receptors (Cys–Cys chemokine receptor 7 [CCR7])) [1–6]. Then, they can reach the lymph nodes, where they prime or cross-prime both microbial- or self-antigen-specific naïve CD4$^+$ or CD8$^+$ T lymphocytes. cDCs can also induce regulatory T (Treg) cells [7], the key cell population involved in the regulation of immune responses and homeostasis [8], either at the steady state condition contributing to peripheral tolerance or modulating excessive and unwanted immune responses in an inflammatory context.

The fate of the enormous number of apoptotic cells that derive from effector T cells undergoing apoptosis after performing their functions during acute or chronic infections remains to be determined [9, 10]. In particular, during acute or chronic HIV, hepatitis B or C virus (HBV or HCV) infections, the corresponding infected tissues (lymphoid tissue for HIV, liver for HBV or HCV) are largely inflamed and infiltrated by several billions of activated lymphocytes (~$3–5 \times 10^8$ per liver in the case of HBV or HCV infection in humans, of which ~50% are activated αβT cells, ~30% NK, ~10% NKT, ~5% γδT cells, etc.). The majority of these infiltrating lymphocytes are not virus specific (that are ~10% of the total), and ~90–95% of them are activated and undergo apoptosis upon performing their effector functions: interestingly, the rate of apoptotic lymphocytes by far exceeds that of apoptotic cells derived from the turnover of tissue cells, during severe inflammatory diseases [11]. In the light of these observations, some logical questions uprise: which is the fate of this huge amount of apoptotic lymphoid cells, whose turnover is sustained over time in inflammatory conditions? Might part of apoptotic lymphocytes escape from the digestion by the phagocytic system, and become source of self-antigens? And hence, might they become relevant in the induction of either tolerance or autoimmunity, particularly upon phagocytosis and cross-presentation by cDCs?

3.2 CD40 Ligand Expression on Apoptotic T Cells Abrogates Tolerance

An additional support for the idea that cDCs carry out opposing functions according to the microenvironment in which they work is provided by studies investigating the complex interplay among cDCs, cross-presentation, apoptotic cells, and stimulatory signals. Apoptosis occurring physiologically during the development of a given tissue should cause tolerance, whereas apoptosis caused by a microbial infection or other inflammatory processes should result in T cell priming [12, 13]. In the steady state conditions, apoptotic cells have been proposed to induce T cell deletion, anergy, immune deviation from Th1 to Th2 responses, or Treg cell induction**,** via a series of not completely clear mechanisms, including the capacity of apoptotic cells to release immunosuppressive cytokines (i.e., IL-10 or TGF-β), or to engage receptors (i.e., CD36 or phosphatidylserine receptors) subverting the

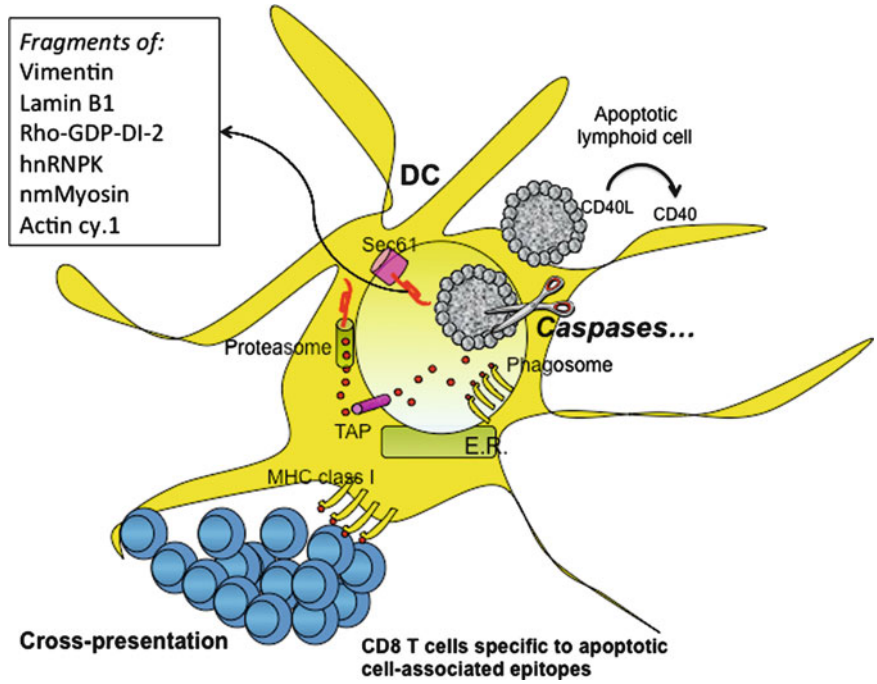

Fig. 3.1 Caspases facilitate cross-presentation of long-lived proteins (LLiPs) within apoptotic cells. CD40L$^+$ apoptotic T cells both deliver activation signals to DCs and are efficiently phagocytosed. Caspases cut fragments from LLiPs that can be released within phagosomes of cDCs and exported into cytosol. Processing of LLiP fragments is mediated by the classical class I pathway, including proteasomes processing fragments in short peptides, and TAPs. The figure emphasizes the model dictating that TAPs retro-transport the resulting apoptotic self-epitopes into phago-endosomal compartments, where they can bind the appropriate class I molecules and can be finally unveiled and cross-presented to the related autoreactive CD8$^+$ T cells

stimulatory DC functions [14–18]. In addition, it has been demonstrated that apoptotic cell-dependent tolerance can be determined by the caspase-induced production of a reactive oxygen species scavenger leading to oxidization and inactivation of the high-mobility group protein B1, a powerful danger signal normally involved in the full cDC activation and immune response initiation [19]. However, the presence of sustained infectious/inflammatory mediators, necrotic cell products, or CD4$^+$ T helper (h) cells can bypass the tolerogenic effects of apoptotic cells and induce cDCs to prime or cross-prime T cells [12, 20–23].

Our previous data proposed that an alternative mechanism of abrogation of the apoptosis-associated tolerance can take place through the expression of CD40L by apoptotic cells [12]. CD40L$^+$ apoptotic cells can derive from activated CD40L$^+$ T cells that undergo apoptosis once they have performed their effector function in a given inflamed tissue or upon infection with viruses infecting activated T cells, such as HIV. CD40L$^+$ apoptotic T cells directly induce cDC maturation and condition them to induce cross-priming of CD8$^+$ T cells specific to apoptotic cell-associated self-antigens, irrespective of additional exogenous signals [12] (Fig. 3.1). In contrast, if apoptotic T cells are CD40L$^-$ (such as those derived from resting T cells), the help of a third party activated T cell or surrogate CD40L molecule is needed for priming. The finding that CD40L$^+$ apoptotic T cells induce cDC maturation and cross-priming without the addition of exogenous stimuli indicates that the surface phenotype and possibly the lineage of origin of apoptotic cells may ultimately dictate the outcome of cross-presentation. This notion may help reconcile several apparently contradictory findings. For example, it explains why apoptotic cells derived from epithelial or resting T cells that are CD40L$^-$

are unable to provide cDC maturation stimuli and are tolerogenic in the absence of sustained infectious/inflammatory signals [12, 19, 20, 24]. Thus, the balance between $CD40L^+$ and $CD40L^-$ apoptotic cells during cross-presentation appears to dictate tolerance or induction of $CD8^+$ T cell responses against T cell-associated epitopes, and to maintain or to stop the related responses in the course of an inflammatory process. As a result, the signals provided by $CD40L^+$ apoptotic cells and not those provided by conventional apoptotic cells have been demonstrate to facilitate the emergence of autoreactive T cell responses to apoptotic self-antigens [12, 25]. In addition, these responses have a critical role in the amplification of chronic inflammation via the continuous bystander effects of inflammatory cytokines produced by T cells specific for the apoptotic T cell-associated antigens [12, 22].

3.3 Apoptotic T Cells Are Source of Immunogenic Self-Antigens

In previous studies, we found that the proteome of apoptotic T cells includes prominent caspase-cleaved cellular proteins and that a high proportion of distinct epitopes in these fragments (apoptotic epitopes) can be cross-presented by cDCs to a wide repertoire of autoreactive $CD8^+$ T cells [22]. In particular, the majority of proteins differently expressed between apoptotic and live T cells belonged to cytoskeleton, cytoplasmic, or nuclear structures (including vimentin, non-muscle myosin, Rho-GDP-DI-2, human nuclear ribonucleoprotein K, lamin B1, actin cytoplasmic 1), with a main difference: those derived from live T cells corresponded to the entire proteins, whereas those derived from apoptotic T cells corresponded to fragments of the same proteins cleaved by caspases [22]. This observation led us to demonstrate a new role for caspases in facilitating cross-presentation of apoptotic (fragmented) proteins that derive from cleavage of long-lived proteins (LLiPs) strictly anchored to cellular structures (e.g., cytoskeleton) (Fig. 3.1). In particular, it is well known that LLiPs [in contrast to short-lived proteins (SLiPs), including defective ribosomal initiation products (DRiPs)] are not efficiently processed and directly presented by live cells expressing them [26]. This rule can be subverted by caspases, by their capability to cut fragments from LLiPs within apoptotic antigen-donor cells (ADCs) that are efficiently phagocytosed by cDCs. As a consequence, LLiP fragments cleaved by caspases within apoptotic ADCs can be released within phagosomes of cDCs and ultimately processed and cross-presented (Fig. 3.1). A proof of principle of this possibility is the demonstration that the down-regulation of caspases 3 and/or 8 expression by mRNA interference or inhibitory chemical treatments impeded formation of LLiP fragments within apoptotic T cells (as detected by proteomic analyses) and cross-presentation of the related apoptotic epitopes [22]. As a control, the same treatment did not affect the cross-presentation of apoptotic cells expressing the short-lived nonstructural (NS)3 protein of HCV to NS3-specific $CD8^+$ T cell clone. Interestingly, processing of LLiP fragments by cDCs leading to cross-presentation is mediated by classical pathway of class I processing, including TAPs and proteasome, and it did not involve the lysosomal pathway of the cDCs. This means that LLiP fragments have to be exported into cytosol after phagocytosis to be processed by classical MHC class I pathway.

Other mechanisms might intervene in favoring cross-presentation of cell-associated antigens, such as macroautophagy leading to the accumulation of autophagosomes [27]. The latter can capture antigenic products within dying ADCs, protect them from excessive degradation in lysosomal compartments, and deliver them to APCs in their intact or semi-intact forms [27]. This possibility may account for our previous observation showing that chloroquine, known to inhibit lysosomal fusion, enhances cross-priming by protecting antigens (likely within autophagosomes) from lysosomal acidification [28].

However, our studies were the first to identify caspase-mediated cleavage of LLiPs as a mechanism for cross-priming $CD8^+$ T cells specific to apoptotic cell-associated self-epitopes. In particular, processing of caspase-cleaved fragments of vimentin, non-muscle myosin, Rho-GDP-DI-2, human nuclear ribonucleoprotein K, lamin B1,

and actin cytoplasmic 1 has been demonstrated to result in the cross-presentation of a huge panel of resulting apoptotic self-epitopes to the related autoreactive CD8⁺ T cells in different pathological conditions. Recent reports have confirmed the role of caspase cleavage in the processing and presentation of epitopes that are derived from apoptotic cells in different models [29–31].

3.4 Relevance of Cross-Presentation of Apoptotic Epitopes in Clinical Setting

Our primal study showed that autoreactive CD8⁺ T cells specific to apoptotic self-epitopes derived from several of the apoptotic T cell-associated LLiP fragments mentioned above are extremely represented in the peripheral blood of patients with chronic HIV infection [22]. They correlated with the proportion of peripheral apoptotic CD4⁺ T cells in vivo and their expansion and function contributed in establishing polyclonal T cell activation, a major feature in HIV-infected individuals that in the long run results in generalized T cell dysfunction/depletion ultimately leading to AIDS [22].

More recently, we questioned about the relevance of CD8⁺ T cells specific to apoptotic self-epitopes in the progression of HCV infection, since it is well known that about 60% of individuals with acute HCV infection progress towards the chronic evolution of infection, whereas the remainder undergoes resolution. Importantly, the multispecificity, magnitude, and polyfunctional (type-1, -2, -17) strength of CD8⁺ T memory effector (ME) cell responses directed to apoptotic self-epitopes were wide and robust during the acute phase of HCV infection, particularly in patients experiencing chronic progression compared with those undergoing infection resolution [32]. The responses were directly correlated with the plasma viral load, the serum ALT levels, or the number of circulating apoptotic T cells, and were then sustained over time in relation to the viral persistence. In addition, similar autoreactive CD8⁺ T cell responses in chronically infected patients are recruited in the inflamed liver, are related with the signs of hepatic damage, and decrease in relation with the decline or the disappearance of the viral load upon antiviral therapy (interferon plus ribavirin®). Altogether these results suggest that strong CD8⁺ T cell responses against apoptotic self-epitopes arise and are maintained in HCV infection, correlate with the progression towards the chronic evolution of infection, and may potentially contribute to the liver immunopathology through the production of high levels of inflammatory cytokines.

The observation that cross-presentation of apoptotic T cells by DCs requires caspase-dependent cleavage of apoptotic self-antigens to promptly activates specific CD8⁺ T_{EM} cells ex vivo indicates that this mechanism might be operative in the induction of the related polyfunctional autoreactive responses in vivo [32]. This possibility is emphasized by the finding that the frequencies of apoptotic epitope-specific CD8⁺ T cells correlated with the number of circulating apoptotic T cells. Cross-presentation of apoptotic cells plays a key role in activating autoreactive CD8⁺ T cells, as caspase-dependent cleavage of cell-associated (long-lived) proteins (such as vimentin, non-muscle myosin, actin, heterogeneous nuclear ribonucleoprotein, and lamin B1) is required to efficiently target the related fragments to the processing machinery of DCs. By contrast, live DCs alone, despite known to express the whole form of the same ubiquitous (long-lived) cellular proteins, are unable to stimulate the related specific CD8⁺ T cells by direct presentation mechanism, likely because they do not possess the caspase-cleavage program required for the presentation of these proteins [22]. Collectively, these data suggest that these autoreactive CD8⁺ T cells may perform their functions through the bystander effect of the pro-inflammatory cytokines upon cross-presentation of apoptotic cells rather than by the direct killing of cells endogenously expressing the related self-antigens. The strong production of IFN-γ and IL-17 may favor the triggering of recruitment of inflammatory cells, which contribute to the immunopathology.

Our study provides a possible explanation for why the enormous expansion of activated T cells, during persisting viral infections, is only minimally attributable to virus-specific T cells [11]. Inflamed tissues (including the HCV-infected

liver) are generally infiltrated by several billions of activated lymphocytes and the rate of apoptotic cells derived from them by far exceeds that originated by the turnover of epithelial cells (i.e., hepatocytes) [11]. The demonstration that apoptotic cells derived from activated T cells (in contrast to those derived from epithelial cells) are $CD40L^+$ and then condition $CD40^+$ DCs to prime T cells [12, 25] suggest that they are the most important source of apoptotic self-antigens capable to cross-prime $CD8^+$ T cell responses in an inflamed microenvironment. However, we cannot exclude that also apoptotic hepatocytes may amplify this phenomenon in an inflammatory context, because they might potentially generate the same caspase-cleaved antigenic fragments described in apoptotic T cells [22], and be cross-presented by DCs.

Recent data have clearly stressed the importance of infections in inducing and maintaining autoimmunity [33]. In particular, the initial emergence of apoptotic antigen-specific T cells in acute HCV infection may be dependent on virus-specific T cells that can provide the first waves of apoptotic substrates, upon performing their effector function. This mechanism may be maintained in patients evolving towards the viral persistence, and be further amplified by the apoptotic antigen-specific T cells themselves providing further waves of apoptotic antigens.

The relevant polyfunctional responses to apoptotic self-epitopes observed in patients undergoing chronic infection suggest that the environmental setting during an acute inflammatory disease seems to be addressed to guarantee the coexisting polarization of type-1, -2, -17, -1/17 $CD8^+$ T_{EM} cells, and even type-2/17 $CD8^+$ T_{EM} cells, likely to limit excessive damage by fine-polarized type-1 or type-17 $CD8^+$ T_{EM} cells [32]. In support of this hypothesis, our study in patients with long-term chronic HCV infection demonstrates that liver-infiltrating $CD8^+$ T_{EM} cells specific to apoptotic self-epitopes produce levels of cytokines significantly lower than patients with acute hepatitis.

An important facet of our findings is that they demonstrate a link between the TCR avidity of autoreactive $CD8^+$ T cells and the difference in the responsiveness of apoptotic epitope-specific $CD8^+$ T cells exhibited by patients experiencing chronic infection and those undergoing infection resolution [32]. The dissociation kinetics of peptide/HLA-A*0201 pentamer binding [34] to antigen-specific $CD8^+$ T cells clearly demonstrated that the $t_{1/2}$ for apoptotic epitope-complexed pentamer staining to $CD8^+$ T cells from patients experiencing chronic infection was significantly longer than the decay of pentamer staining from patients undergoing infection resolution. The $t_{1/2}$ for pentamer staining was detected on freshly isolated $CD8^+$ T cells, suggesting that TCR avidity measured by this system likely reflects what occurs in vivo. Recent studies suggested that in response to different microbial infections, initially naïve T cells with a wide range of avidity are efficiently recruited and expanded [35, 36]; subsequently, those with lower avidity undergo premature contraction, whereas those with higher avidity are selected because of a more prolonged expansion and correlate with protection [35, 36]. Our results provide an additional challenge to this model, and demonstrate that the TCR avidity of autoreactive $CD8^+$ T cells specific for apoptotic self-epitopes was significantly higher in patients undergoing chronic infection than in those resolving infection. The selection of the autoreactive $CD8^+$ T cells with higher avidity likely occurs because of a sustained stimulation by apoptotic antigens [22]. By contrast, lower avidity $CD8^+$ T cells in the presence of weaker stimuli would undergo rapid contraction, as seen in the peripheral blood of patients with self-limited HCV infection. The viral persistence may provide the conditions that influence the availability of sustained apoptotic antigenic stimuli.

Studies in progress suggest that the generation of polyfunctional $CD8^+$ T cell responses to apoptotic self-epitopes may represent a general phenomenon sustaining several pathological conditions. Indeed, we found similar responses in various autoimmune diseases, such as rheumatoid arthritis or multiple sclerosis (MS). Particularly, the frequencies of polyfunctional $CD8^+$ T cells to apoptotic self-epitopes correlate with the disease activity and progression in MS patients, suggesting that they may contribute to the immunopathology and chronic evolution of MS through the production of inflammatory

cytokines (manuscript in preparation). Therefore, irrespective of the nature of the primary triggers initiating central nervous system (CNS) immunopathology (immune responses to self-tissue antigens (e.g., myelin antigens) or antigens of unknown pathogens), they may induce apoptotic antigen-specific T cells by providing them the first boost of apoptotic antigens. Then, the apoptotic antigen-specific T cells can both amplify and sustain the immunopathology through their capacity to produce high levels of inflammatory cytokines and supply further apoptotic substrates for the generation of novel autoreactive responses to apoptotic antigens.

3.5 Conclusion

We can envisage the following scenario. At the onset of an immune-mediated process (initiated and maintained either by a persisting pathogen, such as by HCV, or the breakdown tolerance towards a given organ self-antigen), tissue-resident cDCs could capture either virus-infected or "self-antigen-expressing" cells, according to the different pathological conditions (infection vs. autoimmunity) (Fig. 3.2). Then, they could migrate into the draining lymph nodes (LNs) and cross-prime virus-specific or self-reactive $CD8^+$

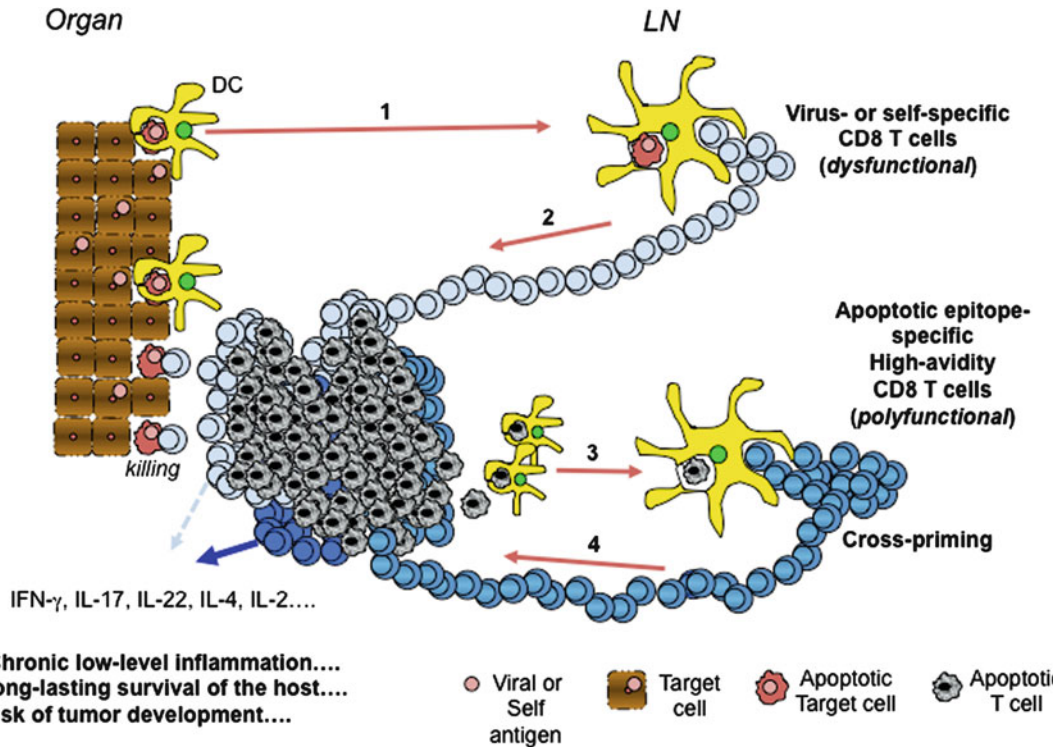

Fig. 3.2 Cross-presentation of apoptotic T cells leading to immunopathology. (1) Tissue-resident cDCs capture dying organ cells (i.e., during viral infection or autoimmunity), migrate into draining LNs, where they cross-prime virus-specific or self-reactive $CD8^+$ T cells, respectively. These responses will result as dysfunctional, because of the persistent antigenic stimuli inducing PD-1-dependent exhaustion, the generation of Treg cells, etc. (2) The continuous migration of these dysfunctional T cells into the target organ leads to the establishment of a state of chronic low-level inflammation. (3) Apoptotic cells derived from these activated T cells are captured by other tissue-resident cDCs that migrate into LNs and cross-prime polyfunctional, high-avidity apoptotic self-epitope-specific $CD8^+$ T cells. (4) In turn, they migrate into inflamed tissue and undergo apoptosis upon performing their polyfunctions, providing hence further apoptotic antigen substrates maintaining continuous activation and migration of apoptotic epitope-specific T cells. In the long run, the simultaneous production of several cytokines (some of which display contrasting functions) together with the involvement of subsequent regulatory mechanisms (i.e., PD-1, Tregs, etc.) contributes to sustain the chronic low-level inflammation, favoring a long-lasting survival of the host, on the one hand, and possibly the development of tumors, on the other hand

T cells, respectively. In the long run, these responses will result as dysfunctional (low cytokine production, low cytotoxic function, etc.), because of the emergence of several mechanisms, including PD-1-dependent exhaustion of chronically stimulated virus-specific or organ self-specific T cells, generation of Treg cells, etc. The viral or the self-antigen persistence will maintain the generation of continuous waves of dysfunctional specific T cells migrating into the organ. Upon performing their effector *dys*functions, they undergo apoptosis and the resulting apoptotic cells can be captured by other tissue-resident cDCs that migrate into LNs and cross-prime apoptotic self-epitope-specific $CD8^+$ T cells (Fig. 3.2). These cells are polyfunctional (producing a vast array of cytokines) and express a high-avidity TCR enabling them to exert strong inflammatory responses in the organ, where they have efficiently migrated. In turn, they will undergo apoptosis upon performing their functions and the resulting apoptotic cells will contribute to shape the apoptotic antigen substrates essential for the maintenance of continuous cross-priming of apoptotic epitope-specific T cells. Therefore, the apoptotic substrates will derive from two sources: the virus-specific or the organ (self)-specific T cells, on the one hand, and the apoptotic epitope-specific T cells themselves, on the other hand. The resulting storm of inflammatory cytokines particularly produced by apoptotic epitope-specific T cells will contribute to the immunopathology persistence. However, the simultaneous production of several cytokines (some of which display contrasting functions) together with the involvement of subsequent regulatory mechanisms (i.e., PD-1, Tregs, etc.) will result in a state of chronic low-level inflammation that is instrumental to favor a long-lasting survival of the host [37, 38]. The chronic low-level inflammation can favor the development of tumors in the long run [37, 38].

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References

1. Bevan MJ (1976) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med* 143: 1283–1288.
2. Heath WR, Carbone FR (2001) Cross-presentation, dendritic cells, tolerance and immunity. *Ann. Rev. Immunol.*19: 47–64.
3. Mellman I, Steinman RM (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106: 255–258.
4. Steinman RM, Hawiger D, Nussenzweig MC (2003) Tolerogenic dendritic cells. Annual review of immunology 21: 685–711.
5. Matzinger P (2002) The danger model: a renewed sense of self. *Science* 296: 301–305.
6. Medzhitov, R. (2010). Inflammation 2010: new adventures of an old flame. *Cell* 140, 771–776.
7. Yamazaki, S., Inaba, K., Tarbell, K.V. and Steinman, R.M. (2006). Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. *Immunol. Rev.* 212, 314–329.
8. Sakaguchi, S., Miyara, M., Costantino, C.M. and Hafler, D.A. (2010). FOXP3+ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 10, 490–500.
9. Green DR, Ferguson T, Zitvogel L, Kroemer G (2009) Immunogenic and tolerogenic cell death. *Nat. Rev. Immunol.* 9: 353–363.
10. Ravichandran KS (2010) Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J. Exp. Med.* 207: 1807–1817.
11. Nuti S, Rosa D, Valiante NM, Saletti G, et al. (1998) Dynamics of intra-hepatic lymphocytes in chronic hepatitis C: enrichment for Valpha24+ T cells and rapid elimination of effector cells by apoptosis. *Eur. J. Immunol.* 28: 3448–3455.
12. Propato, A., Cutrona, G., Francavilla, V., Ulivi, M., et al. (2001). Apoptotic cells overexpress vinculin and induce vinculin-specific cytotoxic T-cell cross-priming. *Nat Med* 7, 807–813.
13. Winau, F., Weber, S., Sad, S., de Diego, J., Hoops, S.L., Breiden, B., Sandhoff, K., Brinkmann, V., Kaufmann, S.H. and Schaible, U.E. (2006). Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* 24, 105–117.

14. Ferguson, T.A., Green, D.R. and Griffith, T.S. (2002). Cell death and immune privilege. *Int Rev Immunol* 21, 153–172.
15. Kurts, C., Heath, W.R., Kosaka, H., Miller, J.F. and Carbone, F.R. (1998). The peripheral deletion of autoreactive CD8+ T cells induced by cross-presentation of self-antigens involves signaling through CD95 (Fas, Apo-1). *J. Exp. Med.* 188, 415–420.
16. Chen, W., Frank, M.E., Jin, W. and Wahl, S.M. (2001). TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 14, 715–725.
17. Gao, Y., Herndon, J.M., Zhang, H., Griffith, T.S. and Ferguson, T.A. (1998). Antiinflammatory effects of CD95 ligand (FasL)-induced apoptosis. *J. Exp. Med.* 188, 887–896.
18. Serhan, C.N. and Savill, J. (2005). Resolution of inflammation: the beginning programs the end. *Nat Immunol* 6, 1191–1197.
19. Kazama, H., Ricci, J.E., Herndon, J.M., Hoppe, G., Green, D.R. and Ferguson, T.A. (2008). Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 29, 21–32.
20. Albert, M.L. (2004). Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nat Rev Immunol* 4, 223–231.
21. Inaba, K., Turley, S., Yamaide, F., Iyoda, et al. (1998). Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* 188, 2163–2173.
22. Rawson PM, Molette C, Videtta M, Altieri L, Franceschini D, et al. (2007) Cross-presentation of caspase-cleaved apoptotic self antigens in HIV infection. *Nat. Med.* 13: 1431–1439.
23. Tesniere, A., Apetoh, L., Ghiringhelli, F., Joza, et al. (2008). Immunogenic cancer cell death: a key-lock paradigm. *Curr Opin Immunol* 20, 504–511.
24. De Vita, L., Accapezzato, D., Mangino, G., Morrone, S., et al. (1998). Defective Th1 and Th2 cytokine synthesis in the T-T cell presentation model for lack of CD40/CD40 ligand interaction. *Eur. J. Immunol.* 28, 3552–3563.
25. Gurung P, Kucaba TA, Ferguson TA, Griffith TS (2009) Activation-induced CD154 expression abrogates tolerance induced by apoptotic cells. *J. Immunol.* 183: 6114–6123.
26. Yewdell, JW, Anton LC, and Bennink JR (1996). Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J. Immunol*. 157, 1823–1826.
27. Joubert P-E, and Albert ML (2012). Antigen cross-priming of cell-associated proteins is enhanced by macroautophagy within antigen donor cells. *Front Immunol.* Doi: 10.3399/fimmu. 2012.00061.
28. Accapezzato D, Visco V, Francavilla V, Molette C, et al. (2005) Chloroquine enhances human CD8+ T cell responses against soluble antigens in vivo. *J. Exp. Med.* 202, 817–828.
29. Pang B, Neijssen J, Qiao X, Janssen L, Janssen H, et al. (2009) Direct antigen presentation and gap junction mediated cross-presentation during apoptosis. *J. Immunol.* 183: 1083–1090.
30. Lopez D, Garcia-Calvo M, Smith GL, Del Val M (2010) Caspases in virus-infected cells contribute to recognition by CD8+ T lymphocytes. *J. Immunol.* 184: 5193–5199.
31. Rock KL, Farfan-Arribas DJ, Shen L (2010) Proteases in MHC class I presentation and cross-presentation. *J. Immunol.* 184: 9–15.
32. Franceschini D, Del Porto P, Piconese S, Trella E et al. (2012). Polyfunctional type-1, -2, and -17 $CD8^+$ T Cell responses to apoptotic self-antigens progress with the chronic evolution of hepatitis C virus infection. *Plos Pathogens*. 2012; 8(6):e1002759. Doi: 10.1371/journal.ppat.1002759. Epub 2012 Jun 21.
33. Wu HJ, Ivanov, II, Darce J, Hattori K, Shima T, et al. (2010) Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 32: 815–827.
34. Savage PA, Boniface JJ, Davis MM (1999) A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity* 10: 485–492.
35. Zehn D, Lee SY, Bevan MJ (2009) Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458: 211–214.
36. van Heijst JW, Gerlach C, Swart E, Sie D, Nunes-Alves C, et al. (2009) Recruitment of antigen-specific CD8+ T cells in response to infection is markedly efficient. *Science* 325: 1265–1269.
37. Barnaba V, Paroli M, Piconese S (2012) The ambiguity in immunology. *Front. Immunol.* 2012; 3:18. doi: 10.3389/fimmu.2012.00018. Epub 2012 Feb 22.
38. Barnaba V. Hepatitis C virus infection: a "liaison a trois" amongst the virus, the host, and chronic low-level inflammation for human survival. *J Hepatol* 53(4):752–61, 2010.

Regulation of Type 2 Immunity by Basophils

4

David Voehringer

Abstract

The immune response against helminths and allergens is generally characterized by high levels of IgE and increased numbers of Th2 cells, eosinophils, and basophils. Basophils represent a relatively rare population of effector cells and their in vivo functions are incompletely understood. Recent studies with basophil-depleting antibodies revealed that these cells might play an important role during the early and late stages of type 2 immune responses. To further characterize the relevance of basophils for protective immunity and orchestration of allergic inflammation, we generated constitutively basophil-deficient mice. We observed a normal Th2 response induced by helminth infections or immunization with alum/OVA or papain/OVA. However, basophils contributed to worm expulsion during secondary helminth infection and mediated an IgE-dependent inflammatory response of the skin. These results argue against a critical role of basophils as antigen-presenting cells for induction of Th2 polarization and highlight their effector cell potential during later stages of a type 2 immune response.

Keywords

Helminths • Allergy • IgE • Th2 cells

4.1 Basophil Development and Homeostasis

Basophils constitute a distinct hematopoietic cell lineage although they are phenotypically and functionally related to mast cells and eosinophils. Mast cells and basophils are the main cell types that express the high-affinity Fc epsilon receptor (FcεRI) with a configuration consisting of the IgE-binding α-chain and the signal-transducing

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β- and γ_2-chains. However, a αβ-configuration of FcεRI can be found in other cell types including eosinophils, monocytes, and subsets of dendritic cells. Mast cells and basophils further express high levels of the IL-3 receptor and produce a similar set of effector molecules including histamine, Th2-associated cytokines, and lipid mediators. However, mast cells can be distinguished from basophils by their expression of the receptor tyrosine kinase c-Kit. On the other hand, basophils share some characteristics with eosinophils including the condensed nuclear morphology, completion of cellular maturation in the bone marrow, and a short lifespan.

A model has been proposed how the eosinophil, basophil, and mast cell lineages may be connected developmentally [1]. All three lineages seem to originate from a granulocyte/monocyte precursor population which expresses the transcription factor c/EBPα. If in addition the transcription factor GATA-2 is expressed in these precursor cells, they start to develop into eosinophils. In contrast, when c/EBPα is down-regulated while GATA-2 is up-regulated the cells differentiate into a basophil/mast cell common precursor population that can give rise to basophils by secondary up-regulation of c/EBPα or to mast cells if c/EBPα remains down-regulated.

Mature basophils have a lifespan of about 60 h under steady-state conditions [2]. However, the lifespan can increase during inflammatory conditions. Helminth infections of mice have shown that basophilia results from increased de novo production of basophils in the bone marrow and probably a reduced rate of apoptosis [2]. The main cytokine that stimulates basophil development is IL-3. T cell-derived IL-3 has been shown to play a critical role for mobilization of basophils and for their recruitment to lymph nodes [3, 4]. More recently, it has been shown that another cytokine of the hematopoietin family named thymic stroma-derived lymphopoietin (TSLP) can also induce basophilia in mice [5]. IL-3- and TSLP-elicited basophils express different sets of genes and may acquire different effector functions. Further studies are required to determine whether these populations represent distinct subsets of basophils.

4.2 The Role of Basophils for Differentiation of Th2 Cells

Basophils have been proposed to play a crucial role for Th2 cell differentiation in mice. A spontaneous Th2 bias can be observed in mice with enhanced basophil numbers like interferon-response factor 2 (IRF2)-deficient [6] or Lyn tyrosine kinase-deficient mice [7]. In vitro cultures have shown that basophil-derived IL-4 promotes Th2 polarization [8]. However, controversial studies were reported with regard to the role of basophils for Th2 polarization in vivo.

Basophils increase in numbers in draining lymph nodes at early time points after allergen administration or helminth infection of mice [9]. They express large amounts of IL-4 and low levels of MHC-II [10]. Antibody-mediated depletion of basophils resulted in poor Th2 development in response to the pro-allergic protease papain [10]. On the other hand depletion of dendritic cells (DCs) by injection of diphtheria toxin in CD11c-DTR mice had no effect. In addition, selective expression of MHC-II on DCs resulted in a poor Th2 response against the helminth *Trichuris muris* suggesting that DCs are not important as antigen-presenting cells for initiation of Th2 responses at least under some experimental conditions [11]. However, other studies showed that DCs are critical for Th2 polarization. It was observed that DCs and basophils cooperate during induction of Th2 cells in response to papain [12]. Using a model of allergic inflammation of the lung, it was further shown that depletion of basophils with the MAR-1 antibody which binds to FcεRI also depletes a subset of DCs. These FcεRI$^+$ DCs appeared critical for Th2 polarization in draining lymph nodes [13]. Another study showed that the Th2 response to *Schistosoma mansoni* eggs required DCs and occurred independently of basophils [14]. We recently generated a genetically basophil-deficient mouse strain (Mcpt8Cre mice) and demonstrated that Th2 polarization in response to papain, alum/OVA, or *Nippostrongylus brasiliensis* infection occurred independently of basophils [15]. These results were also confirmed by

others with another strain of basophil-deficient mice [16].

Taken together, basophils are potent producers of IL-4 but they appear dispensable for Th2 polarization in most experimental models studied so far. However, it remains possible that basophils promote Th2 polarization under certain conditions especially during memory responses [17].

4.3 Basophils as Mediators of Allergic Responses

Basophils can be found in increased numbers in mucosal tissue of patients suffering from allergic rhinitis [18] or atopic asthma [19, 20]. The effector functions of basophils in allergic airway inflammation include the release of vasodilators like histamine or platelet-activating factor and Th2-associated cytokines like IL-4, IL-5, and IL-13. In contrast to mast cells which dominate the early response, basophils appear more important during the late-phase response [21].

Allergic reactions of the skin are also associated with basophil recruitment and activation. A recent study found that basophils were increased in skin biopsies of patients suffering from atopic dermatitis, urticaria, scabies, prurigo, Henoch-Schonlein purpurea, insect bites, eosinophilic pustular folliculitis, and bullous pemphigoid [22]. At present it remains unclear to what extent basophils contribute to inflammation of allergic skin reactions. Murine models have shown that IgE-mediated basophil activation in the skin is essential for a late phase of skin inflammation suggesting that basophils may indeed play a crucial role during the effector phase of allergic responses in the skin [15, 23, 24].

Basophils were also reported to contribute to systemic anaphylactic reactions. In a murine model of anaphylaxis basophils have been shown to release platelet-activating factor (PAF), a potent vasodilator, when stimulated by cross-linking of IgG1 bound to activating Fc gamma receptors on the cell surface [25]. Depletion of basophils with monoclonal antibodies prevented the anaphylactic reaction. However, similar experiments in genetically basophil-deficient mice could not confirm this observation [15]. Furthermore, other cell types like neutrophils or macrophages have also been shown to be important for IgG1-mediated anaphylaxis [26, 27].

4.4 Protective Immunity Against Ticks and Helminths

Ticks of the Ixodid family are ectoparasites that inject their mouth parts through the skin and suck blood from the host. During that process several substances are injected with the tick saliva into the host to avoid blood clotting and inflammation [28]. A study from almost 40 years ago has shown that basophils accumulate in the skin of guinea pigs infested with tick larvae [29]. It was further shown that injection of a basophil-depleting antiserum resulted in loss of resistance against secondary tick infestation [30]. However, it remained unclear whether basophils directly contribute to tick resistance. A recent report demonstrated that depletion of basophils by injection of diphtheria toxin in mice which express the diphtheria toxin receptor exclusively on basophils results in diminished resistance against the tick *Haemaphysalis longicornis* [23]. The authors further observed that mast cells are also required for tick resistance. However, expression of activating Fc receptors was required on basophils and not on mast cells. Whether basophils are involved in limiting dissemination of tick-transmitted pathogens remains to be determined.

Helminths are parasitic worms and comprise a very diverse spectrum of organisms that are generally well adapted to their hosts and can often persist for months to years. Gastrointestinal helminths are transmitted by the oral route or by penetration of larvae through the skin. They induce a type 2 immune response characterized by Th2 polarization, eosinophilia, and high serum IgE levels. Mast cells and basophils are also mobilized and can increase in numbers in infected tissues. The role of basophils for protective immunity against gastrointestinal helminths can be studied in mouse models. Studies with genetically basophil-deficient Mcpt8Cre mice revealed that basophils are not required for worm expulsion

during primary infection with *Nippostrongylus brasiliensis* [15]. Th2 polarization, eosinophilia, and serum IgE levels were also not affected by the absence of basophils. However, protection against secondary infection was compromised in basophil-deficient mice [15]. Basophils were also reported to contribute to expulsion of *Trichuris muris*, a gastrointestinal helminth that mainly populates the large intestine [5]. The protective function of basophils is probably dependent on helminth-specific antibodies that can bind to activating Fc receptors on basophils and thereby mediate activation of basophils upon antigen encounter. Further studies are required to unravel the mode(s) of basophil activation and their effector functions against helminths.

4.5 Conclusion

Based on murine models basophils appear important for protective immunity against ticks and helminths. On the other hand they mediate chronic inflammatory skin reactions. The proposed role of basophils as critical Th2-polarizing antigen-presenting cells could not be confirmed with genetically basophil-deficient mice. A better understanding of basophil biology will hopefully lead to improved treatments of allergic skin reactions and development of vaccines against helminths. Unfortunately, most of our current knowledge about basophil in vivo functions is based on murine models. Whether the results can be translated to the human immune system remains to be determined.

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References

1. Iwasaki H, Mizuno S, Arinobu Y, et al. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. Genes Dev 2006; 20(21):3010–21.
2. Ohnmacht C, Voehringer D. Basophil effector function and homeostasis during helminth infection. Blood 2009; 113(12):2816–25.
3. Shen T, Kim S, Do JS, Wang L, Lantz C, Urban JF, Le Gros G, Min B. T cell-derived IL-3 plays key role in parasite infection-induced basophil production but is dispensable for in vivo basophil survival. Int Immunol 2008; 20(9):1201–9.
4. Kim S, Prout M, Ramshaw H, Lopez AF, LeGros G, Min B. Cutting edge: basophils are transiently recruited into the draining lymph nodes during helminth infection via IL-3, but infection-induced Th2 immunity can develop without basophil lymph node recruitment or IL-3. J Immunol 2010; 184(3):1143–7.
5. Siracusa MC, Saenz SA, Hill DA, et al. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. Nature 2011; 477(7363): 229–33.
6. Hida S, Tadachi M, Saito T, Taki S. Negative control of basophil expansion by IRF-2 critical for the regulation of Th1/Th2 balance. Blood 2005; 106(6):2011–7.
7. Charles N, Watford WT, Ramos HL, et al. Lyn kinase controls basophil GATA-3 transcription factor expression and induction of Th2 cell differentiation. Immunity 2009; 30(4):533–43.
8. Oh K, Shen T, Le Gros G, Min B. Induction of Th2 type immunity in a mouse system reveals a novel immunoregulatory role of basophils. Blood 2007; 109(7):2921–7.
9. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. Nat Immunol 2008; 9(3): 310–8.
10. Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. Nat Immunol 2009; 10(7):713–20.
11. Perrigoue JG, Saenz SA, Siracusa MC, et al. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. Nat Immunol 2009; 10(7):697–705.
12. Tang H, Cao W, Kasturi SP, et al. The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. Nat Immunol 2010; 11(7):608–17.
13. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, Muskens F, Lambrecht BN. Inflammatory dendritic cells–not basophils–are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J Exp Med 2010; 207(10):2097–111.
14. Phythian-Adams AT, Cook PC, Lundie RJ, et al. CD11c depletion severely disrupts Th2 induction and development in vivo. J Exp Med 2010; 207(10): 2089–96.
15. Ohnmacht C, Schwartz C, Panzer M, Schiedewitz I, Naumann R, Voehringer D. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. Immunity 2010; 33(3):364–74.

16. Sullivan BM, Liang HE, Bando JK, Wu D, Cheng LE, McKerrow JK, Allen CD, Locksley RM. Genetic analysis of basophil function in vivo. Nat Immunol 2011; 12(6):527–35.
17. Khodoun MV, Orekhova T, Potter C, Morris S, Finkelman FD. Basophils initiate IL-4 production during a memory T-dependent response. J Exp Med 2004; 200(7):857–70.
18. Iliopoulos O, Baroody FM, Naclerio RM, Bochner BS, Kagey-Sobotka A, Lichtenstein LM. Histamine-containing cells obtained from the nose hours after antigen challenge have functional and phenotypic characteristics of basophils. J Immunol 1992; 148(7):2223–8.
19. Macfarlane AJ, Kon OM, Smith SJ, et al. Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol 2000; 105(1 Pt 1):99–107.
20. Koshino T, Teshima S, Fukushima N, et al. Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma. Clin Exp Allergy 1993; 23(11):919–25.
21. Bascom R, Wachs M, Naclerio RM, Pipkorn U, Galli SJ, Lichtenstein LM. Basophil influx occurs after nasal antigen challenge: effects of topical corticosteroid pretreatment. J Allergy Clin Immunol 1988; 81(3):580–9.
22. Ito Y, Satoh T, Takayama K, Miyagishi C, Walls AF, Yokozeki H. Basophil recruitment and activation in inflammatory skin diseases. Allergy 2011; 66(8): 1107–13.
23. Wada T, Ishiwata K, Koseki H, et al. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. J Clin Invest 2010; 120(8):2867–75.
24. Mukai K, Matsuoka K, Taya C, et al. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. Immunity 2005; 23(2): 191–202.
25. Tsujimura Y, Obata K, Mukai K, et al. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. Immunity 2008; 28(4):581–9.
26. Khodoun M, Strait R, Orekov T, Hogan S, Karasuyama H, Herbert DR, Kohl J, Finkelman FD. Peanuts can contribute to anaphylactic shock by activating complement. The Journal of allergy and clinical immunology 2009; 123(2):342–51.
27. Jonsson F, Mancardi DA, Kita Y, et al. Mouse and human neutrophils induce anaphylaxis. J Clin Invest 2011; 121(4):1484–96.
28. Wikel SK. Host immunity to ticks. Annu Rev Entomol 1996; 41:1–22.
29. Allen JR. Tick resistance: basophils in skin reactions of resistant guinea pigs. Int J Parasitol 1973; 3(2):195–200.
30. Brown SJ, Galli SJ, Gleich GJ, Askenase PW. Ablation of immunity to Amblyomma americanum by anti-basophil serum: cooperation between basophils and eosinophils in expression of immunity to ectoparasites (ticks) in guinea pigs. Journal of immunology 1982; 129(2):790–6.

5 Crosstalk Between Adaptive and Innate Immune Cells Leads to High Quality Immune Protection at the Mucosal Borders

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Abstract

Mucosal effector memory CD8 T cells are located at the epithelium and have a heightened and immediate effector function. By contrast, central memory T cells reside within lymphoid tissues and require proliferation and differentiation to become effector cells that migrate to epithelial surfaces. The accumulation of effector memory T cells at the pathogen entry site(s) is essential for protective immunity, but the mechanisms that drive the differentiation of memory cell subsets are poorly understood. We recently showed that CD8αα, induced selectively on the most highly activated primary CD8αβ T cells, together with its ligand, the thymic leukemia (TL) antigen, induced on mucosal antigen-presenting cells and constitutively expressed on intestinal epithelial cells (IEC), serve as key components to mediate the selective accumulation of the fittest effector cells to form mucosal effector memory T cells. Therefore, the generation of mucosal effector memory is controlled by an innate-adaptive crosstalk that provides for host defense at the body's largest interface.

Keywords

Protective immunity • Effector memory CD8 T cells • Central memory CD8 T cells • TCR affinity • CD8αα • Thymic leukemia antigen TL • Mucosal dendritic cells • Mucosal epithelium

5.1 Introduction

The intestine forms the largest entry port for invading pathogens. Many pathogens infect the host through the expanded epithelium of the intestine. Effective immune memory cells residing at this local mucosal site are of great importance for the initial containment and early control of pathogen infection and re-infection [1–3].

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5.2 Effector and Central Memory T Cells Form Distinct Subsets

Memory T cells can be broadly separated into central memory (T_{CM}) and effector memory (T_{EM}) cells. T_{CM} express CCR7/CD62L and re-circulate through lymphoid tissues. T_{EM} lack CCR7/CD62L and preferentially home to non-lymphoid tissues such as the intestine epithelium and skin [1–5]. T_{CM} are characterized by their ability to proliferate robustly and further differentiate into effector cells upon a secondary stimulation [5], whereas T_{EM} are already fully differentiated effector cells which do not expand much but instead display immediate cytotoxicity and produce functional cytokines upon a secondary challenge [2, 6]. Thus, the efficacy of T_{CM} is based on the quantity of memory cells and the efficacy of T_{EM} is based on the quality of memory cells. Immediate responses from local T_{EM} cells may not be sufficient to provide sterilizing protection, but they can efficiently control the local infection and delay or even prevent the onset of disease, as well as reduce the potential for secondary transmission. Although T_{CM} are thought of as superior memory cells compared to the T_{EM}, most of the data that support this view are based on biased approaches that focus on T_{CM} characteristics and functions, which often bypass or overlook the contribution of T_{EM}. Nevertheless, the unique characteristics of quality-based T_{EM} allow them to rapidly sense the presence of invading pathogens at the mucosal borders and respond immediately, thus preventing the initial infection and/or systemic spreading of the pathogen. The quantity-based T_{CM} on the other hand provide a massive defense once the multiplying pathogens break through the T_{EM} frontline defense or when an infection occurs systemically.

5.3 An Innate-Adaptive Crosstalk Drives the Selective Accumulation of Mucosal T_{EM}

The process that drives the specific development and accumulation of T_{EM} at mucosal sites remains poorly defined. Nevertheless, understanding how to induce preexisting T_{EM} cells at the mucosal interface of the intestine has significant implications for the development of effective vaccines to protect against pathogens that invade via the mucosal interfaces, such as HIV.

Currently much of the knowledge on vaccine-induced T cell-mediated protective immunity has been generated using immunization protocols and analyses that focus on, and favor T_{CM} in lymphoid tissues, which therefore unavoidably miss the specific requirements critical for tissue-resident T_{EM}. Using an oral infection model with *Listeria monocytogenes* (LM), in which bacteria enter the host through the mucosal barrier of the intestine, we investigated the requirements for T_{EM} generation at the intestine epithelium [7]. Typically, conventional CD8αβ$^+$ intraepithelial lymphocytes (IEL) have a T_{EM} phenotype and co-express CD8αα homodimers together with CD8αβ heterodimers [1]. In our recent study [7], we showed that CD8αα, induced on the most optimally activated primary CD8αβ$^+$ effector T cells, together with its high affinity ligand, the thymus leukemia antigen (TL), a non-classical MHC class I-like molecule induced on dendritic cells (DC) and constitutively expressed on intestinal epithelial cells (IEC), selectively control the survival of high affinity memory precursor cells and mucosal T_{EM}, respectively.

5.3.1 Activation-Induced CD8αα Rescues CD8αβ Primary Effector T Cells from AICD

Whereas naïve CD8αβ$^+$ T cell do not express CD8αα homodimers, they are readily and transiently induced upon antigen stimulation [7]. The induction of CD8αα is directly controlled by the TCR signal strength and the stronger the signal, the higher the level of CD8αα induction on primary CD8αβ$^+$ effector cells [7]. Consequently, high CD8αα expression on primed CD8αβ$^+$ T cells marks those effector cells with the highest affinity/avidity. The induction of CD8αα is controlled by an enhancer ($E8_I$) located in the CD8α promoter [8] and high avidity/affinity $E8_I^{-/-}$ CD8αβ T cells with a deletion of the $E8_I$ enhancer fail to induce CD8αα upon activation [7, 9].

Similar to our previous findings using the lymphocytic choriomeningitis virus (LCMV) system [9], also in response to an oral LM infection, antigen-specific $E8_I^{-/-}$ CD8αβ$^+$ T cells did not survive as memory cells in the spleen or the intestine [7]. These observations support the notion that activation-induced CD8αα is required for the rescue of activation-induced cell death (AICD) of high affinity CD8αβ$^+$ memory precursor cells [9].

5.3.2 Activation-Induced CD8αα Rescues CD8αβ Primary Effector T Cells from TICD

The marked presence of CD8αα-expressing CD8αβ$^+$ T cells at the mucosal interface of the intestine suggested that there must be a mechanism in place that drives the selective accumulation of high affinity/avidity T_{EM} at this mucosal border. Because of the high affinity interaction between CD8αα and its ligand TL, induced on the priming mesenteric lymph node (MLN) CCR7$^+$/CD103$^+$ migratory DC [7], and constitutively expressed on the IEC in close proximity of the CD8αα$^+$ IEL [10, 11], we postulated that a crosstalk between the T cells and the innate immune cells (DC and IEC) initiated by this receptor–ligand pair, CD8αα-TL, might be involved in the selective accumulation of the CD8αα$^+$ (high affinity/avidity) effector cells at the mucosal interface. Surprisingly, however, in contrast to the absolute requirement for CD8αα expression, we found that, in TL$^{-/-}$ animals, CD8αβ$^+$ memory cells persist at an increased frequency, indicating a negative role of TL for the survival of activated CD8αβ$^+$ T cells [7]. Consistent with this, in TL-transgenic (TL-Tg) mice, forced expression of a TL transgene driven by an MHC class I promoter interfered with the survival of activated CD8αβ$^+$ T cells and completely prevented the formation of CD8αβ$^+$ memory cells in the intestine as well as in the lymphoid tissues. The TL-induced cell death (TICD) of activated CD8αβ$^+$ T cells also occurred using $E8_I^{-/-}$ CD8αβ$^+$ T cells, indicating that TICD is the result of TL interaction with CD8αβ [7]. The selective survival of CD8αα-expressing CD8αβ$^+$ T cells primed by TL-expressing migratory DC indicated that this adaptive-innate crosstalk drove the rescue of high affinity/avidity CD8αβ$^+$ primary effector cells that home to the intestine.

5.3.3 Activation-Induced CD8αα Rescues CD8αβ Secondary Effector T Cells from TICD

TL constitutively expressed on the IEC in the intestine forms a second checkpoint and eliminates weak secondary effector cells, which escaped TICD during their initial priming by TL negative peripheral antigen-presenting cells (APC). In support of this, we showed that adoptive transfer of sorted CD8αα$^{-/low}$ or CD8αα^{high} effector cells primed in vitro in the absence of TL to either WT or TL$^{-/-}$ hosts resulted in the accumulation of CD8αα^{high} but not CD8αα$^{-/low}$ derived T_{EM} in the TL-expressing intestine epithelium of the WT mice, whereas both CD8αα^{high} and CD8αα$^{-/low}$ effector cells survived as T_{EM} in the intestine of TL$^{-/-}$ recipient mice [7]. This suggested that, in addition to the crosstalk between the primary T cells and the APC, TL expressed on gut epithelial cells forms a second checkpoint to selectively eliminate effector cells with weak affinity/avidity for the antigen and prevent them from accumulating as T_{EM} at the mucosal borders. Consistent with this, we also showed, using altered peptide ligands (APLs) with varied affinities, that priming with low affinity peptides led to memory cells in the lymphoid tissues, but not to the accumulation of T_{EM} in the intestine [7].

5.4 Conclusion

The activation marker CD8αα, induced on high affinity/avidity effector CD8αβ$^+$ T cells together with TL, induced on APC and constitutively expressed on the intestinal epithelium, establish an adaptive-innate crosstalk, which prevents weak primary and secondary effector cells from accumulating at the mucosal interface. This selective

process also preserves the most sensitive effector cells, which are able to respond rapidly and resist the initial infection and consequently also prevent excessive and potentially damaging inflammatory immune responses.

5.5 Future Prospective

Our data demonstrate that an affinity/avidity-based selective crosstalk between innate and adaptive immune cells is essential for the generation and preservation of memory T cells that form the critical first line of defense at mucosal interfaces. These new findings define a fundamental new concept for our understanding of immune memory and for the design of strategies to induce effective vaccine-induced immunity. It is evident from recent advances, including our study here and other published data [4, 7, 12, 13], that in order to generate successful vaccines, it is important to mirror the endogenous immune responses and adapt the design based on the nature of pathogens and the route of infection. For example, most infections, including HIV and SIV infections, are acquired across mucosal barriers, and several studies have demonstrated that local $CD8^+$ CTL responses play a crucial role in the initial containment and early control of pathogen replication [14–18]. Therefore, for successful vaccination-induced preexisting protective immunity, it is of utmost importance to induce a local and highly sensitive and effective $CD8^+$ T_{EM} layer in addition to T_{CM} cells that reside in lymphoid tissues. Based on our findings, this would imply that for strategies, which aim to induce protective immunity at mucosal sites, it is important to screen for vaccines, which mediate strong induction of $CD8\alpha\alpha$ during the initial immunization. This will warrant the selective accumulation of high affinity/avidity mucosal T_{EM} that can sense low doses of the pathogen and respond rapidly and with enhanced efficacy to provide effective protection against pathogens that invade via the largest yet most vulnerable interfaces of the body.

References

1. Cheroutre H, Madakamutil L. Mucosal effector memory T cells: the other side of the coin. Cell Mol Life Sci. 2005 Dec;62(23):2853–66.
2. Masopust D, Vezys V, Marzo AL, Lefrancois L. Preferential localization of effector memory cells in nonlymphoid tissue. Science. 2001 Mar 23;291(5512):2413–7.
3. Masopust D, Vezys V, Wherry EJ, Barber DL, Ahmed R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. J Immunol. 2006 Feb 15;176(4):2079–83.
4. Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. Nature. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. 2012 Mar 8;483(7388):227–31.
5. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999 Oct 14;401(6754):708–12.
6. Wolint P, Betts MR, Koup RA, Oxenius A. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. J Exp Med. [Research Support, Non-U.S. Gov't]. 2004 Apr 5;199(7):925–36.
7. Huang Y, Park Y, Wang-Zhu Y, Larange A, Arens R, Bernardo I, et al. Mucosal memory CD8(+) T cells are selected in the periphery by an MHC class I molecule. Nat Immunol. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. 2011 Nov;12(11):1086–95.
8. Ellmeier W, Sunshine MJ, Losos K, Hatam F, Littman DR. An enhancer that directs lineage-specific expression of CD8 in positively selected thymocytes and mature T cells. Immunity. 1997 Oct;7(4):537–47.
9. Madakamutil LT, Christen U, Lena CJ, Wang-Zhu Y, Attinger A, Sundarrajan M, et al. CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors. Science. 2004 Apr 23;304(5670):590–3.
10. Hershberg R, Eghtesady P, Sydora B, Brorson K, Cheroutre H, Modlin R, et al. Expression of the thymus leukemia antigen in mouse intestinal epithelium. Proc Natl Acad Sci U S A. 1990 Dec;87(24):9727–31.
11. Wu M, van Kaer L, Itohara S, Tonegawa S. Highly restricted expression of the thymus leukemia antigens on intestinal epithelial cells. J Exp Med. 1991 Jul 1;174(1):213–8.
12. Hansen SG, Ford JC, Lewis MS, Ventura AB, Hughes CM, Coyne-Johnson L, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, Non-P.H.S.]. 2011 May 26;473(7348):523–7.
13. Hansen SG, Vieville C, Whizin N, Coyne-Johnson L, Siess DC, Drummond DD, et al. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. Nat Med. 2009 Mar;15(3):293–9.
14. Belyakov IM, Isakov D, Zhu Q, Dzutsev A, Berzofsky JA. A novel functional CTL avidity/activity compartmentalization to the site of mucosal immunization contributes to protection of macaques against simian/human immunodeficiency viral depletion of mucosal CD4+ T cells. J Immunol. 2007 Jun 1;178(11):7211–21.
15. Belyakov IM, Kuznetsov VA, Kelsall B, Klinman D, Moniuszko M, Lemon M, et al. Impact of vaccine-induced mucosal high-avidity CD8+ CTLs in delay of AIDS viral dissemination from mucosa. Blood. 2006 Apr 15;107(8):3258–64.
16. Daucher M, Price DA, Brenchley JM, Lamoreaux L, Metcalf JA, Rehm C, et al. Virological outcome after structured interruption of antiretroviral therapy for human immunodeficiency virus infection is associated with the functional profile of virus-specific CD8+ T cells. J Virol. 2008 Apr;82(8):4102–14.
17. Letvin NL, Walker BD. Immunopathogenesis and immunotherapy in AIDS virus infections. Nat Med. 2003 Jul;9(7):861–6.
18. Vogel TU, Reynolds MR, Fuller DH, Vielhuber K, Shipley T, Fuller JT, et al. Multispecific vaccine-induced mucosal cytotoxic T lymphocytes reduce acute-phase viral replication but fail in long-term control of simian immunodeficiency virus SIVmac239. J Virol. 2003 Dec;77(24):13348–60.

The Interaction Between Filarial Parasites and Human Monocyte/ Macrophage Populations

6

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Abstract

Lymphatic filariasis is a mafor tropical disease affecting approximately 120 million people worldwide. Patent infection, by and large, is clinically asymptomatic but is associated with the inability of T cells to proliferate or produce IFN-γ in response to parasite antigen. Monocyte dysfunction is one hypothesis felt to explain the lack of an antigen-specific T cell response. In fact, monocytes from filaria-infected individuals have been shown to be studded with internalized filarial antigens. Understanding how the phenotype and the function of these monocytes are altered through the internalization of these parasite antigens is one of the areas our laboratory has focused on. In fact, the existence and/or function of alternatively activated macrophages in murine models of filarial infections have been extensively studied. Whether this population of macrophages can be induced in human filarial infections is the main focus of this review.

Keywords

Human • Alternatively activated macrophages • Filarial parasites • Monocytes • Lymphatic filariasis

6.1 Background

6.1.1 Lymphatic Filariasis

Lymphatic filariasis, a mosquito-borne disease and a major cause of morbidity in tropical and subtropical regions of the world, is caused by infection with *Wuchereria bancrofti*, *Brugia malayi*, or *Brugia timori* and affects over 120 million people worldwide with nearly 1.39 billion people at risk of developing the disease [1].

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Lymphatic filariasis is considered one of the most immunologically complex infections for variety of reasons. For example, the life cycle of the parasite causing this disease in humans is complicated and involves two hosts (mosquito and human), with multiple stages of the parasite inducing different immune responses. In addition, there are several clinical manifestations of the disease varying from one endemic area to another with different immunologic criteria (reviewed in [2]). The infection is initiated in humans by mosquito-derived third-stage larvae (L3) deposited in the skin—itself an immunologic organ—containing Langerhans' cells (LC) and keratinocytes, among other cells. The larvae enter the body through the skin, where the parasites evade the primary line of defense. The parasites then undergo a molt to the fourth larval stage (L4) and then mature into lymphatic tissue-dwelling adult male and female worms; a process that takes about 3–12 months. Adult females, after copulation, release progeny microfilariae (mf); a stage believed to be associated with many of the systemic immunologic defects associated with chronic lymphatic filarial infection. Mf released in the lymphatics can travel through the circulation and be picked up by a mosquito at its next blood meal, with the cycle being completed from mf to L3 in the mosquito host. Through this route of travel from skin to lymphatics and lymph nodes (LNs) and finally to the systemic circulation, the various parasite stages interact with different types of antigen-presenting cells (APCs), likely to induce distinct responses.

Patent infection, in large part, is clinically asymptomatic but immunologically associated with the inability of T cells to proliferate or produce IFN-γ in response to parasite antigen [3]. This lack of T cell responsiveness has been shown to be primarily linked to the mf stage of the parasite found in circulation [4]. Of many hypotheses proposed to explain this lack of antigen-specific T cell response [5–9], dysregulation of professional APCs—dendritic cells (DCs), macrophages (MΦs), and monocytes (Mϕs)—has been of interest to our lab. Indeed, because the initiation of infection occurs through the skin and likely conditions the subsequent immune responses, understanding the interaction between filarial L3s and APCs in the skin has been of great importance to us. To that end, we have shown that filarial L3 not only fails to fully activate the human epidermal LCs but, in fact, suppresses the expression of genes involved in antigen presentation and processing, as well as impairing the function of these cells—a mechanism used by the parasites to evade the immune response. Perhaps through this evasion of the first line of defense, the worm can successfully develop to other stages of its life cycle and finally to mf stage, which can encounter other APCs in circulation. The mechanisms underlying the antigen uptake, processing, and presentation of filarial parasites by other APCs such as DCs have not been well studied. While we know that human Mϕ-derived DCs are capable of filarial antigen uptake, how these soluble products are recognized and processed is not fully understood. We have shown that live mf of *B. malayi* induce an apoptotic cell death in human Mϕ-derived DCs but not in the precursor Mϕs. Furthermore, this apoptotic cell death was shown to be dependent on tumor necrosis factor alpha (TNF-α) and TRAIL pathways [10]. Live mf also downregulate Toll-like receptors (TLR) expression and signaling [11], resulting in an impaired function of these cells, perhaps contributing to the T cell hyporesponsiveness observed in microfilaremic individuals.

How mf regulates the function of Mϕ/MΦ populations and what role these cells play in filarial infection are the focus of this review.

6.1.2 Macrophages and Their Role in Inflammation

Mϕs are derived from $CD34^+$ myeloid progenitor cells in the bone marrow, circulated in the blood, and then entered the peripheral tissues, where they mature to MΦs [12, 13], a heterogeneous population of cells that play a critical role in the primary response to intracellular organisms [14].

MΦs play a key role in inflammation. During onset of the inflammatory process, they become activated, resulting in induction of several genes that, in turn, can lead to the increased ability of

these cells to destroy intracellular organisms as well as to regulate other cells to produce cytokines and chemokines important for this destruction. It is in this process that MΦs produce reactive oxygen species, nitric oxide (NO), and proinflammatory cytokines such as TNF-α, interleukin-1 beta (IL-1β), and IL-6 (reviewed in [15]). The duration of this proinflammatory phase is closely related to the balance between the ability of the microorganism to survive and the capacity of MΦs to destroy (reviewed in [15]); however, an unbalanced proinflammatory activation of MΦs can result in inappropriate inflammation leading to tissue damage. The anti-inflammatory phase of MΦs thus has evolved to control the excess activation, leading to deactivation of these cells and finally resulting in resolution of inflammation.

While Th1 cytokines such as IFN-γ can induce proinflammatory activities in MΦs, agents such as IL-4 or glucocorticoids were shown to inhibit expression of proinflammatory cytokines by these cells. Furthermore, while MΦs generally are considered to be phagocytic cells with the ability to engulf unicellular pathogens such as bacteria and protozoa, it is now clear that they can also develop into an alternative, non-phagocytic phenotype [15, 16]. Indeed, initial studies in murine models showed that in vitro treatment of MΦs with a Th2-associated cytokine such as IL-4 resulted in increased expression of mannose receptor or CD206, cellular responses associated with collagen deposition, and reduced antimicrobial iNOs production [17].

In vitro, at least two major MΦ populations have been characterized: IFN-γ-induced type I MΦs—known as classically activated MΦs (CAMΦs) [18]—with a proinflammatory profile and Th2-induced MΦs—known as type II or alternatively activated MΦs (AAMΦs)—which have both anti-inflammatory activities and tissue-repair function [17]. These two MΦ populations have discrete differences in the pathways associated with L-arginine metabolism. CAMΦs produce NO through iNOS upregulation [19], and AAMΦs produce ornithine and urea through the action of arginase 1 (ARG-1) [20]. Typically, there is reciprocal regulation of these two pathways, as induction of arginase is associated with suppression of iNOS and vice versa [21]. AAMΦs have been implicated in responses to helminth infection and in wound repair [22], whereas CAMΦs have antimicrobial and cytotoxic functions. This review focuses on the role of AAMΦs in helminth infection, particularly in filarial infection.

6.2 Induction of AAMΦs in Filarial Infections

6.2.1 Animal Models

The concept of alternative activation was originally introduced by Simon Gordon and colleagues in the early 1990s mainly to describe the in vitro response of MΦs to Th2 cytokines such as IL-4 and IL-13 [16, 23], and the requirement for both of these cytokines was subsequently confirmed in vivo using knockout mice [17, 24–26]. A molecular signature for AAMΦs, which is defined as an IL-4/IL-13-dependent pathway, is represented by three most abundant gene products: YM1, RELMa, and ARG-1. The important aspect of AAMΦ biology has been shown in a range of nematode infection models involving gastrointestinal nematodes along with filarial nematodes such as *B. malayi* (reviewed in [27, 28]). Studies using animal models have suggested that filarial parasites are indeed potent inducers of IL-4 and IL-13 [17]. In fact, the concept of AAMΦs being a feature of helminth infection originally came from studies of *B. malayi* and *Schistosoma mansoni* [24, 25]. Interestingly, while ARG-1 expression in MΦs was shown to be IL-4 dependent in both of the in vivo studies, the novel IL-4-dependent genes such as Ym1 and RELMa/FIZZ1 were shown to be induced by *B. malayi*, as was later confirmed to be the case by other helminth parasites [25]. These researchers showed that if mice are surgically implanted with adult worms of *B. malayi*, the peritoneal exudate cell population in these animals is dominated by MΦs that display IL-4-dependent features; however, these MΦs were found to be responsive to inflammatory stimuli such as LPS and IFN-γ, and

following activation with LPS/IFN-γ were able to effectively control infection with *Leishmania mexicana*. Of interest, these MΦs were able to suppress proliferation and were not able to upregulate IL-12 production [29]. Furthermore, this nematode recruited MΦs when used as APCs in vitro shown to prevent proliferation of a T cell clone with no effect on their cytokine production. Interestingly, this antiproliferative effect was not antigen specific [30, 31] but required IL-4 [32]. While the phagocytic ability of these nematode-elicited MΦs has not been fully understood, the effect of IL-4 treatment on the phagocytic ability of MΦs in general has been studied, revealing some controversy on the issue [33–35]. Indeed, there are reports indicating evidence both for an increase in the phagocytic ability of MΦs for protozoan parasites due to IL-4 [36] and for an impaired phagocytic function of AAMΦs for *Neisseria* meningitis [37].

6.2.2 Humans

A mafor difficulty in the field has been how to translate our understanding of murine AAMΦ to humans, e.g., some mouse-defined AAMΦ markers are not present in the human genome. Furthermore, because it is not feasible to access the relevant tissue in humans, the majority of work has focused on differentiating blood Mϕs to MΦs with MCSF and then polarizing them to AAMΦs or CAMΦs with either IL-4 or LPS, respectively. In fact, transcriptional profiling of these polarized MΦs with IL-4 has shown some similarities to murine AAMΦs, with many differences as well [38] (Table 6.1). For example, it has been shown that while both IL-4 and IL-13 [20, 39] induce expression of the mannose receptor and MΦ galactose-type C-type lectin in human Mϕs and Mϕ-derived MΦs, these cytokines fail to induce ARG-1, which is commonly a hallmark of murine AAMΦs [19]. Whether human MΦs are capable of inducing ARG-1 is a controversial topic in the field. Indeed, ARG-1 can be induced by IL-4 in human MΦ cell lines but not in alveolar MΦs of healthy volunteers [40]; however, combining IL-4 with agents to induce cAMP levels can induce arginase activity in human AMMΦs. In our laboratory, culturing primary human Mϕs with IL-4 for either 48 h or differentiating the cells with MCSF for 7 days and then polarizing them with IL-4 for 48 h did not alter the mRNA or activity of arginase (data not shown). Even manipulation with cAMP agonists such as forskolin in combination with IL-4 did not induce arginase activity (Barron et al., personal communication). Indeed, the explanation for these discrepancies between mouse and man could be due to limitation of this enzyme in humans or to not having access to the relevant tissues.

In humans, similar to mice, AAMΦs express mannose receptor CD206 and chemokines such as CCL17 and CCL22. Unlike the murine system, in humans the expression of DC-SIGN (CD209), CCL18, and CCL26 can be used to

Table 6.1 Characteristics of alternatively activated macrophages

	Murine	Human
Gene expression		
Arginase-1	++++	–/?
YM1	++++	–
Fizz1	++++	–
iNOS	–	–
CCL2	++++	–
CCL7	++++	–
CCL13	–	++++
CCL15	–	++++
CCL18	–	++++
CCL26	–	++++
Fizz3	–	+/?
CD209	–	++++
CCL17	++++	++++
CCL22	++++	++++
CD206 (mannose receptor)	++++	++++
E-cad	++++	++++
CD301 (C-lectin family)	++++	++++
PDL1	++++	+++/?
PDL2	++++	+++/?
Stat6	++++	++++
MGL1	++++	++++
Function		
Ability to phagocytose	Diminished	?
Ability to promote T cell proliferation	Diminished	?

identify AAMΦs [41]. Furthermore, we have done extensive in vitro studies showing that Mϕs need not be differentiated prior to polarization with IL-4. In fact, short-term exposure of Mϕs to IL-4 is sufficient to induce the AAMΦ phenotype in human cells [42]. In addition, the mf stage of *B. malayi* or soluble factors from mf induce a programmed response very similar to that induced by IL-4, resulting in upregulation of MRC1 or CD206, CCL15, CCL17, CCL18, CCL22, CD273, and CD274. Association of CCL17, CCL18, and CCL22 with the alternative activation phenotype has been observed in alveolar MΦs in patients with lung fibrosis [43]. Furthermore, both IL-4- and mf-exposed Mϕs had significantly lower ability to phagocytose opsonized *Escherichia coli* bioparticles as compared to MCSF-treated Mϕs [42], suggesting that in humans, the phagocytic ability of AAMΦs is different from that of CAMΦs. While mf exhibited similarities to IL-4 in inducing an alternative activation phenotype in human Mϕs, there are major differences, as well. One important difference between IL-4- and mf-induced effects on human Mϕs was in expression of IDO, as mf upregulated and IL-4 downregulated IDO expression [42]. IDO expression is regulated and induced by cytokines such as IFN-γ, type I interferons, and IL-10 and is an important immunosuppressive factor for Th1-mediated immune responses [44]. The implications of this difference—and how this mf-positive regulation of IDO plays a role in human T cell function—are areas that our laboratory is investigating.

Another difference between IL-4- and mf-exposed Mϕs was upregulation of RETN, which was significantly induced by mf but not IL-4 [42]. RETN is homologous to mouse resistin-like genes that have been seen as markers for murine AAMΦs [45]. This gene was also upregulated in Mϕs from filaria-infected individuals, with higher expression than in uninfected individuals.

We also found that while exposure of Mϕs to IL-4 diminished the ability of these cells to promote CD4 and CD8 T cell proliferation in vitro and in response to anti-CD3 activation, exposure to mf did not cause these effects—further evidence of the different effects of mf and IL-4 on Mϕs [42].

Given that AMMΦs are important in immunity against extracellular pathogens and that CAMΦs are important primarily in immunity against intracellular pathogens, one must wonder whether they have different TLR profiles. We have shown that IL-4 downregulates mRNA expression of some TLRs such as TLR2, TLR3, TLR5, TLR7, and TLR8, and also downregulates their response to the TLR2 ligand [42]. This could be explained by the fact that different APCs may have different responses to the same pathogen [46], a finding that can explain differing expression/function of TLRs. In fact, there are data suggesting that human alveolar Mϕs as well as lung interstitial MΦs vary in their response to TLR4 and TLR9 agonists, with one inducing more proinflammatory cytokines and the other more regulatory cytokines [47]. Furthermore, alveolar MΦs and circulating Mϕs from the same individuals have differences in TLR2 expression and function [48]. While mf contact does not change the TLR profile in Mϕs, soluble factors from this parasite, similar to IL-4, downregulate the mRNA expression of some of the TLRs [42].

6.3 Implications in Clinical Settings

The existence and/or function of AAMΦs in human filarial infections has not been fully explored. In fact, we know that Mϕs from filaria-infected individuals are laden with filarial antigen, exhibit diminished expression of genes involved in antigen presentation and processing, and produce fewer proinflammatory cytokines in response to surface receptor crosslinking [49]. In addition, Mϕs from filaria-infected individuals have a lower expression of TLRs, leading to diminished cytokine production in response to TLR engagement [50]. Data from our laboratory suggest that Mϕs from individuals with patent human filaria exhibit a phenotype associated with alternative activation with a higher basal mRNA expression of ARG-1 and lower basal mRNA expression of NOS2 compared with filaria-uninfected individuals from the same endemic region [51]. Furthermore, CD206 and CCL18 were shown to be upregulated in patients with filarial infection compared with levels in uninfected individuals [51]. Perhaps the fact that

these individuals are chronically infected and that the Mϕs of these individuals are studded with internalized filarial antigens may explain the difference we see in the mRNA expression of ARG1 in infected individuals but not in vitro differentiated Mϕs.

One important question is how best to define induction of AAMΦs in the context of a Th2 environment caused by helminth parasites in humans. As it is not feasible to access tissue MΦs from filaria-infected individuals, the most logical alternative is to isolate circulating Mϕs and examine the phenotype and function of these cells and assess their similarity to the AAMΦ population described in vitro or in tissue MΦs. The caveat, however, still remains as to whether these parasite-exposed Mϕs in circulation are indeed similar to the tissue MΦs in mf individuals.

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References

1. World Health Organization. (2011). Global programme to eliminate lymphatic filariasis: progress report on mass drug administration, 2010. Wkly Epidemiol Rec *35*, 377–388.
2. Nutman, T.B. (2001). Lymphatic filariasis: new insights and prospects for control. Curr Opin Infect Dis *14*, 539–546.
3. Maizels, R.M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M.D., and Allen, F.E. (2004). Helminth parasites—masters of regulation. Immunol Rev *201*, 89–116.
4. Mahanty, S., Luke, H.E., Kumaraswami, V., Narayanan, P.R., Vifayshekaran, V., and Nutman, T.B. (1996). Stage-specific induction of cytokines regulates the immune response in lymphatic filariasis. Exp Parasitol *84*, 282–290.
5. King, C.L., Mahanty, S., Kumaraswami, V., Abrams, F.S., Regunathan, F., Fayaraman, K., Ottesen, E.A., and Nutman, T.B. (1993). Cytokine control of parasite-specific anergy in human lymphatic filariasis. Preferential induction of a regulatory T helper type 2 lymphocyte subset. F Clin Invest *92*, 1667–1673.
6. Loke, P., MacDonald, A.S., and Allen, F.E. (2000a). Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naive $CD4^+$ T cells. Eur F Immunol *30*, 1127–1135.
7. Whelan, M., Harnett, M.M., Houston, K.M., Patel, V., Harnett, W., and Rigley, K.P. (2000). A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. F Immunol *164*, 6453–6460.
8. Semnani, R.T., Sabzevari, H., Iyer, R., and Nutman, T.B. (2001). Filarial antigens impair the function of human dendritic cells during differentiation. Infect Immun *69*, 5813–5822.
9. Fenson, F.S., O'Connor, R., Osborne, F., and Devaney, E. (2002). Infection with *Brugia* microfilariae induces apoptosis of $CD4^+$ T lymphocytes: a mechanism of immune unresponsiveness in filariasis. Eur F Immunol *32*, 858–867.
10. Semnani, R.T., Venugopal, P.G., Mahapatra, L., Skinner, F.A., Meylan, F., Chien, D., Dorward, D.W., Chaussabel, D., Siegel, R.M., and Nutman, T.B. (2008b). Induction of TRAIL- and TNF-α-dependent apoptosis in human monocyte-derived dendritic cells by microfilariae of *Brugia malayi*. F Immunol *181*, 7081–7089.
11. Semnani, R.T., Venugopal, P.G., Leifer, C.A., Mostbock, S., Sabzevari, H., and Nutman, T.B. (2008a). Inhibition of TLR3 and TLR4 function and expression in human dendritic cells by helminth parasites. Blood *112*, 1290–1298.
12. Hume, D.A., Robinson, A.P., MacPherson, G.G., and Gordon, S. (1983). The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Relationship between macrophages, Langerhans cells, reticular cells, and dendritic cells in lymphoid and hematopoietic organs. F Exp Med *158*, 1522–1536.
13. Grage-Griebenow, E., Flad, H.D., and Ernst, M. (2001). Heterogeneity of human peripheral blood monocyte subsets. F Leukoc Biol *69*, 11–20.
14. Stein, M., and Keshav, S. (1992). The versatility of macrophages. Clin Exp Allergy *22*, 19–27.
15. Valledor, A.F., Comalada, M., Santamaria-Babi, L.F., Lloberas, F., and Celada, A. (2010). Macrophage proinflammatory activation and deactivation: a question of balance. Adv Immunol *108*, 1–20.
16. Stein, M., Keshav, S., Harris, N., and Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. F Exp Med *176*, 287–292.
17. Gordon, S. (2003). Alternative activation of macrophages. Nat Rev Immunol *3*, 23–35.

18. Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., and Stewart, T.A. (1993). Multiple defects of immune cell function in mice with disrupted interferon-γ genes. Science *259*, 1739–1742.
19. Munder, M., Eichmann, K., and Modolell, M. (1998). Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by $CD4^+$ T cells correlates with Th1/Th2 phenotype. F Immunol *160*, 5347–5354.
20. Munder, M., Mollinedo, F., Calafat, F., Canchado, F., Gil-Lamaignere, C., Fuentes, F.M., Luckner, C., Doschko, G., Soler, G., Eichmann, K., et al. (2005). Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. Blood *105*, 2549–2556.
21. Modolell, M., Corraliza, I.M., Link, F., Soler, G., and Eichmann, K. (1995). Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by T_H1 and T_H2 cytokines. Eur F Immunol *25*, 1101–1104.
22. Kreider, T., Anthony, R.M., Urban, F.F. Fr., and Gause, W.C. (2007). Alternatively activated macrophages in helminth infections. Curr Opin Immunol *19*, 448–453.
23. Doyle, A.G., Herbein, G., Montaner, L.F., Minty, A.F., Caput, D., Ferrara, P., and Gordon, S. (1994). Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-γ. Eur F Immunol *24*, 1441–1445.
24. Hesse, M., Modolell, M., La Flamme, A.C., Schito, M., Fuentes, F.M., Cheever, A.W., Pearce, E.F., and Wynn, T.A. (2001). Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. F Immunol *167*, 6533–6544.
25. Loke, P., Nair, M.G., Parkinson, F., Guiliano, D., Blaxter, M., and Allen, F.E. (2002). IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. BMC Immunol *3*, 7.
26. Linehan, S.A., Coulson, P.S., Wilson, R.A., Mountford, A.P., Brombacher, F., Martinez-Pomares, L., and Gordon, S. (2003). IL-4 receptor signaling is required for mannose receptor expression by macrophages recruited to granulomata but not resident cells in mice infected with *Schistosoma mansoni.* Lab Invest *83*, 1223–1231.
27. Horsnell, W.G., and Brombacher, F. (2010). Genes associated with alternatively activated macrophages discretely regulate helminth infection and pathogenesis in experimental mouse models. Immunobiology *215*, 704–708.
28. Fenkins, S.F., and Allen, F.E. (2010). Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes. F Biomed Biotechnol *2010*, 262609.
29. Mylonas, K.F., Nair, M.G., Prieto-Lafuente, L., Paape, D., and Allen, F.E. (2009). Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. F Immunol *182*, 3084–3094.
30. Allen, F.E., Lawrence, R.A., and Maizels, R.M. (1996). APC from mice harbouring the filarial nematode, *Brugia malayi*, prevent cellular proliferation but not cytokine production. Int Immunol *8*, 143–151.
31. Loke, P., MacDonald, A.S., Robb, A., Maizels, R.M., and Allen, F.E. (2000b). Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. Eur F Immunol *30*, 2669–2678.
32. MacDonald, A.S., Maizels, R.M., Lawrence, R.A., Dransfield, I., and Allen, F.E. (1998). Requirement for in vivo production of IL-4, but not IL-10, in the induction of proliferative suppression by filarial parasites. F Immunol *160*, 1304–1312.
33. Cipriano, I.M., Mariano, M., Freymuller, E., and Carneiro, C.R. (2003). Murine macrophages cultured with IL-4 acquire a phenotype similar to that of epithelioid cells from granulomatous inflammation. Inflammation *27*, 201–211.
34. Gratchev, A., Kzhyshkowska, F., Utikal, F., and Goerdt, S. (2005). Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scand J Immunol *61*, 10–17.
35. Wainszelbaum, M.F., Proctor, B.M., Pontow, S.E., Stahl, P.D., and Barbieri, M.A. (2006). IL-4/PGE2 induction of an enlarged early endosomal compartment in mouse macrophages is Rab5-dependent. Exp Cell Res *312*, 2238–2251.
36. Wirth, F.F., Kierszenbaum, F., and Zlotnik, A. (1989). Effects of IL-4 on macrophage functions: increased uptake and killing of a protozoan parasite (*Trypanosoma cruzi*). Immunology *66*, 296–301.
37. Varin, A., Mukhopadhyay, S., Herbein, G., and Gordon, S. (2010). Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion. Blood *115*, 353–362.
38. Martinez, F.O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. F Immunol *177*, 7303–7311.
39. Raes, G., Brys, L., Dahal, B.K., Brandt, F., Grooten, F., Brombacher, F., Vanham, G., Noel, W., Bogaert, P., Boonefaes, T., et al. (2005). Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. F Leukoc Biol *77*, 321–327.
40. Erdely, A., Kepka-Lenhart, D., Clark, M., Zeidler-Erdely, P., Polfakovic, M., Calhoun, W.F., and Morris, S.M. Fr. (2006). Inhibition of phosphodiesterase 4 amplifies cytokine-dependent induction of arginase in macrophages. Am F Physiol Lung Cell Mol Physiol *290*, L534–539.
41. Martinez, F.O., Helming, L., and Gordon, S. (2009). Alternative activation of macrophages: an immunologic

functional perspective. Annu Rev Immunol *27*, 451–483.
42. Semnani, R.T., Mahapatra, L., Moore, V., Sanprasert, V., and Nutman, T.B. (2011). Functional and phenotypic characteristics of alternative activation induced in human monocytes by interleukin-4 or the parasitic nematode *Brugia malayi*. Infect Immun *79*, 3957–965.
43. Pechkovsky, D.V., Prasse, A., Kollert, F., Engel, K.M., Dentler, F., Luttmann, W., Friedrich, K., Muller-Quernheim, F., and Zissel, G. (2010). Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. Clin Immunol *137*, 89–101.
44. Xu, H., Zhang, G.X., Ciric, B., and Rostami, A. (2008). IDO: a double-edged sword for T_H1/T_H2 regulation. Immunol Lett *121*, 1–6.
45. Allen, F.E., and Loke, P. (2001). Divergent roles for macrophages in lymphatic filariasis. Parasite Immunol *23*, 345–352.
46. Reyes, F.L., Terrazas, C.A., Vera-Arias, L., and Terrazas, L.I. (2009). Differential response of antigen presenting cells from susceptible and resistant strains of mice to *Taenia crassiceps* infection. Infect Genet Evol *9*, 1115–1127.
47. Hoppstadter, F., Diesel, B., Zarbock, R., Breinig, T., Monz, D., Koch, M., Meyerhans, A., Gortner, L., Lehr, C.M., Huwer, H., and Kiemer, A.K. (2010). Differential cell reaction upon Toll-like receptor 4 and 9 activation in human alveolar and lung interstitial macrophages. Respir Res *11*, 124.
48. Fuarez, E., Nunez, C., Sada, E., Ellner, F.F., Schwander, S.K., and Torres, M. (2010). Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. Respir Res *11*, 2.
49. Semnani, R.T., Keiser, P.B., Coulibaly, Y.I., Keita, F., Diallo, A.A., Traore, D., Diallo, D.A., Doumbo, O.K., Traore, S.F., Kubofcik, F., et al. (2006). Filaria-induced monocyte dysfunction and its reversal following treatment. Infect Immun *74*, 4409–4417.
50. Babu, S., Blauvelt, C.P., Kumaraswami, V., and Nutman, T.B. (2005). Diminished expression and function of TLR in lymphatic filariasis: a novel mechanism of immune dysregulation. F Immunol *175*, 1170–1176.
51. Babu, S., Kumaraswami, V., and Nutman, T.B. (2009). Alternatively activated and immunoregulatory monocytes in human filarial infections. F Infect Dis *199*, 1827–1837.

Innate-Like B Cells and Their Rules of Engagement

7

Nicole Baumgarth

Abstract

Antibodies are an integral part of the immune system. They are produced in response to an infection or insult but are also present prior to any encounter with antigen as so-called natural antibodies. This review focuses on the tissues and cellular origins of natural antibodies. It summarizes recent data showing that B-1 cells, an innate-like B cell population distinct in development, repertoire, and tissue location from the majority conventional or B-2 cells, are the main contributors of natural antibodies in mice in steady state. Furthermore, they show that natural IgM production appears largely confined to B-1 cell populations in the spleen and bone marrow. In contrast, B-1 cells in the body cavities, sites of predominance of this population, harbor B-1 cells that do not constitutively produce antibodies. Instead, these cells act as rapid immune responders that relocate to secondary lymphoid tissues and differentiate to cytokine and antibody-secreting cells shortly after an infection. Thus, the process of B-1 cell response participation is distinct from that of B-2 cell activation as the accumulation of effector B-1 cells does not rely on extensive clonal expansion, but instead on their rapid migration and redistribution, a process that appears under the control of infection-induced innate signals.

Keywords

Antibody secretion • B-1 cells • Body cavities • Natural IgM • Innate-like responses

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7.1 Introduction

The immune system is an exquisite and rapid response system. Specific antibody production is induced rapidly, sometimes in less than 2–3 days following encounter with a pathogen or a noxious agent. These antibodies are generated after

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conventional bone marrow-derived B cells have bound to pathogen-derived antigens via surface antigen receptor (B cell receptor, BCR), which induces their clonal expansion and differentiation to antibody-secreting plasma blasts and plasma cells. These activation and differentiation pathways are increasingly well mapped (reviewed in [1]). They often involve interaction of the responding B cells with antigen-presenting cells and CD4 T helper cells and occur in specialized secondary lymphoid tissues optimized to enhance potential interaction with these cells [2]. Induction of these adaptive immune responses also leads to long-term changes of the humoral immune system, causing the development of memory B cells and long-lived plasma cells. The latter produce specific antibodies continuously for years, if not decades, after that initial insult.

While conventional B cells do not generate antibodies unless stimulated, serum antibodies and antibody-forming cells are readily found prior to encounter with pathogens, and even in gnotobiotic mice held in the complete absence of antigens [3–5]. In fact, much of the circulating IgM serum antibodies are "natural antibodies." These antibodies do not require foreign antigenic stimulation and thus the signaling and differentiation pathways that induce natural antibody production must be distinct from those inducing antigen-specific B cell responses. Little is known about the regulation of natural antibodies and natural antibody-secreting cells. Identification of these processes is of significance, however, as natural antibodies provide crucial immune protection against both viral and bacterial pathogens [6–9].

Here I will provide a summary of recent data from our group and others that show that B-1 cells, a cell population distinct in development, repertoire, and tissue location, are the main contributors of natural antibodies in mice in steady state and that natural IgM production appears confined largely to B-1 cell populations in the spleen and bone marrow. In response to influenza virus infection, antibody-producing B-1 cells are activated and effector cells redistribute without extensive clonal expansion to secondary lymphoid tissues, a process that seems controlled by innate-like signals.

7.2 B Cells and Their Contribution to the Antibody Pools

Based on developmental origins, B cells can be divided into two lineages: B-1 and B-2. B-1 cells appear first in ontogeny, hence their name B-1, and are derived from distinct precursors found in the splanchnopleura of developing embryos, the fetal liver, and the bone marrow. The latter precursors do not contribute significantly to B-1 cell pools during adulthood, unless stimulated as occurs during severe lymphopenia, such as after sublethal or lethal whole-body irradiation (reviewed in [10]). This reactivation of B-1 cell development after irradiation has contributed to the earlier debates about the origins of B-1 cells. However, the recent studies by Montecino-Rodriguez and colleagues [11, 12], showing distinct origins and developmental periods of B-1 and B-2 cell development, are consistent with many of the early data on B-1 cells that suggested a restricted developmental period for B-1 cells in mice that spans the early fetal life until 3–6 weeks after birth. Furthermore, the isolation of precursors with B-1 cell-restricted potential clearly supports the different lineage origins for B-1 and B-2 cells.

Bone marrow B-2 cell precursors give rise to both follicular B cells and marginal zone B cell populations. Follicular B cells are the main populations responding to antigen exposure with formation of germinal centers and thus long-lived responses. They are the majority B cell population in the host and are predominant in all major lymphoid tissues. Marginal zone B cells in the mouse are restricted to the splenic marginal zone, while their counterparts in humans appear to be found also circulating in the blood. MZ B cells respond rapidly to blood-borne infections with formation of extrafollicular foci, which harbor short-lived plasma cells. While MZ B cells can also participate in germinal center responses, their contributions to these centers appear to be small and to occur only late after immunization [13].

B-1 cells (previously known as Ly-1 B cells or $CD5^+$ B cells) are divided into two subsets, B-1a and B-1b, based on their expression of CD5. $CD5^+$ B-1a cells and $CD5^-$ B-1b cells seem to

share developmental precursors. Apart from the differential expression of CD5 they are phenotypically similar, and clear functional differences are also not apparent. They do seem to differ, however, in the type of antigen or infectious agent they respond to. For example, in mouse models of *Streptococcus pneumonia,* B-1a and B-1b cells seem to recognize and respond to distinct antigens on the bacteria [14]. During infections with the relapsing fever agent, *Borrelia hermsii*, B-1b but not B-1a cells contribute to IgM-mediated humoral immunity during recall responses [15], while in influenza virus infection only B-1a cells respond to the infection with redistribution to local draining lymph nodes and IgM production [16].

B-1 cells are rare in secondary lymph nodes (about 0.2–0.3%), and represent only about 1% of splenic B cells. They are, however, the major B cell population in the peritoneal and pleural cavities (reviewed in [17]). Depending on the strain, age, and sex of the mouse, B-1 cells comprise 30–60% of total lymphocytes at these sites. Due to their unique tissue-distribution and low frequencies in other tissues, most information about B-1 cells is based on studies with peritoneal cavity (PerC) B-1 cells. However, as we will outline below, differences exist between body cavity and spleen B-1 cells, which remain to be fully evaluated.

The distribution of B-1 cells is unusual and distinct from that of B-2 cells and suggests significant functional differences between B-1 and B-2 cells. Indeed, studies with chimeric mice, created by treating newborn mice with host-allotype-specific IgM to deplete host B-1 cells and reconstituting them with congenic but allotype-mismatched B-1 cells, demonstrated that B-1 cells are the major source of natural IgM in the serum of noninfected mice [8, 18] and in the mucosal tissues of the intestinal [19] and the respiratory tract [16]. Thus, B-1 cells appear to be uniquely positioned to produce antibodies in the absence of foreign antigenic signals. Understanding the biology and physiology of their responses and the regulation of their antibody production is thus likely to uncover also the regulation of natural antibody production.

7.3 Role and Function of Natural Antibodies

Natural antibodies are important in host defense. Numerous studies showed that following infections with both viral and bacterial pathogens, preexisting IgM antibodies directly neutralize and inhibit early pathogen replication, in part via complement binding, and thereby increase survival from infection [6, 9, 20–23]. With mice lacking secreted IgM, it was shown that the presence of natural IgM enhances the ensuing pathogen-specific IgG responses [21, 24], possibly via the formation of antibody–antigen complexes for their deposition on follicular dendritic cells [21, 25], although other mechanisms are likely [25]. Analogous "natural" poly-specific IgA antibodies exist at mucosal surfaces, where they might act as a first layer of immune defense [19, 26]. Thus, natural antibodies constitute an important component of preexisting protective immunity. These antibodies are often polyreactive, meaning they will bind to multiple antigens. This interaction is usually a low-affinity interaction ($Kd = 10^{-3}$–10^{-7} mol l^{-1}); however, given the pentameric structure of the IgM, overall affinities may nonetheless be considerable [27], supported by data on their protective effects on host survival following infections.

Another function of natural antibodies is their involvement in "housekeeping" functions: the maintenance of tissue integrity and homeostasis. They facilitate removal of apoptotic cells via their binding to surface antigens such as phosphatidylcholine (PtC), annexin IV [28], phosphorylcholine [29], and malondialdehyde, the latter a reactive aldehyde degradation product of polyunsaturated lipids [29–32] and xenoantigens [33]. This seems to facilitate increased phagocytosis by immature dendritic cells [31], while also limiting tissue inflammation [31]. The genetic ablation of secreted IgM results in increased autoimmunity, with accelerated, pathogenic IgG responses and resulting disease progression [34], further suggesting that IgM participates in the removal of self-antigens to lower the likelihood of autoimmune responses. Inappropriate and/or

enhanced local secretion of natural IgM secretion and ensuing IgM-self-antigen complex formation can result in local activation of the complement cascade and tissue damage, as seen during ischemia-reperfusion injury [28, 35]. Natural antibody binding to self-antigens seems to be involved also in atherosclerosis development, where these antibodies contribute to plaque formation via their binding to oxidation-specific epitopes on low-density lipoproteins and cardiolipins [29, 32]. Thus, natural antibody secretion and activation must be carefully controlled to ensure their beneficial effects, while avoiding the potential dangers of their inappropriate activation.

7.4 Tissue Origins of Natural Antibodies

Early studies by Benner and colleagues followed the development of spontaneous antibody production in gnotobiotic and SPF-housed mice and demonstrated the largely antigen and T cell-independent development of spontaneous IgM-secreting cells in two tissues: the spleen and the bone marrow [36, 37]. However, their phenotype and lineage origins were not defined at that time, and the body cavities were not investigated as sources for natural antibody-producing cells. In support of the early studies by Benner, natural IgM-secreting B cells were shown by others to be present in the steady-state spleen [38, 39]. Erythrocyte-autoantibody transgenic mice lack splenic B-1 cells [40, 41] and B-1 cells were the major if not the only source of natural IgM in the spleen of Ig-allotype-chimeric mice [18].

Since B-1 cells are the predominant B cell subsets in the peritoneal cavity (PerC), natural IgM was thought to originate from there [7, 42]. Recently Rothstein and colleagues further suggested significant spontaneous IgM secretion by PerC B-1 cells [43]. A number of studies indicate, however, that PerC B-1 cells do not spontaneously produce natural IgM, either in vivo or ex vivo [38, 39, 44]. Instead, the data suggested that activation signals such as cytokines (IL-5 and IL-10) or mitogenic activation with LPS activated IgM secretion by PerC B-1 cells [40, 41]. Indeed, injection of bacteria or LPS into the PerC did not induce antibody production in the PerC. Instead it caused the migration [45] and differentiation [46] of PerC B-1 cells to IgM-secreting cells in the spleen via TLR4-mediated activation [45]. The spleen and the bone marrow are also sites of long-lived antibody production by B-2 cell-derived plasma cells following vaccination or infection [47, 48].

Because of these discrepancies in the literature and the importance of univocally identifying the B cells that produce natural antibodies, we recently revisited the question of the tissues and cellular origins of spontaneous IgM secretion [49]. For that we screened various tissues, including PerC, spleen, bone marrow, and lymph nodes for the presence of spontaneously IgM-secreting cells via allotype-specific ELISPOT. The results demonstrated that spleen and bone marrow, but not PerC (or pleural cavity) cells, contained large numbers of IgM-secreting cells, prior to immunization or infection. Since the bone marrow had never been shown to harbor B-1 cells, we then sought to identify the phenotype of these secreting cells. We demonstrated that spontaneous IgM-secreting cells were enriched among $CD19^{hi}$ $CD43^{+}$ IgM + cells, a phenotype consistent with that of B-1 cells. Further analysis showed that bone marrow B-1 cells resembled their counterparts in the spleen, but not the PerC by phenotype, as they lacked CD11b expression, a hallmark of body cavity B-1 cells, and they were IgM^{hi} IgD^{lo} $CD19^{hi}$ $CD43^{+}$. Finally, studies using allotype-chimeric mice confirmed that most of the spontaneous IgM-secreting cells in both spleen and bone marrow are B-1 cell derived. Remarkably, when B and T cell-deficient $RAG^{-/-}$ mice were reconstituted with bone marrow depleted of IgM + cells, thus depleting all B-1 cells, these mice had no detectable levels of serum IgM 6 weeks after reconstitution, compared to mice reconstituted with complete bone marrow, despite significant reconstitution of their B cell populations [49]. This data thus demonstrate that the bone marrow niche harbors IgM-secreting B-1 cells, which together with splenic B-1 cells are major contributors of serum IgM. The transfer of adult IgM^{-} bone marrow precursors, however, does not easily replenish these cells.

Collectively the data suggest that while B-1 cells are most prevalent in the body cavities, PerC cells do not appear to spontaneously secrete IgM. In contrast, B-1 cells in bone marrow and spleen, two major sites also of B-2 cell-derived plasma cells, instead support the production of natural IgM by B-1 cells. While body cavity B-1 cells do not generate steady-state natural IgM, they can nonetheless be rapidly activated to migrate to the spleen, and possibly the bone marrow, although the latter has not been experimentally demonstrated, to differentiate to IgM-secreting cells. Whether the repertoire of the splenic and bone marrow natural IgM is similar to that of IgM secretion by body cavity B-1 cells following their activation is currently unclear. Earlier studies on B-1a cells' ability to bind to PtC, visualized with PtC-liposomes by flow cytometry, showed higher frequencies of PtC-binders among the PerC than spleen B-1 cells. Clearly, more work is required to investigate the relationship between truly "natural" IgM-secreting B-1 cells and those that can be induced to differentiate and secrete IgM in response to an infection or other outside stimulus.

7.5 Regulation of Natural Antibody Production

The molecular mechanisms governing B-1 cell differentiation to antibody-secreting cells are somewhat controversial. Studies with B-2 cells have revealed the main regulators of the B cell differentiation pathway (reviewed in [1]). In B-2 cells, BLIMP-1 (B lymphocyte-induced maturation protein) induced during differentiation to Ig(M)-secreting plasma cells [50–52] is thought to suppress the transcriptional repressor PAX-5 (paired box protein) [53–55]. Removal of PAX-5 induces activation of XBP-1 (X-box-binding protein). XBP-1 and spliced XBP-1 (sXBP-1) regulate the unfolded protein response in the endoplasmic reticulum of antibody-secreting plasma cells [56, 57]. Ectopic expression of either BLIMP-1 or XBP-1 in B cell lines and primary B cells drives plasma cell differentiation [53, 57, 58].

The role of BLIMP-1 in the differentiation of B-1 cells to natural IgM-secreting cells is less clear. Calame and colleagues reported that BLIMP-1 is essential for B-1 cell-derived natural IgM production and protection from influenza infection, but is dispensable for the development and self-replenishment of B-1 cells in BLIMP-1 knockout mice [59]. In support of these findings, Tarlinton and colleagues reported that B-1 cells in the PerC express low levels of BLIMP-1 and do not spontaneously produce IgM, but could be induced to express BLIMP-1 and secrete IgM after LPS-stimulation [44]. In contrast, Tumang et al. concluded that B-1 cells do not depend on BLIMP-1 expression to differentiate into natural IgM-secreting cells in vitro as they found IgM production, but not BLIMP-1 expression by PerC B-1 cells [43].

The studies by Tumang et al. [43] were done with PerC B-1 cells, which they suggested produce IgM, but which we and others do not find to spontaneously produce significant amounts of IgM [38, 39, 44, 49]. While the reasons for these discrepant findings with regard to PerC B-1 IgM secretion are unknown, it is possible that the process of isolating B-1 cells from the PerC may cause their rapid activation and differentiation. This could explain spontaneous IgM secretion by PerC B-1 cells, possibly influenced by the method of isolation. In support, we observed that while total PerC cell cultures result in little IgM production when plated straight from harvest onto ELISPOT plates, aliquots of the same PerC cells, but stained and B-1 cells then sorted by FACS, will show enhanced IgM secretion, even in the absence of significant BLIMP-1 expression, as measured with cells from BLIMP-1 reporter mice (unpublished observation). Also, this "spontaneous" IgM secretion by PerC B-1 cells does not occur within the first day of culture, but increases with time of culture, indicating nonspecific activation in the cultures (unpublished observations). In addition, an earlier study had shown that the presence of prostaglandin (PG) E_2-producing macrophages can inhibit differentiation and IgM secretion of B-1 cells in the PerC [60] and that LPS stimulation of macrophages increases PGE_2

production. This would further suggest that isolation of B-1 cells may not only lead to their stimulation, the removal of PGE_2-producing cells during the isolation procedure may further enhance their spontaneous differentiation and IgM production.

Collectively, these studies indicate to us that B-1 cell in the body cavities are not contributing directly to the steady-state pool of natural antibodies, but that they can be stimulated rapidly to produce IgM. Indeed, as discussed above, multiple studies have shown the rapid relocation of PerC B-1 cells to the spleen following exposure to cytokines or mitogenic signals, consistent with the notion that PerC B-1 cells are ready to rapidly respond to an insult. Furthermore, it is possible that they but may not always fully differentiate to plasma cells to do so. Thus, most studies support the notion that the body cavity B-1 cells act like "armed soldiers" ready to go into combat zones (spleen or lymph nodes, possibly also mucosal sites) upon an infection-induced signal. While TLR4 has been shown to be one such signal, it is likely that other innate signals can have similar effects, based on the type of infection the host is encountering. This infection-induced production of body cavity-derived B-1 cell IgM must not be confused with the steady-state production of natural antibodies, mainly in bone marrow and spleen, which do not require outside signals for their production. Having identified true "natural" IgM-producing cells in the bone marrow of mice, their molecular characterization can now be used to define the differentiation stage and molecular mechanisms underlying natural antibody production.

7.6 B-1 Cell Migration

The scenario outlined above indicates that one important outcome of B-1 cell activation is their altered migratory behavior. However, the mechanisms of their migration to and from the body cavities and between body cavities and secondary lymphoid tissues are incompletely understood. B-1 cells are readily found in the blood of mice, where they represent about 0.5% of B cells; thus redistribution of B-1 cells via the bloodstream is a possible mechanism of their distribution throughout the body and into the body cavities. In support, Cyster and colleagues showed that mice deficient in the chemokine CXCL13 lack B-1 and B-2 cells in the body cavities while the numbers of B-1 cells in the blood of these mice were significantly elevated compared to wild-type mice [61]. The spleen populations appeared unaffected. Further support for considerable recirculation of B-1 cells into and out of the body cavities was provided in the same study with parabiosis experiments. Two months after fusion of the blood circulation between two mice, there was significant mixing of B-1a and B-1b cells in the PerC, albeit not as complete as that of B-2 cells. Thus, B-1 cells can circulate to and from body cavities, possibly via entry through the blood and omentum [61] in adult mice. It is likely that the increased migration of PerC B-1 cells, observed following TLR4-mediated activation, represents an enhancement of this otherwise rather slow recirculation process. Importantly, in the latter case, B-1 cells seem to lodge themselves into the spleen where they begin to secrete antibodies. Thus, activating signals seem to (a) enhance the migration of B-1 cells from the body cavities and (b) facilitate the enhanced entry of activated B-1 cells into secondary lymphoid tissues and (c) initiate their differentiation (Fig. 7.1).

7.7 Innate-Like B Cell Responses to Influenza Virus Infection

To study the responses and contribution of B-1 cells during an immune response, we began a number of years ago to focus on B-1 cell responses during influenza virus infection. Influenza virus infection in humans and other mammals is typically a respiratory tract infection, transmitted via droplets into the upper respiratory tract. Epithelial cells in the nasal cavity and/or upper pharynx are likely the first targets of seasonal influenza virus strains, although more pathogenic strains might directly infect lower parts of the lung. The local draining lymph nodes act as major sites of B cell response induction following influenza infection

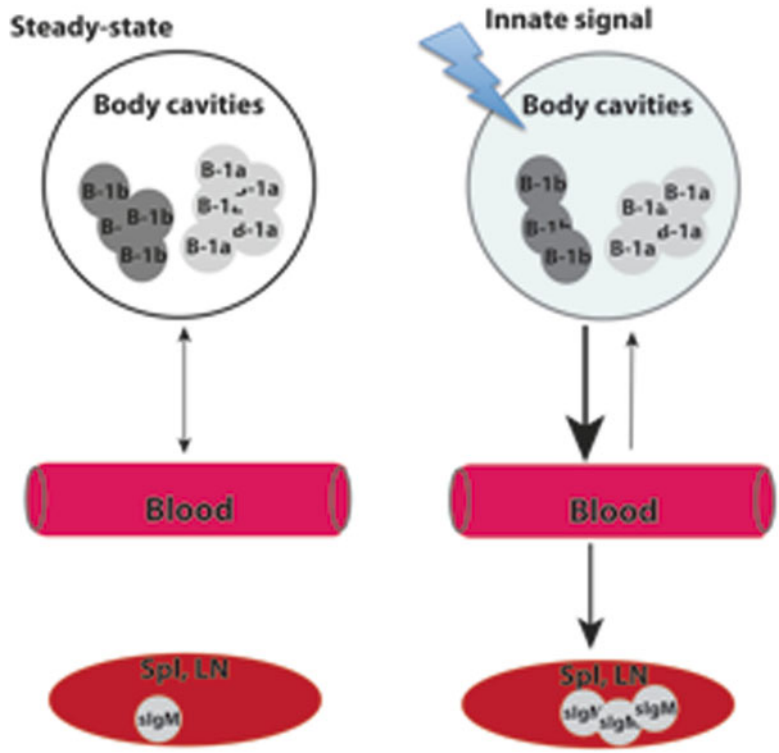

Fig. 7.1 Alteration in B-1 cell migration following an infection or insult. In steady state (*left*) there is a slow migration of B-1 cells into and out of the pleural and peritoneal cavity, likely via the omentum and similar structures in the pleural cavity into the blood. From there B-1 cell may recirculate back into the body cavities. IgM-secreting B-1 cells are also found in the spleen (and bone marrow, not depicted), providing steady-state natural IgM. In response to an infection (*right*), B-1 cell rapidly leave the body cavities, in the short-term lowering the numbers of B-1 cells at those sites. The cells then redistribute to spleen and other secondary lymphoid tissues, where they differentiate to IgM-producing cells

[62, 63]. In both mice [16, 63, 64] and ferrets [65], the gold standard influenza virus infection animal model, antibody-secreting cells are identified as early as 3 days after initial infection in the cervical and mediastinal lymph nodes as antibody titers rise in nasal washes and lung lavages. Antibody-secreting cells are identified in the cervical and mediastinal lymph nodes of mice and ferrets, as early as 3 days after initial infection [62, 63]. Since mice are usually infected directly into the lung tissues, lower respiratory tract draining mediastinal lymph nodes are the main secondary lymphoid tissues involved in the response.

Initial studies by us and others showed that B-1 cells contribute to protection from influenza virus infection even prior to any encounter with the virus by generating protective IgM antibodies that are generated constitutively in the absence of influenza exposure [8, 20, 66]. More recently, studies on their responses to influenza infection highlighted the ability of B-1 cells, specifically B-1a cells, to respond to infection in an innate-like manner. $CD5^+$ B-1a cells, but not $CD5^-$ B-1b cells, increased in frequency locally in the draining lymph nodes during acute influenza virus infection (days 5–10) and they secreted increased amounts virus-binding IgM into the airways [16], consistent with the above-outlined migration after B-1 cell activation. Importantly, influenza virus-binding IgM represented only a small fraction (roughly 10%) of the overall increases in IgM production by B-1a cells in lymph nodes and airways, and the relative amounts of influenza-binding

natural antibodies did not increase over time compared to non-influenza-binding IgM. This apparent lack of clonal expansion was further supported by BrdU-labeling studies, which indicated a complete lack of B-1 cell clonal expansion over the course of the infection [16].

Thus, B-1 cells respond to influenza infection with redistribution to and differentiation at the site of infection, while maintaining steady-state levels natural serum antibody levels [8, 16, 20]. It is tempting to speculate that the redistribution of B-1 cells, which our data show occurs at least in part from the pleural cavity, is a consequence of systemically elaborated innate cytokines. Consistent with this, influenza infection-induced type I IFN can profoundly affect leukocytes at distant sites [67], and our data thus far with type I IFNR$^{-/-}$ mice support a role for IFN in regulating B-1 cell migration via the activation of surface integrins (Waffarn, EE. Hastey, CJ, Dixit, N., Simon SI, and Baumgarth, N, in preparation). While we expected that the B-1 cell migration would result in the accumulation of lung IgM-secreting B-1 cells, we did not find evidence for that. Instead, the major accumulation of IgM-secreting B-1 cells occurred in the draining mediastinal lymph nodes [16], raising the question what additional functions, apart from IgM secretion, B-1 cells might play during the initiation of the adaptive immune response in the draining lymph nodes. There is evidence that B-1 cells and secreted IgM might contribute to immune protection against influenza other than by virus neutralization [16, 20, 22]. For example, B-1 cell-derived IgM is required for maximal induction of (B-2 cell-derived) influenza virus-specific IgG [20]. B-1 cells are also known as strong producers of IL-10 [68] and GM-CSF [69] thus could be involved in the regulation of local immune responses, similar to a recently identified regulatory role proposed for a B cell subset that shares some phenotypic characteristics with B-1 cells [70]. Increasing our understanding on the mechanisms that regulates B-1 cell migration and their function might help the development of therapies or prophylaxes to garner the help of these fascinating lymphocytes in immune protection.

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References

1. Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. Nature Rev Immunol. 2005;5:230–42.
2. Qi H. From SAP-less T cells to helpless B cells and back: dynamic T-B cell interactions underlie germinal center development and function. Immunol Rev. 2012 May;247(1):24–35.
3. Hooijkaas H, Benner R, Pleasants J, Wostmann B. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. Eur J Immunol. 1984;14:1127–30.
4. Bos N, Kimura H, Meeuwsen C, De Visser H, Hazenberg M, Wostmann B, et al. Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. Eur J Immunol. 1989;19:2335–9.
5. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur J Immunol. 1997;27:1557–63.
6. Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 Streptococcus pneumoniae. J Exp Med. 1981;153:694–705.
7. Masmoudi H, Mota-Santos T, Huetz F, Coutinho A, Cazenave PA. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. Int Immunol. 1990;2:515–20.
8. Baumgarth N, Herman OC, Jager GC, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are provided by distinct arms of the immune system. Proc Natl Acad Sci USA. 1999;96:2250–5.
9. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and bacterial distribution and disease by natural antibodies. Science. 1999;286:2156–8.
10. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. Immunity. 2012 Jan 27;36(1):13–21.
11. Montecino-Rodriguez E, Dorshkind K. Formation of B-1 B cells from neonatal B-1 transitional cells exhibits NF-kappaB redundancy. J Immunol. 2011 Dec 1;187(11):5712–9.
12. Montecino-Rodriguez E, Lethers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. Nature Immunol. 2006;3:293–301.

13. Song H, Cerny J. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. J Exp Med. 2003 Dec 15;198(12):1923–35.
14. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. Immunity. 2005 Jul;23(1):7–18.
15. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. Immunity. 2004;21:379–90.
16. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. J Exp Med. 2008 Dec 22;205(13):3053–64.
17. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat Rev Immunol. 2011 Jan;11(1):34–46.
18. Stall AM, Wells SM, Lam KP. B-1 cells: unique origins and functions. Sem Immunol. 1996;8:45–59.
19. Kroese FGM, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg LA. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. Int Immunol. 1989;1:75–84.
20. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. J Exp Med. 2000 Jul 17;192(2):271–80.
21. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J Exp Med. 1998 Dec 21;188(12):2381–6.
22. Jayasekera JP, Moseman EA, Carroll MC. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. J Virol. 2007 Apr;81(7):3487–94.
23. Zhou ZH, Zhang Y, Hu YF, Wahl LM, Cisar JO, Notkins AL. The broad antibacterial activity of the natural antibody repertoire is due to polyreactive antibodies. Cell Host Microbe. 2007 Mar 15;1(1):51–61.
24. Baumgarth N, Chen J, Herman OC, Jager GC, Herzenberg LA. The role of B-1 and B-2 cells in immune protection from influenza virus infection. Curr Top Microbiol Immunol. 2000;252:163–9.
25. Baumgarth N. A two-phase model of B-cell activation. Immunol Rev. 2000 Aug;176:171–80.
26. Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science. 2000 Jun 23;288(5474):2222–6.
27. Notkins A. Polyreactivity of antibody molecules. Trends Immunol. 2004;25:174–9.
28. Kulik L, Fleming SD, Moratz C, Reuter JW, Novikov A, Chen K, et al. Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury. J Immunol. 2009 May 1;182(9):5363–73.
29. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J Clin Invest. 2000 Jun;105(12):1731–40.
30. Binder CJ, Silverman GJ. Natural antibodies and the autoimmunity of atherosclerosis. Springer Semin Immunopathol. 2005 Mar;26(4):385–404.
31. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science. 2009 Mar 27;323(5922):1722–5.
32. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. J Clin Invest. 2009 May;119(5):1335–49.
33. Parker W, Lundberg-Swanson K, Holzknecht ZE, Lateef J, Washburn SA, Braedehoeft SJ, et al. Isohemagglutinins and xenoreactive antibodies: members of a distinct family of natural antibodies. Hum Immunol. 1996 Feb;45(2):94–104.
34. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. Proc Natl Acad Sci USA. 2000 Feb 1;97(3):1184–9.
35. Zhang M, Alicot EM, Chiu I, Li J, Verna N, Vorup-Jensen T, et al. Identification of the target self-antigens in reperfusion injury. J Exp Med. 2006 Jan 23;203(1):141–52.
36. Hooijkaas H, van der Linde-Preesman AA, Benne S, Benner R. Frequency analysis of the antibody specificity repertoire of mitogen-reactive B cells and "spontaneously" occurring "background" plaque-forming cells in nude mice. Cell Immunol. 1985 Apr 15;92(1):154–62.
37. Van Oudenaren A, Haaijman JJ, Benner R. Frequencies of background cytoplasmic Ig-containing cells in various lymphoid organs of athymic and euthymic mice as a function of age and immune status. Immunology. 1984 Apr;51(4):735–42.
38. Ohdan H, Swenson KG, Kruger Gray HS, Yang Y-G, Xu Y, Thall AD, et al. Mac-1-negative B-1b phenotype of natural antibody-producing cells, including those responding to Galα1,3Gal epitopes in α1,3-galactosyltransferase-deficient mice. J Immunol. 2000;165:5518–29.
39. Kawahara T, Ohdan H, Zhao G, Yang Y-G, Sykes M. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. J Immunol. 2003;171:5406–14.
40. Murakami M, Tsubata T, Shinkura R, Nisitani S, Okamoto M, Yoshioka H, et al. Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. J Exp Med. 1994 Jul 1;180(1):111–21.

41. Nisitani S, Tsubata T, Murakami M, Honjo T. Administration of interleukin-5 or -10 activates peritoneal B-1 cells and induces autoimmune hemolytic anemia in anti-erythrocyte autoantibody-transgenic mice. Eur J Immunol. 1995;25:3047–52.
42. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. Annu Rev Immunol. 2002;20:253–300.
43. Tumang JR, Frances R, Yeo SG, Rothstein TL. Cutting edge: Spontaneously Ig-secreting B-1 cells violate the accepted paradigm for expression of differentiation-associated transcription factors. J Immunol. 2005;174:3173–7.
44. Fairfax KA, Corcoran LM, Pridans C, Huntington ND, Kallies A, Nutt SL, et al. Different kinetics of Blimp-1 induction in B cell subsets revealed by reporter gene. J Immunol. 2007;178:4104–11.
45. Ha S, Tsuji M, Suzuki K, Meek B, Yasuda N, Kaisho T, et al. Regulation of B1 cell migration by signals through toll-like receptors. J Exp Med. 2006;203: 2541–50.
46. Yang Y, Tung JW, Ghosn EEB, Herzenberg LA, Herzenberg LA. Division and differentiation of natural antibody-producing cells in mouse spleen. Proc Natl Acad Sci USA. 2007;104:4542–6.
47. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity. 1998;8:363–72.
48. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature. 2002;421:282–7.
49. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. European journal of immunology. 2012 Jan;42(1):120–9.
50. Shaffer AL, Lin K-I, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity. 2002;17:51–62.
51. Kallies A, Hasbold J, Fairfax K, Pridans C, Emslie D, McKenzie BS, et al. Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. Immunity. 2007;26:555–66.
52. Sciammas R, Davis MM. Blimp-1; immunoglobulin secretion and the switch to plasma cells. Curr Top Microbiol Immunol. 2005;290:201–24.
53. Lin K-I, Angelin-Duclos C, Kuo TC, Calame K. Blimp-1 dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. Mol Cell Immunol. 2002;22:4771–80.
54. Delogu A, Schebesta A, Sun Q, Aschenbrenner K, Perlot T, Busslinger M. Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. Immunity. 2006;24:269–81.
55. Nera K-P, Kohonen P, Narvi E, Peippo A, Mustonen L, Terho P, et al. Loss of Pax5 promotes plasma cell differentiation. Immunity. 2006;24:283–93.
56. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, et al. Plasma cell differentiation requires the transcription factor XBP-1. Nature. 2001;412:300–7.
57. Iwakoshi NN, Lee A-H, Vallabhajosyula P, Otipoby K, Rajewsky K, Glimcher LH. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nature Immunol. 2003;4:321–9.
58. Sciammas R, Davis MM. Modular nature of Blimp-1 in the regulation of gene expression during B cell maturation. J Immunol. 2004;172:5427–40.
59. Savitsky D, Calame K. B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. J Exp Med. 2006;203:2305–14.
60. Chace JH, Fleming AL, Gordon JA, Perandones CE, Cowdery JS. Regulation of differentiation of peritoneal B-1a (CD5+) B cells: Activated peritoneal macrophages release prostaglandin E_2, which inhibits IgM secretion by peritoneal B-1a cells. J Immunol. 1995;154:5630–6.
61. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. Immunity. 2002 Jan;16(1): 67–76.
62. Sangster MY, Riberdy JM, Gonzalez M, Topham DJ, Baumgarth N, Doherty PC. An early CD4+ T cell-dependent immunoglobulin A response to influenza infection in the absence of key cognate T-B interactions. J Exp Med. 2003 Oct 6;198(7):1011–21.
63. Sealy R, Surman S, Hurwitz JL, Coleclough C. Antibody response to influenza infection of mice: different patterns for glycoprotein and nucleocapsid antigens. Immunology. 2003 Apr;108(4):431–9.
64. Coro ES, Chang WL, Baumgarth N. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. J Immunol. 2006 Apr 1;176(7):4343–51.
65. McLaren C, Butchko GM. Regional T- and B-cell responses in influenza-infected ferrets. Infect Immun. 1978 Oct;22(1):189–94.
66. Savitsky D, Calame K. B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. J Exp Med. 2006 Oct 2;203(10):2305–14.
67. Hermesh T, Moltedo B, Moran TM, Lopez CB. Antiviral instruction of bone marrow leukocytes during respiratory viral infections. Cell host & microbe. 2010 May 20;7(5):343–53.
68. O'Garra A, Howard M. IL-10 production by CD5 B cells. Annals of the New York Academy of Sciences. 1992 May 4;651:182–99.
69. Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, et al. Innate response activator B cells protect against microbial sepsis. Science. 2012 Feb 3;335(6068):597–601.
70. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. Immunological reviews. 2008 Aug;224:201–14.

The CD28/B7 Pathway: A Novel Regulator of Plasma Cell Function

8

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Abstract

The CD28/B7 pathway is pivotal for the activation, optimal function, and regulation of T cell function. While the CD28 receptor and its ligands B7.1/B7.2 are also expressed on plasma cells, little is known of the role of the CD28/B7 pathway in plasma cell function. In this chapter we discuss the recent studies that have examined the role of CD28 expression on plasma cell function. Both stimulatory and inhibitory effects of CD28 on plasma cells have been reported. Based on our findings we propose that under homeostatic conditions the CD28/B7 interaction mediates regulation of plasma cell function whereas during inflammation this pathway can be perturbed to ramp up Ab production from existing plasma cells.

Keywords

Plasma cells • CD28 • B cells • Antibody • CD28/B7

Abbreviations

Abs	Antibodies
PD	Programmed death
PDL	Programmed death ligand
BAFF	B cell activating factor
BAFF-R	B cell activating factor receptor
APRIL	A proliferation-inducing ligand
TACI	Transmembrane activator, calcium modulator, and cyclophilin ligand interactor
NOD	Non-obese diabetic mice

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8.1 Introduction

Immunological memory is an important feature of the adaptive immune response; it is the ability to remember previously encountered antigens and respond promptly and more effectively than during the primary response. When naive B or T cells encounter cognate antigen, they differentiate into effectors that facilitate antigen clearance.

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The majority of these effectors die while a subset of the antigen-experienced lymphocytes differentiate into long-lived memory cells [1]. Upon subsequent encounter with the same antigen these memory B and T cells mediate a rapid and effective immune response which prevents disease [2]. These anamnestic responses can provide immunological protection for a lifetime, and thus are the cornerstone for effective vaccination.

Most of the effective vaccines require B cell memory to provide protection [3]; this exemplifies the importance of the memory B cell compartment. Memory B cells and plasma cells contribute to the anamnestic humoral responses. They arise from the germinal center B cells, after undergoing isotype switching, rigorous selection, and affinity maturation [4, 5]. Memory B cells remain quiescent and reside mainly in the marginal zone of the spleen; upon reencounter with antigen they differentiate into antibody-secreting plasma cells [6, 7]. Plasma cells secrete high-affinity antibodies (Abs); most of the long-lived plasma cells reside in the bone marrow [8], although tissue-specific plasma cells have also been reported [8, 9]. Plasma cells continuously secrete Abs; the presence of specific Abs serves as the first line of the memory response, resulting in the control and often the elimination of invading pathogen.

Recent studies have made good progress in identifying the potential molecular interactions involved in the generation and maintenance of plasma cells. Studies conducted in murine knockout models indicate that the engagement of complement receptor 1/2 (CR1/2) [10], programmed death ligand 1/2 (PDL-1/2) [11], CD80 [12], and IL-21R [13] on germinal center B cells with their counter-receptors on follicular dendritic cells/T helper cells is critical for the optimal production of plasma cells, whereas their maintenance is attributed to their expression of B cell maturation antigen (BCMA) [14], B cell activating factor receptor (BAFF-R) [15], and transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) [16] which interact with survival cytokines a proliferation-inducing ligand (APRIL) and BAFF.

The molecular interactions involved in the regulation of plasma cells are less well understood. Since plasma cells are terminally differentiated, their regulation is generally underappreciated. Nonetheless, in autoimmune disease conditions manifested by rampant antibody production against self-antigens it becomes evident that under normal conditions perhaps plasma cells are under homeostatic regulation. In support of this notion, a recent study suggests that autoantibody-producing cells may be regulated via the interaction between PD-1 on B cells and PD-1-PDL1/2 on regulatory T cells [17]. Further studies are warranted to investigate the regulation of plasma cells under steady state conditions. In this chapter we examine the possible role of CD28/B7 pathway in regulation of plasma cell function.

8.2 CD28/B7 Costimulation in T Cell Function

CD28 is a 44 kDa glycoprotein expressed on the surface of T cells, plasma cells, and NK cells [18–20]. It binds to B7.1 (CD80) and B7.2 (CD86), which are expressed on dendritic cells, macrophages, B cells, and T cells [21–23]. The interaction between CD28 on T cells and B7.1/B7.2 molecules on antigen-presenting cells provides a crucial costimulatory signal required for the activation and optimal function of T cells [21, 22]. Since the CD28/B7 pathway has been extensively studied in T cells [24, 25], we will begin with an overview of how this pathway modulates T cell function. CD28 signaling in T cells leads to the activation of AKT, which stimulates numerous molecules including NF-κB, IL-2, and Bcl-XL; these molecules subsequently enhance activation, proliferation, and survival of T cells that have encountered antigen [26–28]. Moreover, the CD28/B7 costimulation is also involved in promoting metabolism in activated T cells so as to facilitate their effector functions [29]. Given the central role of CD28/B7 costimulation in T cell activation it is inevitable that T cells that recognize antigen without concurrent CD28 signaling become anergic.

The CD28/B7 costimulatory pathway was first identified as the critical second signal required for naive T cells activation [18] and its role in primary responses has been thoroughly investigated. Relatively fewer studies assessed the role of CD28/B7 on memory T cell responses, in part due to the widely held belief that memory T cells do not require costimulation. This belief was based on results from studies using in vitro reactivation memory T cells, which showed that unlike naive T cells, memory T cells can be activated by antigen without B7.1/B7.2 costimulation [30, 31]. A few in vivo studies corroborated the in vitro observation concluding that memory T cells do not require costimulation [32, 33]. In one of these studies by Suresh et al. they infected and rechallenged *Cd28*$^{-/-}$ mice with LCMV and assessed their primary and secondary T cell responses. They showed that memory CD8 responses did not require CD28 costimulation [33]. Interestingly these mice exhibited a normal primary response suggesting that the LCMV infection system used here obviates the CD28 requirement for naive T cells; thus it is possible that this system bypasses the CD28 requirements for memory T cells as well. Conversely, a couple of studies demonstrated that memory T cells require dendritic cells for their optimal responses to infections with *Listeria monocytogenes*, vesicular stomatitis virus, and influenza virus [34, 35]. Given that the major role of DC in naive T cell activation is to provide costimulation and cytokines, the enhanced memory T cell responses in the presence of DCs suggested that perhaps memory T cells still need costimulation.

Indeed recent studies suggest that memory T cells need CD28 costimulation for optimal function [36]. This phenomenon was initially reported by Farber and colleagues; in this study they rechallenged adoptive hosts of influenza virus-specific memory CD4 T cells in the presence of CD28/B7 blocking agent- CTLA4-Ig or isotype control. They discovered that memory CD4 T cells responding in the presence of CTLA4-Ig despite upregulating CD25 and CD69—early activation markers—exhibited diminished proliferation and differentiation in comparison to CD4 T cells from isotype control-treated hosts [37]. Subsequently, Borowski et al. used HSV and Influenza A virus infection models to assess the CD28 requirements for memory CD8 T cells. They demonstrated that in the absence of CD28 signaling memory CD8 T cells exhibit reduced expansion resulting in impaired protection. The diminished memory CD8 T cell responses in the absence of CD28 were due to cell cycle arrest and failure to down-modulate Bcl2 [38]. Unlike the earlier in vivo studies [32, 33], these recent studies [37–40] were able to decipher the role of CD28/B7 costimulation in memory T cells likely due to the use of memory T cells generated in a CD28-sufficient environment; specific targeting of the CD28/B7 pathway using specific CD28, B7.1/B7.2 blocking antibodies, or CTLA4-Ig; and assessment of in vivo proliferation rather than ex vivo expansion. Taken together, these studies showed that both memory CD4 and CD8 T cells responding to secondary challenge require CD28 signaling to undergo robust proliferation and differentiation.

In addition to its costimulatory role, the CD28/B7 pathway is also important for the regulation of T cell function via promotion of thymic development and peripheral maintenance of regulatory T cells [41]. Regulatory T cells play a pivotal role in maintaining self-tolerance thus preventing T cell responses against self-antigens. This phenomenon is exemplified in studies which showed that the depletion of Tregs in non-autoimmune prone Balb/c mice leads to spontaneous development of T cell-mediated autoimmune disease [42]. The importance of the CD28/B7 pathway in regulatory T cell-mediated tolerance is demonstrated in non-obese diabetic (NOD) mice that are bred with *Cd28*$^{-/-}$ or *B7.1/B7.2*$^{-/-}$ mice. NOD mice spontaneously develop diabetes at 10–12 weeks of age; however, when they lack CD28 or B7.1/B7.2 these mice develop diabetes earlier, at a higher frequency, and increased disease severity in comparison to their littermates. The severe diabetes in NOD *Cd28*$^{-/-}$ and NOD *B7.1/B7.2*$^{-/-}$ mice is attributed to the depletion of Tregs in these hosts [43]. Thus the CD28/B7 costimulatory pathway is important for the optimal activation and regulation of T cell function.

8.3 CD28/B7 in B Cell Responses

Due to the pivotal role of CD28/B7 costimulation on T cells, this pathway is also important for T-dependent B cell responses. Upon encounter with cognate antigen, the primed B cells require an interaction with activated T cells in order to enter a germinal center reaction and undergo affinity maturation to produce memory B cells and long-lived plasma cells. T cell activation usually occurs when antigen-presenting cells—mainly DCs—present antigen and provide B7-CD28 costimulation to the CD4 T cells. CD28/B7 costimulation enhances the expression of ICOS [44] and CD40L [45, 46] on the T cells which are critical for the subsequent activation of B cells [47–51]. Thus, mice that are deficient in CD28 expression have poor T cell responses resulting in impaired T-dependent B cell responses including isotype switching and germinal center formation.

8.4 CD28/B7 in Plasma Cell Function

Interestingly, upon plasma cell differentiation BLIMP-1 suppresses PAX5, the transcription factor essential for B cell lineage commitment, and this induces CD28 expression [52]. Therefore, plasma cells express both CD28 and its ligands B7.1/B7.2. The role of the CD28 expression on plasma cells has not been fully explored; thus far most of the studies have been done in transformed plasma cells from multiple myeloma patients. CD28 expression in multiple myeloma cells correlates with tumor expansion [53] and prolonged survival [54, 55]. Additionally, in these patients, the level of CD28 expression on their plasma cells correlates with poor disease prognosis [53, 55, 56]. Taken together, these observations suggest that CD28 expression enhances plasma cell proliferation and survival. However, there has also been a report indicating that CD28 signaling in multiple myeloma cells induces apoptosis [57]. The precise role of CD28 in plasma cells, particularly normal antigen-driven plasma cells, is still under investigation.

The initial report on the effect of CD28 deficiency on plasma cells was from a study by Busslinger et al.; they reconstituted lethally irradiated *Rag2*$^{-/-}$ hosts with bone marrow cells from *Cd28*$^{-/-}$ or WT hosts and B cell-deficient conditional *Pax5*$^{-/-}$ mice at a 1:1 ratio. The reconstituted hosts were immunized with NIP-OVA in complete freund's adjuvant and plasma cell responses were examined 2 weeks after immunization. The NIP-IgG1 titer of the *Cd28*$^{-/-}$-reconstituted hosts was significantly lower than the wild-type controls [52]. Similar observations have been reported by Rozanski et al. In their study they lethally irradiated B6.SJL.Ly5.1 mice and reconstituted them with bone marrow from *Cd28*$^{-/-}$ or WT hosts and B cell-deficient μMT hosts at a 60:40 ratio. In the reconstituted host, the B cells would be derived from the *Cd28*$^{-/-}$ or WT bone marrow cells, whereas the other hematopoietic cells would be derived from both *Cd28*$^{-/-}$or WT and μMT bone marrow cells. They immunized the reconstituted hosts with NIP-OVA in complete freund's adjuvant and rechallenged the mice a week later with NIP-OVA in incomplete freund's adjuvant. They report a significant decline in plasma cell numbers and IgG1 titers in the hosts that received *Cd28*$^{-/-}$ bone marrow cells in comparison to the WT controls [58]. These reports suggest that CD28 is critical for plasma cell function and survival.

We have also investigated the role of CD28 expression on normal plasma cells using various models. In the first model, we adoptively transferred mature splenic B cells from either *Cd28*$^{-/-}$ or WT hosts into B cell-deficient μMT hosts. In this system CD28 expression is intact in all the cells, except the transferred B cells from *Cd28*$^{-/-}$ mice. We then immunized these hosts with NP-CGG in alum and examined their antibody levels over a time course of 6 months; we found that adoptive hosts that received *Cd28*$^{-/-}$ B cells produced higher NP-CGG-specific IgM than their wild-type counterparts (Fig. 8.1a). We also found that at 6 months postimmunization, these mice had a higher frequency of plasma cells than the WT controls (Fig. 8.1b). These observations suggested that CD28 deficiency enhances plasma cell function. This is in contradiction with previous reports from multiple myeloma cells and the studies

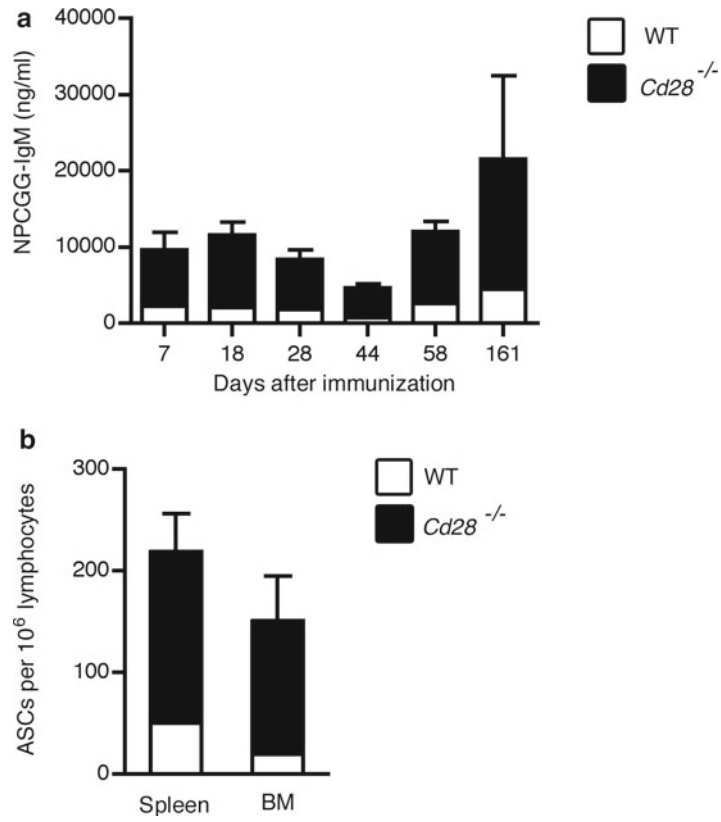

Fig. 8.1 CD28 deficiency in plasma cells leads to upregulation of antibody production and plasma cell frequency. Cohorts of B cell-deficient μMT mice were reconstituted with splenic B cells from wild-type C57BL/6 or *Cd28*$^{-/-}$ mice and immunized with 50 μg NP-CGG/alum and the B cell responses were examined for 6 months. (**a**) ELISA results showing serum anti-NP-CGG IgM levels on the indicated time course. (**b**) The frequency of plasma cells secreting NP-CGG-specific IgM in the spleen and bone marrow (BM) as determined by ELISPOT, 6 months postimmunization

using model antigen discussed above. Therefore, to ascertain these observations we examined this phenomenon in bone marrow chimeric hosts. We reconstituted lethally irradiated B6.SJL.Ly5.1 mice with *Cd28*$^{-/-}$ or WT bone marrow cells and μMT bone marrow cells at a ratio of 40:60. In the reconstituted hosts, most of the T cells expressed CD28 whereas the plasma cells from the *Cd28*$^{-/-}$ recipients did not. We immunized these hosts with NP-CGG in alum and *Cd28*$^{-/-}$ recipients exhibit higher NP-CGG IgG titers and maintain a higher frequency of plasma cells than the wild-type controls [59]. Finally, we assessed whether CD28 expression would have an impact on plasma cells responding to a physiological infection. We adoptively transferred *Cd28*$^{-/-}$ or WT B cells and WT T cells into *Rag1*$^{-/-}$ hosts and infected these hosts with influenza virus. Consistent with our previous observations, *Cd28*$^{-/-}$ hosts had higher hemagglutination inhibition and neutralization titers than the WT controls [59]. Collectively, these observations suggest that in normal plasma cells generated in response to antigen CD28 expression serves as a negative regulator of their function.

The discrepancy between our observations and the previous reports could be due to several technical variables in the experiment design. First, we used different adjuvants; we used alum while both of the studies associating a decline in plasma cell function with CD28 deficiency used complete freund's adjuvant/incomplete freund's adjuvant. Second, in the study by Busslinger et al. they assessed plasma cell function only at 2 weeks postimmunization; at this time point Abs from short-lived plasma cells would still be detected. To exclusively detect the long-lived plasma cell titers one has to wait for at least a month after immunization. Third, in the Rozanski study, they rechallenged the mice 1 week after initial immunization; the presence of antigen at this time point is known to disrupt germinal centers [60] and thus plasma cell output. However, in real life all these scenarios are possible: We get

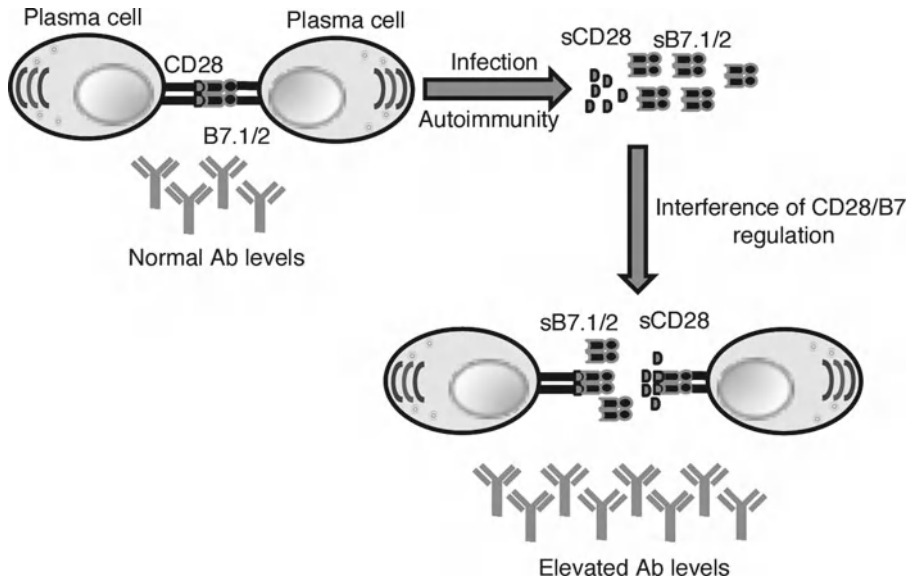

Fig. 8.2 Model for CD28/B7-mediated regulation of plasma cell function. Schematic diagram showing proposed regulation of plasma cells under steady state conditions via the CD28/B7 interaction. The production of soluble CD28 or B7.1/B7.2 molecules may ensure infection or an autoimmune disorder and could possibly interrupt the CD28/B7 interaction on plasma cells resulting in elevated antibody production and frequency of plasma cells

vaccinated with different adjuvants, we encounter different types of pathogens which could mimic different adjuvants, their antigens persist for variable lengths of time, and in immunocompromised patients and in disease-endemic regions individuals may get re-infected by the same pathogen within a short time interval. It appears that all these factors may determine the role of CD28 in plasma cell function.

Activated B cells express B7.1/B7.2 which facilitate their activation [51, 53, 54]; further analysis of B7.1- and B7.2-CD28 signaling revealed that B7.2 is important for the initiation of the immune response whereas B7.1 is involved in sustaining the response, mediating isotype switching and germinal center formation [61]. Interestingly, Rau et al. have shown that while global *B7.1/B7.2*$^{-/-}$ drastically affects germinal center formation in response to influenza virus infection, B7.1/B7.2 deficiency exclusively on B cells does not affect germinal center formation [62]. Additionally, B7.1 signaling has also been implicated in the regulation of B cell function [63, 64]. Plasma cells retain B7.1/B7.2 expression; the role of these molecules in plasma cell function is not well understood. Recently, it has been proposed that B7.1/B7.2 expression on plasma cells may facilitate their interaction with follicular helper T cells [65]. Since plasma cells express both CD28 and its ligands B7.1/B7.2, there is a possibility of autonomous CD28/B7 interaction. This made us wonder whether B7.1/B7.2 deficiency in plasma cells would mimic CD28 deficiency and likewise impair the CD28-mediated regulation. To investigate the role of B7.1/B7.2 in plasma cell function, we adoptively transferred *B7.1/B7.2*$^{-/-}$ B cells into a μMT B cell-deficient host and immunized these hosts with NP-CGG in alum. We followed their plasma cell responses for 5 months and we find a significant increase in the NP-CGG-specific IgM titers and frequency of plasma cells in the *B7.1/B7.2*$^{-/-}$ recipients in comparison to their wild-type counterparts [59]. These results are similar to those observed in *Cd28*$^{-/-}$ recipients, suggesting that the CD28/B7 interaction between plasma cells regulates their antibody production.

Based on our observations, we propose the following model for CD28-mediated regulation of plasma cells. We envision that under normal conditions, CD28 on plasma cells is engaged with B7.1/B7.2 resulting in the maintenance of antibody secretion at normal levels. Interference of this CD28/B7 interaction would then lead to an increase in antibody production and plasma cell frequency (Fig. 8.2). It turns out that CD28 and B7 molecules can also be expressed as secreted soluble molecules; these lack the transmembrane domain due to alternative mRNA splicing [66, 67]. The production of soluble CD28/B7.1/B7.2 may transiently relieve the CD28/B7 regulation of plasma cells, thus, boosting antibody production. Interestingly, a recent study has shown that during hepatitis B infection the levels of soluble CD28 increase [68]. This suggests that perhaps during infection the rise in soluble CD28 disrupts the CD28/B7 regulation so as to boost antibody production and control infection. Conversely, several antibody-driven autoimmune diseases such as systemic lupus erythematosus [69] and rheumatoid arthritis [70] are associated with a dramatic increase of soluble CD28, B7.1, and/or B7.2. We conjecture that the soluble molecules may disrupt the CD28/B7 plasma cell regulation leading to excessive autoantibody production. Studies are underway to validate these hypotheses.

8.5 Concluding Remarks

Here, we have reviewed studies that examined the role of CD28 expression on plasma cell function. It is clear that CD28 expression on plasma cells has an impact on their antibody function and perhaps survival. However, the evidence on the precise role of CD28 in plasma cell function is contradictory; both stimulatory and inhibitory effects have been reported. Further studies investigating this phenomenon using CD28 conditional knockout mice may provide more definitive evidence. Based on our experience we propose that the CD28/B7 pathway plays a regulatory role in plasma cell function and maintenance.

References

1. Gourley TS, Wherry EJ, Masopust D, Ahmed R. Generation and maintenance of immunological memory. Semin Immunol. 2004 Oct;16(5):323–33.
2. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. Science. 1996 Apr 5;272(5258):54–60.
3. McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. Nat Rev Immunol. 2012 Jan;12(1):24–34.
4. Jacob J, Przylepa J, Miller C, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. J Exp Med. 1993 Oct 1;178(4):1293–307.
5. Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. Nature. 1991 Dec 5;354(6352):389–92.
6. Chappell CP, Dauner J, Jacob J. Ontogeny of the secondary antibody response: origins and clonal diversity. Adv Exp Med Biol. 2009;633:27–41.
7. McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. Annu Rev Immunol. 2005;23:487–513.
8. Slifka MK, Ahmed R. Long-term antibody production is sustained by antibody-secreting cells in the bone marrow following acute viral infection. Ann N Y Acad Sci. 1996 Oct 25;797:166–76.
9. Jones PD, Ada GL. Persistence of influenza virus-specific antibody-secreting cells and B-cell memory after primary murine influenza virus infection. Cell Immunol. 1987 Oct 1;109(1):53–64.
10. Gatto D, Pfister T, Jegerlehner A, Martin SW, Kopf M, Bachmann MF. Complement receptors regulate differentiation of bone marrow plasma cell precursors expressing transcription factors Blimp-1 and XBP-1. J Exp Med. 2005 Mar 21;201(6):993–1005.
11. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nat Immunol. 2010 Jun;11(6):535–42.
12. Good-Jacobson KL, Song E, Anderson S, Sharpe AH, Shlomchik MJ. CD80 expression on B cells regulates murine T follicular helper development, germinal center B cell survival, and plasma cell generation. J Immunol. 2012 May 1;188(9):4217–25.
13. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J Exp Med. 2010 Feb 15;207(2):365–78.
14. O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. J Exp Med. 2004 Jan 5;199(1):91–8.

15. Mackay F, Browning JL. BAFF: a fundamental survival factor for B cells. Nat Rev Immunol. 2002 Jul;2(7):465–75.
16. Marsters SA, Yan M, Pitti RM, Haas PE, Dixit VM, Ashkenazi A. Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI. Curr Biol. 2000 Jun 29;10(13): 785–8.
17. Gotot J, Gottschalk C, Leopold S, Knolle PA, Yagita H, Kurts C, et al. Regulatory T cells use programmed death 1 ligands to directly suppress autoreactive B cells in vivo. Proc Natl Acad Sci USA. 2012 Jun 11.
18. Aruffo A, Seed B. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc Natl Acad Sci USA. 1987 Dec;84(23): 8573–7.
19. June CH, Ledbetter JA, Linsley PS, Thompson CB. Role of the CD28 receptor in T-cell activation. Immunol Today. 1990 Jun;11(6):211–6.
20. Kozbor D, Moretta A, Messner HA, Moretta L, Croce CM. Tp44 molecules involved in antigen-independent T cell activation are expressed on human plasma cells. J Immunol. 1987 Jun 15;138(12):4128–32.
21. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J Exp Med. 1991 Mar 1;173(3):721–30.
22. Freeman GJ, Gribben JG, Boussiotis VA, Ng JW, Restivo VA, Jr., Lombard LA, et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science. 1993 Nov 5;262(5135): 909–11.
23. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005;23:515–48.
24. Allison JP. CD28-B7 interactions in T-cell activation. Curr Opin Immunol. 1994 Jun;6(3):414–9.
25. Sharpe AH. Mechanisms of costimulation. Immunological reviews. 2009 May;229(1):5–11.
26. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. ARI. 1996;14: 233–58.
27. Thompson CB, Lindsten T, Ledbetter JA, Kunkel SL, Young HA, Emerson SG, et al. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. Proc Natl Acad Sci USA. 1989 Feb;86(4):1333–7.
28. Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity. 1995 Jul;3(1):87–98.
29. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol. 2005 Nov;5(11):844–52.
30. Croft M, Bradley LM, Swain SL. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J Immunol. 1994 Mar 15;152(6):2675–85.
31. London CA, Lodge MP, Abbas AK. Functional responses and costimulator dependence of memory CD4+ T cells. J Immunol. 2000 Jan 1;164(1): 265–72.
32. Kim SK, Schluns KS, Lefrancois L. Induction and visualization of mucosal memory CD8 T cells following systemic virus infection. J Immunol. 1999 Oct 15;163(8):4125–32.
33. Suresh M, Whitmire JK, Harrington LE, Larsen CP, Pearson TC, Altman JD, et al. Role of CD28-B7 interactions in generation and maintenance of CD8 T cell memory. J Immunol. 2001 Nov 15;167(10):5565–73.
34. Zammit DJ, Cauley LS, Pham QM, Lefrancois L. Dendritic cells maximize the memory CD8 T cell response to infection. Immunity. 2005 May;22(5): 561–70.
35. Belz GT, Wilson NS, Smith CM, Mount AM, Carbone FR, Heath WR. Bone marrow-derived cells expand memory CD8+ T cells in response to viral infections of the lung and skin. Eur J Immunol. 2006 Feb;36(2):327–35.
36. Boesteanu AC, Katsikis PD. Memory T cells need CD28 costimulation to remember. Semin Immunol. 2009 Apr;21(2):69–77.
37. Ndejembi MP, Teijaro JR, Patke DS, Bingaman AW, Chandok MR, Azimzadeh A, et al. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. J Immunol. 2006 Dec 1;177(11):7698–706.
38. Borowski AB, Boesteanu AC, Mueller YM, Carafides C, Topham DJ, Altman JD, et al. Memory CD8+ T cells require CD28 costimulation. J Immunol. 2007 Nov 15;179(10):6494–503.
39. Garidou L, Heydari S, Truong P, Brooks DG, McGavern DB. Therapeutic memory T cells require costimulation for effective clearance of a persistent viral infection. J Virol. 2009 Sep;83(17):8905–15.
40. Fuse S, Zhang W, Usherwood EJ. Control of memory CD8+ T cell differentiation by CD80/CD86-CD28 costimulation and restoration by IL-2 during the recall response. J Immunol. 2008 Jan 15;180(2):1148–57.
41. Liston A, Rudensky AY. Thymic development and peripheral homeostasis of regulatory T cells. Curr Opin Immunol. 2007 Apr;19(2):176–85.
42. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell. 2000 May 26;101(5):455–8.
43. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity. 2000;12(4):431–40.
44. McAdam AJ, Chang TT, Lumelsky AE, Greenfield EA, Boussiotis VA, Duke-Cohan JS, et al. Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells. J Immunol. 2000 Nov 1;165(9):5035–40.
45. de Boer M, Kasran A, Kwekkeboom J, Walter H, Vandenberghe P, Ceuppens JL. Ligation of B7 with CD28/CTLA-4 on T cells results in CD40 ligand

expression, interleukin-4 secretion and efficient help for antibody production by B cells. Eur J Immunol. 1993 Dec;23(12):3120–5.
46. Johnson-Leger C, Christensen J, Klaus GG. CD28 costimulation stabilizes the expression of the CD40 ligand on T cells. Int Immunol. 1998 Aug;10(8):1083–91.
47. Snapper CM, Kehry MR, Castle BE, Mond JJ. Multivalent, but not divalent, antigen receptor cross-linkers synergize with CD40 ligand for induction of Ig synthesis and class switching in normal murine B cells. A redefinition of the TI-2 vs T cell-dependent antigen dichotomy. J Immunol. 1995 Feb 1;154(3):1177–87.
48. Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, et al. ICOS is essential for effective T-helper-cell responses. Nature. 2001 Jan 4;409(6816):105–9.
49. McAdam AJ, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, et al. ICOS is critical for CD40-mediated antibody class switching. Nature. 2001 Jan 4;409(6816):102–5.
50. Dong C, Temann UA, Flavell RA. Cutting edge: critical role of inducible costimulator in germinal center reactions. J Immunol. 2001 Mar 15;166(6):3659–62.
51. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc Natl Acad Sci USA. 1992 Jul 15;89(14):6550–4.
52. Delogu A, Schebesta A, Sun Q, Aschenbrenner K, Perlot T, Busslinger M. Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. Immunity. 2006 Mar;24(3):269–81.
53. Robillard N, Jego G, Pellat-Deceunynck C, Pineau D, Puthier D, Mellerin MP, et al. CD28, a marker associated with tumoral expansion in multiple myeloma. Clin Cancer Res. 1998 Jun;4(6):1521–6.
54. Nair JR, Carlson LM, Koorella C, Rozanski CH, Byrne GE, Bergsagel PL, et al. CD28 expressed on malignant plasma cells induces a prosurvival and immunosuppressive microenvironment. J Immunol. 2011 Aug 1;187(3):1243–53.
55. Bahlis NJ, King AM, Kolonias D, Carlson LM, Liu HY, Hussein MA, et al. CD28-mediated regulation of multiple myeloma cell proliferation and survival. Blood. 2007 Jun 1;109(11):5002–10.
56. Pellat-Deceunynck C, Bataille R, Robillard N, Harousseau JL, Rapp MJ, Juge-Morineau N, et al. Expression of CD28 and CD40 in human myeloma cells: a comparative study with normal plasma cells. Blood. 1994 Oct 15;84(8):2597–603.
57. Qiu YH, Sun ZW, Shi Q, Su CH, Chen YJ, Shi YJ, et al. Apoptosis of multiple myeloma cells induced by agonist monoclonal antibody against human CD28. Cell Immunol. 2005 Jul–Aug;236(1–2):154–60.
58. Rozanski CH, Arens R, Carlson LM, Nair J, Boise LH, Chanan-Khan AA, et al. Sustained antibody responses depend on CD28 function in bone marrow-resident plasma cells. J Exp Med. 2011 Jul 4;208(7):1435–46.
59. Njau MN, Kim JH, Chappell C, Ravindran R, Thomas L, Pulendran B, Jacob J. CD28-B7 interaction modulates long-lived and short-lived plasma cell function. J Immunol. 2012 Sep 15;189(6):2758–67.
60. Pulendran B, Smith KG, Nossal GJ. Soluble antigen can impede affinity maturation and the germinal center reaction but enhance extrafollicular immunoglobulin production. J Immunol. 1995 Aug 1;155(3):1141–50.
61. Borriello F, Sethna MP, Boyd SD, Schweitzer AN, Tivol EA, Jacoby D, et al. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. Immunity. 1997 Mar;6(3):303–13.
62. Rau FC, Dieter J, Luo Z, Priest SO, Baumgarth N. B7-1/2 (CD80/CD86) direct signaling to B cells enhances IgG secretion. J Immunol. 2009 Dec 15;183(12):7661–71.
63. Suvas S, Singh V, Sahdev S, Vohra H, Agrewala JN. Distinct role of CD80 and CD86 in the regulation of the activation of B cell and B cell lymphoma. J Biol Chem. 2002 Mar 8;277(10):7766–75.
64. Sethna MP, van Parijs L, Sharpe AH, Abbas AK, Freeman GJ. A negative regulatory function of B7 revealed in B7-1 transgenic mice. Immunity. 1994 Aug;1(5):415–21.
65. Pelletier N, McHeyzer-Williams LJ, Wong KA, Urich E, Fazilleau N, McHeyzer-Williams MG. Plasma cells negatively regulate the follicular helper T cell program. Nat Immunol. 2010 Dec;11(12):1110–8.
66. Magistrelli G, Jeannin P, Elson G, Gauchat JF, Nguyen TN, Bonnefoy JY, et al. Identification of three alternatively spliced variants of human CD28 mRNA. Biochem Biophys Res Commun. 1999 May 27;259(1):34–7.
67. Jeannin P, Magistrelli G, Aubry JP, Caron G, Gauchat JF, Renno T, et al. Soluble CD86 is a costimulatory molecule for human T lymphocytes. Immunity. 2000 Sep;13(3):303–12.
68. Cao J, Zhang L, Huang S, Chen P, Zou L, Chen H, et al. Aberrant production of soluble co-stimulatory molecules CTLA-4 and CD28 in patients with chronic hepatitis B. Microb Pathog. 2011 Oct;51(4):262–7.
69. Hebbar M, Jeannin P, Magistrelli G, Hatron PY, Hachulla E, Devulder B, et al. Detection of circulating soluble CD28 in patients with systemic lupus erythematosus, primary Sjogren's syndrome and systemic sclerosis. Clin Exp Immunol. 2004 May;136(2):388–92.
70. Hock BD, O'Donnell JL, Taylor K, Steinkasserer A, McKenzie JL, Rothwell AG, et al. Levels of the soluble forms of CD80, CD86, and CD83 are elevated in the synovial fluid of rheumatoid arthritis patients. Tissue Antigens. 2006 Jan;67(1):57–60.

Memory CD8+ T Cell Protection

9

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Abstract

Memory CD8+ T cells play an essential role in controlling pathogenic infections. Therefore generating protective memory CD8+ T cells by vaccination is an attractive strategy for preventing and treating a variety of human diseases. Understanding what comprises a protective memory CD8+ T cell response will help optimize vaccine-induced CD8+ T cell immunity. Here we discuss essential antiviral effector functions and highlight how recall expansion of memory CD8+ T cells may affect the primary response.

Keywords

Viral immunity • T cell memory • CD8+ T cells

9.1 Introduction

Pathogens are a bane on mankind's existence, causing pain, stress, disability, physical or mental dysfunction, economic burden, and death. Well before the discovery of the germ-theory and of the immune system, immunization had already been a method to prevent pathogen-induced diseases as performed in the Far East in ancient times with the practice of variolation [1], where people were purposefully infected with variola virus (VARV) in a controlled manner to minimize the severity of smallpox and also to prevent reinfection. Variolation was first reported in the Western medical literature in 1671 by Heinrich Vollgnad [2] and later replaced by vaccination with less pathogenic but antigenically similar cowpox (CPXV) or vaccinia (VACV) virus. This resulted in the successful worldwide eradication of smallpox using live VACV [3]. Vaccines are also used very effectively to control many other human infections such as measles, mumps, chickenpox, rubella, rotavirus, etc. Despite this enormous success, we still lack a complete understanding of the mechanisms of vaccine protection and this is a hindrance to our progress in designing effective ones for those pathogens for which they are not available, or to produce safer ones in the case when they result in frequent complications.

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The two major effector arms of the adaptive immune system are antibodies (Abs) and T cells, and both are important for resistance to primary infections. However, because high titers of neutralizing Abs correlate with resistance, they are widely regarded as the dominant player and even esteemed by some as being singularly responsible for most successful vaccines including those against smallpox, measles, and yellow fever [4, 5]. Still, memory CD8$^+$ T cells have been found to be protective in animal models of several infections [6–10] including ectromelia virus (ECTV) [11], a natural mouse infection that is probably the best animal model for human smallpox. Furthermore, it is now widely considered that in order to combat pathogens like human immunodeficiency virus [12], malaria, and *Mycobacterium tuberculosis* (Mtb), it is necessary to target cellular immunity because Abs alone may not be sufficient to control these devastating diseases (Freitas do [13–15]). Indeed, in a murine model of malaria infection, failure to maintain long-term protective immunity was due to a gradual decline in malaria-specific memory T cell responses despite persistence of B cell memory and circulating Abs (Freitas do [13]). Generating vaccines that target cellular immunity necessitates in-depth studies of T cell activation, differentiation, memory maintenance, trafficking, essential effector functions, and T cell subsets, to name a few.

9.2 Primary CD8$^+$ T Cell Responses to Pathogens

T lymphocytes play a crucial role in the adaptive immune response. They include CD4$^+$ and CD8$^+$ T cells, named after the glycoprotein co-receptor expressed at their cell surface [16–18]. CD4$^+$ T cells are mainly considered regulators while CD8$^+$ T cells are considered mostly effectors of the immune response.

Naïve CD8$^+$ T cells circulate the host and survey for potential threats. However, different to innate natural killer (NK) cells which are prearmed to kill their targets and readily produce cytokines such as IFN-γ; CD8$^+$ T cells do not constitutively express cytolytic components or have a transcriptionally open IFN-γ gene.

During infection, CD8$^+$ T cells recognize antigens (Ag) as small peptides bound to major histocompatibility complex class I (MHC I) molecules. The vast majority of these peptides derive from the cytosolic degradation of pathogen proteins by the proteasome [19, 20]. When naïve Ag-specific CD8$^+$ T cells encounter foreign Ag as peptide-MHC I complexes presented by the Ag-presenting cell (APC), the process of activation ensues. To become activated, naïve CD8$^+$ T cell require the engagement of the T cell receptor (TCR) with peptide-MHC I and additional secondary signals provided by costimulatory molecules and cytokines [21]. Following activation, CD8$^+$ T cells undergo rapid expansion and effector differentiation, increasing in numbers by up to 50,000-fold [22–24]. The effector CD8$^+$ T cells develop cytolytic activity and produce chemokines, inflammatory, and antiviral cytokines thereby becoming equipped to fight invading pathogens [25]. Primary CD8$^+$ T cell responses were shown to be essential for resistance to ECTV [26], LCMV [27], acute clearance of γ-herpesvirus [28], and defense against primary Mtb and control of latent Mtb [29–31]. Furthermore, humans deficient in CD8$^+$ T cells have been reported to develop severe combined immunodeficiency with features of autoimmunity caused by a nonfunctional CD3-y truncation [32]. Additionally, a patient with a CD8$^+$ T cell deficiency but normal percentage and absolute numbers of CD4 T cells, B cells, and NK cells suffered repeated respiratory bacterial infections [33].

The main mechanism whereby activated CD8$^+$ T cells kill Ag-bearing cells is through granule-exocytosis (GE) which requires perforin (Prf) and granzymes (Gz) [34]. Although the cellular and molecular functions of Prf remain controversial, it is clear that mediators of cell death such as Gz are packaged into granules, and that Prf is responsible for its effective delivery to the target cell, resulting in caspase-mediated or caspase-independent apoptosis. Prf-mediated cytotoxicity induces cell death within minutes; this function rapidly limits pathogen replication and spread [35–38]. In addition to GE, activated CD8$^+$ T cells

can kill targets by means of secreted tumor necrosis factor alpha (TNF-α) or the cell membrane bound TNF family members Fas ligand (FasL) and TNF1-related apoptosis-inducting ligand (TRAIL) (Istrail et al.) that respectively bind to the death receptors TNF1R, Fas, and TRAIL-R at the surface of target cells [39–42]. It is important to note, however, that killing through GE and TNF family members is not exclusive of CD8+ cells but also a property of NK cells and, in some instances CD4+ T cells [43–46].

CD8+ T cells also induce an antiviral state through the production of cytokines like IFN-γ, TNF-α, and chemokines [47]. Cytokines may directly interfere with pathogen entry and/or gene expression, or restrict intracellular replication [40]. In addition to CD8+ T cells, IFN-γ is also produced by activated NK, NK-T, and CD4+ T cells [45, 48]. Because all nucleated cells express the IFN-γ receptor (IFN-γ R) they are all subject to IFN-γ stimulation [49, 50]. In addition to directly inhibiting viral replication, IFN-γ induces a large number of genes with immunoregulatory functions [51]. For example, IFN-γ increases the expression of genes involved in chemotaxis and inflammation including CXCL10, TNF-α, and inducible nitric oxide synthase. Additionally IFN-γ activates macrophages and dendritic cells and stimulates increased expression of MHC-peptide complexes [49]. Thus, IFN-γ plays a key role in resistance and recovery from many intracellular bacterial and viral infections [52, 53].

Even though CD8+ T cells can use various mechanisms to kill infected cells and also produce many cytokines, GE-mediated killing and IFN-γ production are thought to be the major effector mechanisms whereby CD8+ T cells clear viral infections [40]. The use of mice deficient in effector molecules, alone or in combination, allowed elucidation of their importance in primary resistance to various infections. For instance, Prf has been shown to be important for resistance to noncytopathic lymphocytic choriomeningitis virus (LCMV) and intracellular pathogens such as *Listeria monocytogenes* (Lm) and Mtb [35, 54–56]. Prf, GzA, and GzB have been shown to be essential for natural resistance to cytophatic ECTV [57–59]. On the other hand, Prf is not essential for the clearance of vesicular stomatitis virus (VSV) and VACV [55]. For IFN-γ, its importance has been demonstrated in the defense against primary ECTV [60], VACV, Theiler's virus, Mtb, Lm, and Leishmania infections [61–63]. For TNF-α, it has been shown to be essential for resistance to fatal Leishmania [64], mycobacterial infection [65], and disseminated lethal *Streptococcus pneumoniae* infection [66]. However, given that all of these molecules are also expressed by NK cells and/or CD4+ T cells, the absence of genetic deficiencies specifically targeted to CD8+ T cells has made it difficult to firmly establish their role in protection by effector CD8+ T cells.

9.3 Memory CD8+ T Cells

As the pathogen is eliminated, the number of effector CD8+ T cells declines. Typically 90–95% of the initial clonal burst size undergoes apoptosis. The remaining 5–10% differentiate into memory CD8+ T cells [24], which may be maintained for the life of the host by homeostatic proliferation with the aid of cytokines such as IL-7 and IL-15 [67]. Along with their increased expression of adhesion molecules such as CD2, CD44, and LFA-1 [68, 69] and different CD45 isoforms [70, 71], memory T cells also differ from naïve cells in that they can be activated by relatively low numbers of peptide-MHC complexes and with low or no requirements for costimulatory signals [72]. Moreover, epigenetic modifications allow memory cells to express effector molecules such as IFN-γ and Gz more readily than naïve T cells [73–75].

9.3.1 Memory CD8+ T Cell Heterogeneity in Protection

Different to naïve CD8+ T cells, which recirculate between lymphoid tissues and blood, memory CD8+ T cells are heterogeneous and can populate lymphoid as well as non-lymphoid tissues. Central memory T cells (TCM) survey lymphoid tissues while effector (TEM) and tissue resident

memory CD8+ T cells (Trm) remain in the periphery such as the skin, lungs, intestinal, and genital tract [76–79]. Understanding the importance of these various populations in protection from different infections is important for the development of novel vaccines as the success of a memory T cell response in the control of a pathogenic infection may depend on the anatomical location of the T cells and the microbe [80–83]. For instance, skin resident memory T cells may be important to control herpes simplex virus (HSV) flares [76, 77]. Protection by different populations of memory CD8+ T cells has been studied in several murine models including LCMV [84], Sendai virus [85, 86], and VACV [87]. Using adoptive transfers of TCR transgenic CD8+ T cells specific for the immunodominant LCMV gp33 epitope, Wherry et al. demonstrated that on a per cell basis, TCM control LCMV and VACV replication better than TEM irrespective of the route of infection [84]. Still, protection conferred by TCM, EM, or Trm cells could change according to the mode of viral spread. As an example, fatal mousepox is caused by ECTV, a cytopathic virus belonging to the genus Orthopoxvirus (OPV) which also includes VACV and VARV, the virus in the smallpox vaccine, and the causative agent of smallpox, respectively [3]. Similar to many other systemic infections, OPVs spread in their natural hosts following a lympho-hematogenous (LH) route whereby infection originates in the periphery, disseminates to local draining lymph node (LN), and then spreads systemically through efferent lymphatics and the bloodstream. Different than most other mouse models of viral infection where inoculation is performed systemically, ECTV is traditionally inoculated into its natural route of entry, the footpad. This permits the study of pathogenesis and the immune response during the course of LH spread. Using adoptive transfers into naïve mice, we found that memory CD8+ T cells control early virus dissemination from the draining LN to vital organs [11]. While in these experiments we did not determine whether protection involve TCM or TEM, it is very likely that TCM are involved as they are the main population that resides in the LN.

9.3.2 Effector Functions and Quality of the Memory CD8+ T Cells in Protection

Studies that elucidate the necessary essential effector functions of memory T cells after vaccination are scarce. While it has traditionally been thought that the capacity of memory CD8+ T cells to protect is largely a function of their absolute numbers [88, 89], a topic of major attention in recent years has been the "quality" of the memory T cells. Quality can be broadly defined as the ability of the memory T cell to perform multiple functions, such as kill their targets and produce multiple cytokines. Thus, it has become a common view that the more effector molecules a memory T cell express, the better its quality [90, 91]. While this criteria may serve as a checklist to investigate whether specific effector functions are required for memory CD8+ T cell protection against a particular pathogen, the need for particular effector functions are likely to vary for different pathogens. Indeed, Darrah et al. prospectively showed that multifunctional T_H1 cells correlated with protection and have enhanced effector function against Leishmania major [92]. Thus, to aid us in vaccine design, it is important to determine which specific effector molecules a memory CD8+ T cells must be equipped with in order to protect.

Several studies have attempted to identify the essential effector molecules required for protection by memory CD8+ T cells against various infections. The Woodland laboratory demonstrated that an early recruitment of circulating memory CD8+ T cells was required for protection from influenza A virus (IAV) reinfection [93]. The early virus control correlated with memory CD8+ T cell lytic activity and GzB expression induced before recruitment by systemic inflammatory signals including type I IFN [93]. Another study used a heterologous vaccination scheme with plasmid DNA prime/recombinant adenovirus 5 boost to protect mice against the human parasite Trypanosoma cruzi and found that perforin and IFN-γ expression were required for a protective CD4+ and CD8+ T cell immune response [94]. In a murine model of New World

leishmaniasis, vaccination of Prf and IFN-γ deficient mice showed that Prf and IFN-γ were critical for protective memory CD8+ T cell responses [95]. However, Pf/Gz-mediated cytolysis and IFN-γ are not restricted to CD8+ T cells. Hence, in these studies, the function of other immune cell was compromised limiting the conclusions that can be made regarding the need for expression of the effector molecules by the memory CD8+ T cells themselves. A better understanding of the role in protection of particular effector molecules expressed by memory CD8+ T cell without debilitating other immune components may require the adoptive transfer of memory CD8+ T cells defective in a particular effector molecule, ideally polyclonal and not TCR transgenic, into naïve WT mice.

9.3.3 Naïve T Cell Responses in the Presence of Memory CD8+ T Cells

During immune responses CD8+ T cells could either compete or promote among themselves and this could affect disease progression. Competition between primary T cells for APC access has been demonstrated by transferring chicken ovalbumin (OVA) specific TCR transgenic OT-1 CD8+ T cells and infecting with VACV-OVA, which resulted in complete inhibition of the host Ag-specific T cells to the same or irrelevant Ag by the OT-1 cells [96]. Another study found that after heterologous prime-boost immunization with replication-deficient adenovirus and modified vaccinia Ankara virus vectors expressing two different Ags from Plasmodium falciparum, CD8+ T cells competed for space to expand [97, 98]. In the case of memory CD8+ T cells, it has been postulated that low-affinity naïve may be outnumbered by high-affinity memory CD8+ T cells and be at a disadvantage to compete for cytokines, Ag, APCs, and physiological space [99]. A caveat is that the loss of an endogenous naïve response may be the result of transferring too many high-affinity cells [100] and/or that it occurs in instances of limited Ag/APC availability such as in the case of inoculation of inert antigens [101]. Indeed, there are several reports using *Mtb*, LCMV, or IAV [102, 103] where a naïve response was observed and at times necessary to reconstitute the Ag-specific T cell pool. By using adoptive transfer of memory T cells in conjunction with congenic markers, it is possible not only to examine protection [11] but also to study the memory and concomitant primary T cell response, including competition among virus-specific T cells for signals such as cytokines, costimulation, or Ag. These types of studies also make it possible to examine how memory CD8 T cells affect the naïve response. Using this system we have found that during infection with rapidly replicating and highly pathogenic ECTV, otherwise unresponsive naïve CD8+ T cells are recruited to the response and play an essential role in protection from lethal mousepox, in particular when the memory CD8+ T cells are suboptimal. Hence, by transiently controlling virus replication and spread memory CD8+ T cells can open the way for a de novo response that is otherwise being overwhelmed by the pathogen.

9.4 Concluding Remarks

Memory CD8+ T cells equip the host to better fight secondary infections due to their enhanced frequency, wide tissue distribution, ability to rapidly expand, mobilize their effector functions, swiftly kill their targets, and, in some situations, rescue a naïve response [40, 70, 99, 104–107]. In light of the demand for preventative and therapeutic T cell vaccines against stubborn, emerging and reemerging pathogens, it is imperative that we determine essential antiviral effector functions and learn how recall expansion of memory CD8+ T cells affect the primary response. Each study that furthers our understanding of protective T cell immunity will bring us one step closer to treating some of the worst ailments of mankind. It is indeed "in the power of man to cause all parasitic diseases to disappear from the world" [108].

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References

1. Alivisatos, C.N. (1934). The First Immunologist, James Pylarino (1659–1718), and the Introduction of Variolation: (Section of the History of Medicine). Proc R Soc Med *27*, 1099–1104.
2. Brown, J.R., and Mc, L.D. (1962). Smallpox–a retrospect. Can Med Assoc J *87*, 765–767.
3. Fenner, F., Henderson, D.A., Arita, I., Jezek, Z., Ladnyi, D., and Organization, W.H. (1988). Smallpox and its eradication (Geneva: World Health Organization).
4. Zinkernagel, R.M., Bachmann, M.F., Kundig, T.M., Oehen, S., Pirchet, H., and Hengartner, H. (1996). On immunological memory. Annu Rev Immunol *14*, 333–367.
5. Zinkernagel, R.M., and Hengartner, H. (2006). Protective 'immunity' by pre-existent neutralizing antibody titers and preactivated T cells but not by so-called 'immunological memory'. Immunol Rev *211*, 310–319.
6. Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M., and Sadoff, J. (1990). Oral Salmonella: malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. J Exp Med *172*, 1083–1090.
7. Badovinac, V.P., Messingham, K.A., Jabbari, A., Haring, J.S., and Harty, J.T. (2005). Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nat Med *11*, 748–756.
8. Topham, D.J., Castrucci, M.R., Wingo, F.S., Belz, G.T., and Doherty, P.C. (2001). The role of antigen in the localization of naive, acutely activated, and memory CD8(+) T cells to the lung during influenza pneumonia. J Immunol *167*, 6983–6990.
9. van der Most, R.G., Murali-Krishna, K., Whitton, J.L., Oseroff, C., Alexander, J., Southwood, S., Sidney, J., Chesnut, R.W., Sette, A., and Ahmed, R. (1998). Identification of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. Virology *240*, 158–167.
10. van der Most, R.G., Sette, A., Oseroff, C., Alexander, J., Murali-Krishna, K., Lau, L.L., Southwood, S., Sidney, J., Chesnut, R.W., Matloubian, M., and Ahmed, R. (1996). Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during acute and chronic lymphocytic choriomeningitis virus infection. J Immunol *157*, 5543–5554.
11. Xu, R.H., Fang, M., Klein-Szanto, A., and Sigal, L.J. (2007). Memory CD8+ T cells are gatekeepers of the lymph node draining the site of viral infection. Proc Natl Acad Sci U S A *104*, 10992–10997.
12. Derjuga, A., Gourley, T.S., Holm, T.M., Heng, H.H., Shivdasani, R.A., Ahmed, R., Andrews, N.C., and Blank, V. (2004). Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus. Mol Cell Biol *24*, 3286–3294.
13. Freitas do Rosario, A.P., Muxel, S.M., Rodriguez-Malaga, S.M., Sardinha, L.R., Zago, C.A., Castillo-Mendez, S.I., Alvarez, J.M., and D'Imperio Lima, M.R. (2008). Gradual decline in malaria-specific memory T cell responses leads to failure to maintain long-term protective immunity to Plasmodium chabaudi AS despite persistence of B cell memory and circulating antibody. J Immunol *181*, 8344–8355.
14. Halwani, R., Doroudchi, M., Yassine-Diab, B., Janbazian, L., Shi, Y., Said, E.A., Haddad, E.K., and Sekaly, R.P. (2006). Generation and maintenance of human memory cells during viral infection. Springer Semin Immunopathol *28*, 197–208.
15. Seder, R.A., and Hill, A.V. (2000). Vaccines against intracellular infections requiring cellular immunity. Nature *406*, 793–798.
16. Cantor, H., and Boyse, E.A. (1975). Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly+ cells in the generation of killer activity. J Exp Med *141*, 1390–1399.
17. Kisielow, P., Hirst, J.A., Shiku, H., Beverley, P.C., Hoffman, M.K., Boyse, E.A., and Oettgen, H.F. (1975). Ly antigens as markers for functionally distinct subpopulations of thymus-derived lymphocytes of the mouse. Nature *253*, 219–220.
18. Shiku, H., Kisielow, P., Bean, M.A., Takahashi, T., Boyse, E.A., Oettgen, H.F., and Old, L.J. (1975). Expression of T-cell differentiation antigens on effector cells in cell-mediated cytotoxicity in vitro. Evidence for functional heterogeneity related to the surface phenotype of T cells. J Exp Med *141*, 227–241.
19. York, I.A., Goldberg, A.L., Mo, X.Y., and Rock, K.L. (1999). Proteolysis and class I major histocompatibility complex antigen presentation. Immunol Rev *172*, 49–66.
20. York, I.A., and Rock, K.L. (1996). Antigen processing and presentation by the class I major histocompatibility complex. Ann. Rev. Immunol. *14*, 369–396.
21. Williams, M.A., and Bevan, M.J. (2007). Effector and memory CTL differentiation. Annu Rev Immunol *25*, 171–192.
22. Blattman, J.N., Antia, R., Sourdive, D.J., Wang, X., Kaech, S.M., Murali-Krishna, K., Altman, J.D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med *195*, 657–664.
23. Butz, E., and Bevan, M.J. (1998). Dynamics of the CD8+ T cell response during acute LCMV infection. Adv Exp Med Biol *452*, 111–122.

24. Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177–187.
25. Doherty, P.C., and Christensen, J.P. (2000). Accessing complexity: the dynamics of virus-specific T cell responses. Annu Rev Immunol *18*, 561–592.
26. Fang, M., and Sigal, L.J. (2005). Antibodies and CD8+ T cells are complementary and essential for natural resistance to a highly lethal cytopathic virus. Journal of immunology *175*, 6829–6836.
27. Fung-Leung, W.P., Kundig, T.M., Zinkernagel, R.M., and Mak, T.W. (1991). Immune response against lymphocytic choriomeningitis virus infection in mice without CD8 expression. J Exp Med *174*, 1425–1429.
28. Ehtisham, S., Sunil-Chandra, N.P., and Nash, A.A. (1993). Pathogenesis of murine gammaherpesvirus infection in mice deficient in CD4 and CD8 T cells. J Virol *67*, 5247–5252.
29. Behar, S.M., Dascher, C.C., Grusby, M.J., Wang, C.R., and Brenner, M.B. (1999). Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. J Exp Med *189*, 1973–1980.
30. Muller, I., Cobbold, S.P., Waldmann, H., and Kaufmann, S.H. (1987). Impaired resistance to Mycobacterium tuberculosis infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. Infect Immun *55*, 2037–2041.
31. Sousa, A.O., Mazzaccaro, R.J., Russell, R.G., Lee, F.K., Turner, O.C., Hong, S., Van Kaer, L., and Bloom, B.R. (2000). Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. Proc Natl Acad Sci U S A *97*, 4204–4208.
32. Arnaiz-Villena, A., Timon, M., Corell, A., Perez-Aciego, P., Martin-Villa, J.M., and Regueiro, J.R. (1992). Brief report: primary immunodeficiency caused by mutations in the gene encoding the CD3-gamma subunit of the T-lymphocyte receptor. N Engl J Med *327*, 529–533.
33. de la Calle-Martin, O., Hernandez, M., Ordi, J., Casamitjana, N., Arostegui, J.I., Caragol, I., Ferrando, M., Labrador, M., Rodriguez-Sanchez, J.L., and Espanol, T. (2001). Familial CD8 deficiency due to a mutation in the CD8 alpha gene. J Clin Invest *108*, 117–123.
34. Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994b). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science *265*, 528–530.
35. Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. (1996). Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol *14*, 207–232.
36. Russell, J.H., and Dobos, C.B. (1980). Mechanisms of immune lysis. II. CTL-induced nuclear disintegration of the target begins within minutes of cell contact. J Immunol *125*, 1256–1261.
37. Trapani, J.A., Jans, D.A., Jans, P.J., Smyth, M.J., Browne, K.A., and Sutton, V.R. (1998). Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. J Biol Chem *273*, 27934–27938.
38. Trapani, J.A., and Smyth, M.J. (2002). Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol *2*, 735–747.
39. Alejo, A., Ruiz-Arguello, M.B., Ho, Y., Smith, V.P., Saraiva, M., and Alcami, A. (2006). A chemokine-binding domain in the tumor necrosis factor receptor from variola (smallpox) virus. Proc Natl Acad Sci U S A *103*, 5995–6000.
40. Harty, J.T., Tvinnereim, A.R., and White, D.W. (2000). CD8+ T cell effector mechanisms in resistance to infection. Annu Rev Immunol *18*, 275-308.
41. Istrail, S., Florea, L., Halldorsson, B.V., Kohlbacher, O., Schwartz, R.S., Yap, V.B., Yewdell, J.W., and Hoffman, S.L. (2004). Comparative immunopeptidomics of humans and their pathogens. Proc Natl Acad Sci U S A *101*, 13268–13272.
42. Smyth, M.J., and Trapani, J.A. (1998). The relative role of lymphocyte granule exocytosis versus death receptor-mediated cytotoxicity in viral pathophysiology. J Virol *72*, 1–9.
43. Brown, D.M., Lee, S., Garcia-Hernandez, M.D., and Swain, S.L. (2012). Multi-functional CD4 cells expressing IFN-gamma and perforin mediate protection against lethal influenza infection. J Virol.
44. Fang, M., Siciliano, N.A., Hersperger, A.R., Roscoe, F., Hu, A., Ma, X., Shamsedeen, A.R., Eisenlohr, L.C., and Sigal, L.J. (2012). Perforin-dependent CD4+ T-cell cytotoxicity contributes to control a murine poxvirus infection. Proc Natl Acad Sci U S A *109*, 9983–9988.
45. French, A.R., and Yokoyama, W.M. (2003). Natural killer cells and viral infections. Curr Opin Immunol *15*, 45–51.
46. Marshall, N.B., and Swain, S.L. (2011). Cytotoxic CD4 T cells in antiviral immunity. J Biomed Biotechnol *2011*, 954602.
47. Harty, J.T., and Bevan, M.J. (1999). Responses of CD8(+) T cells to intracellular bacteria. Curr Opin Immunol *11*, 89–93.
48. Schoenborn, J.R., and Wilson, C.B. (2007). Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol *96*, 41–101.
49. Boehm, U., Klamp, T., Groot, M., and Howard, J.C. (1997). Cellular responses to interferon-gamma. Annu Rev Immunol *15*, 749–795.
50. Farrar, M.A., and Schreiber, R.D. (1993). The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol *11*, 571–611.

51. Saha, B., Jyothi Prasanna, S., Chandrasekar, B., and Nandi, D. (2010). Gene modulation and immunoregulatory roles of interferon gamma. Cytokine *50*, 1–14.
52. Casanova, J.L., and Ochs, H. (1999). Interferon-gamma receptor deficiency: An expanding clinical phenotype? J Pediatr *135*, 543–545.
53. Tau, G., and Rothman, P. (1999). Biologic functions of the IFN-gamma receptors. Allergy *54*, 1233–1251.
54. Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994a). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31–37.
55. Kagi, D., Seiler, P., Pavlovic, J., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. (1995). The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. Eur J Immunol *25*, 3256–3262.
56. Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., et al. (1998). An antimicrobial activity of cytolytic T cells mediated by granulysin. Science *282*, 121–125.
57. Mullbacher, A., Ebnet, K., Blanden, R.V., Hla, R.T., Stehle, T., Museteanu, C., and Simon, M.M. (1996). Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. Proc Natl Acad Sci U S A *93*, 5783–5787.
58. Mullbacher, A., Hla, R.T., Museteanu, C., and Simon, M.M. (1999a). Perforin is essential for control of ectromelia virus but not related poxviruses in mice. J Virol *73*, 1665–1667.
59. Mullbacher, A., Waring, P., Tha Hla, R., Tran, T., Chin, S., Stehle, T., Museteanu, C., and Simon, M.M. (1999b). Granzymes are the essential downstream effector molecules for the control of primary virus infections by cytolytic leukocytes. Proc Natl Acad Sci U S A *96*, 13950–13955.
60. Karupiah, G., Fredrickson, T.N., Holmes, K.L., Khairallah, L.H., and Buller, R.M. (1993). Importance of interferons in recovery from mousepox. J Virol *67*, 4214–4226.
61. Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M., and Aguet, M. (1993). Immune response in mice that lack the interferon-gamma receptor. Science *259*, 1742–1745.
62. Remakus, S., and Sigal, L.J. (2011). Gamma interferon and perforin control the strength, but not the hierarchy, of immunodominance of an antiviral CD8+ T cell response. Journal of virology *85*, 12578–12584.
63. van den Broek, M.F., Muller, U., Huang, S., Zinkernagel, R.M., and Aguet, M. (1995). Immune defence in mice lacking type I and/or type II interferon receptors. Immunol Rev *148*, 5–18.
64. Fromm, P.D., Kling, J., Mack, M., Sedgwick, J.D., and Korner, H. (2012). Loss of TNF Signaling Facilitates the Development of a Novel Ly-6Clow Macrophage Population Permissive for Leishmania major Infection. J Immunol.
65. Jacobs, M., Marino, M.W., Brown, N., Abel, B., Bekker, L.G., Quesniaux, V.J., Fick, L., and Ryffel, B. (2000). Correction of defective host response to Mycobacterium bovis BCG infection in TNF-deficient mice by bone marrow transplantation. Lab Invest *80*, 901–914.
66. Kirby, A.C., Raynes, J.G., and Kaye, P.M. (2005). The role played by tumor necrosis factor during localized and systemic infection with Streptococcus pneumoniae. J Infect Dis *191*, 1538–1547.
67. Surh, C.D., Boyman, O., Purton, J.F., and Sprent, J. (2006). Homeostasis of memory T cells. Immunol Rev *211*, 154–163.
68. Mackay, C.R., Marston, W.L., and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med *171*, 801–817.
69. Sanders, M.E., Makgoba, M.W., Sharrow, S.O., Stephany, D., Springer, T.A., Young, H.A., and Shaw, S. (1988). Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. J Immunol *140*, 1401–1407.
70. Ahmed, R., and Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. Science *272*, 54–60.
71. Dianzani, U., Luqman, M., Rojo, J., Yagi, J., Baron, J.L., Woods, A., Janeway, C.A., Jr., and Bottomly, K. (1990). Molecular associations on the T cell surface correlate with immunological memory. Eur J Immunol *20*, 2249–2257.
72. Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89–95.
73. Fitzpatrick, D.R., Shirley, K.M., and Kelso, A. (1999). Cutting edge: stable epigenetic inheritance of regional IFN-gamma promoter demethylation in CD44highCD8+ T lymphocytes. J Immunol *162*, 5053–5057.
74. Mullen, A.C., High, F.A., Hutchins, A.S., Lee, H.W., Villarino, A.V., Livingston, D.M., Kung, A.L., Cereb, N., Yao, T.P., Yang, S.Y., and Reiner, S.L. (2001). Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science *292*, 1907–1910.
75. Mullen, A.C., Hutchins, A.S., High, F.A., Lee, H.W., Sykes, K.J., Chodosh, L.A., and Reiner, S.L. (2002). Hlx is induced by and genetically interacts with

T-bet to promote heritable T(H)1 gene induction. Nat Immunol *3*, 652–658.
76. Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R., and Carbone, F.R. (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat Immunol *10*, 524–530.
77. Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S., et al. (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. J Exp Med *207*, 553–564.
78. Masopust, D., Vezys, V., Marzo, A.L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science *291*, 2413–2417.
79. Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708–712.
80. Gourley, T.S., Wherry, E.J., Masopust, D., and Ahmed, R. (2004). Generation and maintenance of immunological memory. Semin Immunol *16*, 323–333.
81. Jameson, S.C., and Masopust, D. (2009). Diversity in T cell memory: an embarrassment of riches. Immunity *31*, 859–871.
82. Masopust, D., Kaech, S.M., Wherry, E.J., and Ahmed, R. (2004). The role of programming in memory T-cell development. Curr Opin Immunol *16*, 217–225.
83. Verhoeven, D., Teijaro, J.R., and Farber, D.L. (2008). Heterogeneous memory T cells in antiviral immunity and immunopathology. Viral Immunol *21*, 99–113.
84. Wherry, E.J., Teichgraber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H., and Ahmed, R. (2003). Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol *4*, 225–234.
85. Hikono, H., Kohlmeier, J.E., Takamura, S., Wittmer, S.T., Roberts, A.D., and Woodland, D.L. (2007). Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. J Exp Med *204*, 1625–1636.
86. Roberts, A.D., Ely, K.H., and Woodland, D.L. (2005). Differential contributions of central and effector memory T cells to recall responses. J Exp Med *202*, 123–133.
87. Bachmann, M.F., Wolint, P., Schwarz, K., Jager, P., and Oxenius, A. (2005). Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol *175*, 4686–4696.
88. Harty, J.T., and Badovinac, V.P. (2008). Shaping and reshaping CD8+ cell memory. Nat Rev Immuno *8*, 107–119.
89. Sallusto, F., Lanzavecchia, A., Araki, K., and Ahmed, R. (2008). From vaccines to memory and back. Immunity *33*, 451–463.
90. Kannanganat, S., Ibegbu, C., Chennareddi, L., Robinson, H.L., and Amara, R.R. (2007). Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. J Virol *81*, 8468–8476.
91. Seder, R.A., Darrah, P.A., and Roederer, M. (2008). T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol *8*, 247–258.
92. Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W., Davey, D.F., Flynn, B.J., Hoff, S.T., Andersen, P., Reed, S.G., Morris, S.L., et al. (2007). Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med *13*, 843–850.
93. Kohlmeier, J.E., Cookenham, T., Roberts, A.D., Miller, S.C., and Woodland, D.L. (2010). Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. Immunity *33*, 96–105.
94. de Alencar, B.C., Persechini, P.M., Haolla, F.A., de Oliveira, G., Silverio, J.C., Lannes-Vieira, J., Machado, A.V., Gazzinelli, R.T., Bruna-Romero, O., and Rodrigues, M.M. (2009). Perforin and gamma interferon expression are required for CD4+ and CD8+ T-cell-dependent protective immunity against a human parasite, Trypanosoma cruzi, elicited by heterologous plasmid DNA prime-recombinant adenovirus 5 boost vaccination. Infect Immun *77*, 4383–4395.
95. Colmenares, M., Kima, P.E., Samoff, E., Soong, L., and McMahon-Pratt, D. (2003). Perforin and gamma interferon are critical CD8+ T-cell-mediated responses in vaccine-induced immunity against Leishmania amazonensis infection. Infect Immun *71*, 3172–3182.
96. Kedl, R.M., Rees, W.A., Hildeman, D.A., Schaefer, B., Mitchell, T., Kappler, J., and Marrack, P. (2000). T cells compete for access to antigen-bearing antigen-presenting cells. J Exp Med *192*, 1105–1113.
97. Forbes, E.K., Biswas, S., Collins, K.A., Gilbert, S.C., Hill, A.V., and Draper, S.J. (2011). Combining liver- and blood-stage malaria viral-vectored vaccines: investigating mechanisms of CD8+ T cell interference. J Immunol *187*, 3738–3750.
98. van den Broek, M.E., Kagi, D., Ossendorp, F., Toes, R., Vamvakas, S., Lutz, W.K., Melief, C.J., Zinkernagel, R.M., and Hengartner, H. (1996). Decreased tumor surveillance in perforin-deficient mice. J Exp Med *184*, 1781–1790.
99. Welsh, R.M., Selin, L.K., and Szomolanyi-Tsuda, E. (2004). Immunological memory to viral infections. Annu Rev Immunol *22*, 711–743.
100. Zehn, D., Lee, S.Y., and Bevan, M.J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. Nature *458*, 211–214.
101. Guarda, G., Hons, M., Soriano, S.F., Huang, A.Y., Polley, R., Martin-Fontecha, A., Stein, J.V., Germain, R.N., Lanzavecchia, A., and Sallusto, F. (2007). L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph

nodes and kill dendritic cells. Nat Immunol *8*, 743–752.
102. Badovinac, V.P., Messingham, K.A., Hamilton, S.E., and Harty, J.T. (2003). Regulation of CD8+ T cells undergoing primary and secondary responses to infection in the same host. J Immunol *170*, 4933–4942.
103. Turner, S.J., Cross, R., Xie, W., and Doherty, P.C. (2001). Concurrent naive and memory CD8(+) T cell responses to an influenza A virus. J Immunol *167*, 2753–2758.
104. Barber, D.L., Wherry, E.J., and Ahmed, R. (2003). Cutting edge: rapid in vivo killing by memory CD8 T cells. J Immunol *171*, 27–31.
105. Kaech, S.M., Hemby, S., Kersh, E., and Ahmed, R. (2002). Molecular and functional profiling of memory CD8 T cell differentiation. Cell *111*, 837–851.
106. Lalvani, A., Brookes, R., Hambleton, S., Britton, W.J., Hill, A.V., and McMichael, A.J. (1997). Rapid effector function in CD8+ memory T cells. J Exp Med *186*, 859–865.
107. Selin, L.K., and Welsh, R.M. (1997). Cytolytically active memory CTL present in lymphocytic choriomeningitis virus-immune mice after clearance of virus infection. J Immunol *158*, 5366–5373.
108. Otis, E.O. (1909). The great white plague, tuberculosis (New York,: Crowell).

Human T Follicular Helper Cells: Development and Subsets

10

Nathalie Schmitt and Hideki Ueno

Abstract

Antibody response constitutes one of the key immune protection mechanisms. T follicular helper (Tfh) cells represent the major CD4+ T cell subset that provides help to B cells to induce antibody response. How Tfh cells develop and how Tfh cells or their associated subsets regulate antibody response in humans remains largely unknown. In this review, we will summarize the recent discoveries on the biology of Tfh cells, with a particular focus on human Tfh cells.

Keywords

Human • T follicular helper cells • CD4+ T cell subsets • Antibody response • Tonsils • Blood • CXCR5 • Autoimmune diseases

10.1 Introduction

T follicular helper (Tfh) cells are the major CD4+ T cell subset that provides help to B cells to induce antibody response [1, 2]. Tfh cells are essential for the formation of germinal centers (GCs), the site of the selection of high-affinity B cells, and their differentiation into memory B cells or long-lived plasma cells. Similar to other CD4+ T cell subsets such as Th1, Th2, and Th17 cells, the magnitude and the duration of Tfh response need to be controlled by the immune system, because exaggerated Tfh response causes autoimmunity [3]. Recent studies including ours identified similarities and differences in the biology of Tfh cells between humans and mice. Determining the biology and developmental pathways of human Tfh cells is of great significance, as it will provide direct insights regarding the development of novel vaccine design and therapeutic strategies for human autoimmune diseases.

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10.2 T Follicular Helper Cells: The CD4+ T Cell Subset Specialized for B Cell Help

In secondary lymphoid organs, CD4+ T cells primed by dendritic cells (DCs) loaded with antigens interact with antigen-primed naïve B cells at extrafollicular sites [4], typically at the border of T cell zone and primary follicles [5]. This interaction initiates the B cell differentiation process towards two different paths: extrafollicular plasma cells and cells forming GCs [6–8]. Extrafollicular plasma cells contribute to the early generation of specific antibodies after antigen exposure [9]. Germinal center B (GC-B) cells subsequently differentiate into either high-affinity long-lived plasma cells or memory B cells after an extensive selection step [10, 11] (Fig. 10.1).

Together with GC-B cells and follicular dendritic cells, Tfh cells constitute essential cell compartments for GC formation. In GCs, Tfh cells play an important role in the selection of high-affinity B cells and in the induction of the differentiation of selected B cells. While no unique markers have been reported, Tfh cells can be identified by the combination of several markers that are directly associated with their functions. The chemokine (C-X-C) receptor 5 (CXCR5) is important for their initial migration into B cell follicles [12–15]. Tfh cells express PD-1, which was shown to play a role in the selection of high-affinity B cells in GCs [16]. Inducible co-stimulator (ICOS) is critical for the development [17, 18] and functions [15, 19, 20] of Tfh cells. CD40 ligand (CD40L) expressed by Tfh cells provides essential signals to B cells through CD40 for their differentiation and class-switching [21]. Tfh cells and their precursors secrete IL-21 [22–24], a γc-family cytokine which potently promotes the growth, differentiation, and class-switching of B cells [25]. IL-21 delivers activation signals to B cells via STAT3 (signal transducer and activator of transcription 3), and accordingly STAT3-deficient

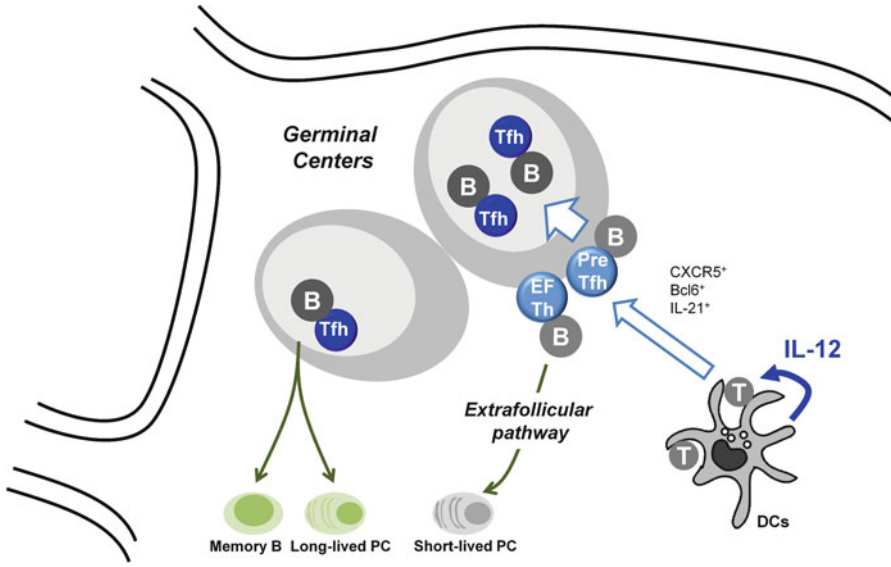

Fig. 10.1 Generation of human Tfh cells. In secondary lymphoid organs, naïve CD4+ T cells interact with DCs loaded with antigens. IL-12 secreted by the DCs induces naïve CD4+ T cells to initiate the program to differentiate into the Tfh lineage. These cells migrate towards B cell follicles, and interact with B cells. T and B cell interaction initiates the B cell differentiation process towards two different paths: extrafollicular plasma cells and cells forming GCs. How Tfh precursors and extrafollicular helper (EF) T cells overlap remains unclear in humans. Inside the GCs, Tfh cells provide help to high-affinity B cells and support their differentiation into long-lived plasma cells or memory B cells

patients show severely impaired antibody responses including a decreased generation of memory B cells [26].

10.3 Development of Tfh Cells

Differentiation of naïve CD4+ T cells towards conventional helper T cell (Th) subsets (including Th1, Th2, and Th17 cells) is regulated by the signals that they receive from DCs and from microenvironment [27, 28]. Recent mouse studies indicate that this is also the case for Tfh cell generation. Tfh cells express large amounts of B cell lymphoma 6 (Bcl-6) [23], the transcription repressor that is necessary and sufficient for Tfh cell generation in vivo [29–31]. Initial commitment towards Tfh cells occurs by upregulation of Bcl-6 expression when CD4+ T cells encounter with DCs [32–35]. The subsequent interaction between T and B cells appears to be important for the maintenance of Bcl-6 expression in Tfh cells. However, Bcl-6 does not regulate IL-21 secretion in mouse [30, 31] or human CD4+ T cells [36]. This is in contrast to other transcription factors engaged in the differentiation of other conventional Th subsets, regulating the secretion of cytokines typical of each subset. Thus, Tfh cell generation occurs through a highly orchestrated process, and likely requires other transcription factors in addition to Bcl-6.

Which types of DCs do promote Tfh cell generation? DCs are endowed with enormous functional plasticity, which permits them to induce different immune responses according to the microenvironment. In addition, the DC system is composed of subsets associated with the induction of different types of immunity [37, 38]. Our study on human skin DC subsets demonstrated that CD14+ dermal DCs are one of the most efficient DC subsets at inducing human naïve CD4+ T cells to become Tfh-like cells in vitro [39]. CD4+ T cells primed by CD14+ dermal DCs, but not by epidermal Langerhans cells, strongly induce naïve B cells to become antibody secreting plasma cells producing IgM, as well as to switch isotypes towards IgG and IgA [39]. Furthermore, in vitro-generated DCs sharing properties with CD14+ dermal DCs induce the differentiation of CD40-activated naïve B cells into IgM-producing plasma cells through direct DC and B cell interactions [40]. These observations suggest that CD14+ dermal DCs display unique properties to promote the development of antibody responses in humans. Notably, in mice, activated dermal DCs migrate into the outer paracortex just beneath the B cell follicles, whereas LCs migrate into the T cell rich inner paracortex [41]. This suggests that also in mice, dermal DCs, rather than LCs, are one of the major DC subsets associated with the development of humoral immunity.

Mouse studies showed that STAT3 signaling delivered by IL-6 and IL-21 contributes to Tfh cell development [42–45]. Similarly, STAT3-deficient human subjects (Hyper IgE syndrome) were shown to have altered Tfh response [46], although whether this is T cell intrinsic or secondary to defective B cell response [26] remains unclear. We and others showed that the IL-12-STAT4 pathway is one of the major pathways in humans by which DCs promote the development of IL-21-producing Tfh-like cells [47, 48]. IL-6 and IL-21 are much less potent than IL-12 in vitro at inducing human naïve CD4+ T cells to express Tfh-associated molecules, including IL-21, CXCR5, ICOS, and Bcl-6 [47, 49, 50]. Indeed, dermal CD14+ DCs, but not LCs, express IL-12 upon CD40L stimulation [39], which explains at least in part why dermal CD14+ DCs are efficient at inducing Tfh-like cells. Interestingly, there is evidence that the IL-12-STAT4 pathway contributes to the development of Tfh cells also in mice [51, 52]. Thus both STAT3 and STAT4 are involved in the generation of Tfh cells in mice and humans, while the extent of contribution by each pathway and/or cytokine might be different. It will be important to address whether IL-12 and/or IL-23 indeed contribute to in vivo Tfh and GC response in humans. Also it will be important to determine how the initial lineage commitment towards Th1 and Tfh cells is regulated in humans, because the IL-12/STAT4 axis also potently promotes Th1 cell generation through the upregulation of T-bet [53]. In mice, the effect of IL-12 on CD4+ T cells for the expression of Tfh-associated molecules is short-lived and eventually dominated by T-bet-driven Th1 cell generation [51].

10.4 Human Tfh Subsets

10.4.1 Tonsillar Tfh Subsets

Whereas Tfh cells are considered to help the selection and differentiation of B cells in GCs, the identity of $CD4^+$ T cells interacting with B cells outside GCs was unknown in humans. We recently identified a Tfh-committed subset that is exclusively localized outside GCs in human tonsils [24]. This subset can be identified by the expression of IL-7 receptor, and low levels of CXCR5 and ICOS ($CXCR5^{lo}ICOS^{lo}$). The expression of *BCL6* and *PRDM1* transcripts are comparable between $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. Interestingly, these two Tfh-lineage subsets differentially help B cells. $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells are efficient at helping GC-B cells. Reciprocally, GC-B cells are able to maintain the survival of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells are far more efficient than GC-Tfh cells at inducing naïve B cells to proliferate and differentiate into Ig-producing cells. Notably, $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells lack the capacity to help GC-B cells and induce the apoptosis of GC-B cells through the FAS/FAS-ligand (FAS-L) interaction. Thus, $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ tonsillar $CD4^+$ T cells likely represent extrafollicular helper cells engaged in inducing the differentiation of B cells into extrafollicular plasma cells and/or represent precursors of GC-Tfh cells (Pre-Tfh cells).

10.4.2 Blood Circulating $CXCR5^+$ $CD4^+$ T Cell Subsets

Human tonsillar Tfh cells display distinct phenotype and gene profiles from other conventional Th subsets [15, 23, 54]. Discovery of Bcl-6 as a "master regulator" of Tfh cell generation further supports the concept that Tfh cells represent an independent $CD4^+$ T cell subset. However, mouse Tfh cells are indeed heterogeneous, and encompass distinct subsets secreting cytokines characteristic of Th1, Th2, and Th17 cells [55–59]. Furthermore, mouse Th2 [57] and T regs [60] were shown to be convertible into Tfh cells in vivo. Thus, the type of Tfh precursors also remains elusive and the relationship between Tfh cells and other Th subsets still remains unclear.

A fraction of human blood memory $CD4^+$ T cells expresses CXCR5 [61]. Several observations suggest the relationship between $CXCR5^+$ $CD4^+$ T cells and Tfh cells. For example, humans who show severely impaired GC formation through deficiency of CD40-ligand or ICOS display significantly less circulating $CXCR5^+$ $CD4^+$ T cells [18]. On the contrary, $CXCR5^+$ $CD4^+$ T cells expressing ICOS are present at a higher frequency in blood of lupus patients [62]. Our studies on human blood $CXCR5^+$ $CD4^+$ T cells conclude that they share functional properties with Tfh cells from secondary lymphoid organs, and likely represent their circulating memory compartment [63]. In concordance with Tfh cells, blood $CXCR5^+$ $CD4^+$ T cells induce naïve and memory B cells to become Ig-producing cells via IL-21, IL-10, ICOS, and secrete CXCL13. At variance with Tfh cells, blood $CXCR5^+$ $CD4^+$ T cells barely express CD69 and ICOS, and express PD-1 only at low levels [14, 48, 62], suggesting that they are in a resting state. Consistently, blood $CXCR5^+$ $CD4^+$ T cells required cell activation to provide help to B cells through cognate interaction. In contrast to GC-Tfh cells, blood $CXCR5^+$ $CD4^+$ T cells express CCR7 and CD62L, suggesting their capacity to migrate into secondary lymphoid organs.

Importantly, blood $CXCR5^+$ $CD4^+$ T cells comprise three subsets; Th1, Th2, and Th17 cells [63] (Fig. 10.2). These subsets can be defined according to the expression of chemokine receptors, expression of transcription factors, and the type of cytokine secretion patterns. Th2 and Th17 cells within $CXCR5^+$ compartment efficiently induce naïve B cells to produce immunoglobulins and to switch isotypes through IL-21 secretion. While $CXCR5^+$ Th2 cells promote IgG and IgE secretion, $CXCR5^+$ Th17 cells are efficient at promoting IgG and, in particular, IgA secretion. In contrast, $CXCR5^+$ Th1 cells lack the capacity to help naïve B cells. These findings suggest that Tfh cells associated with conventional Th subsets differentially shape the quality of human humoral immunity, with $CXCR5^+$ Th2 cells favoring IgE responses and $CXCR5^+$ Th17

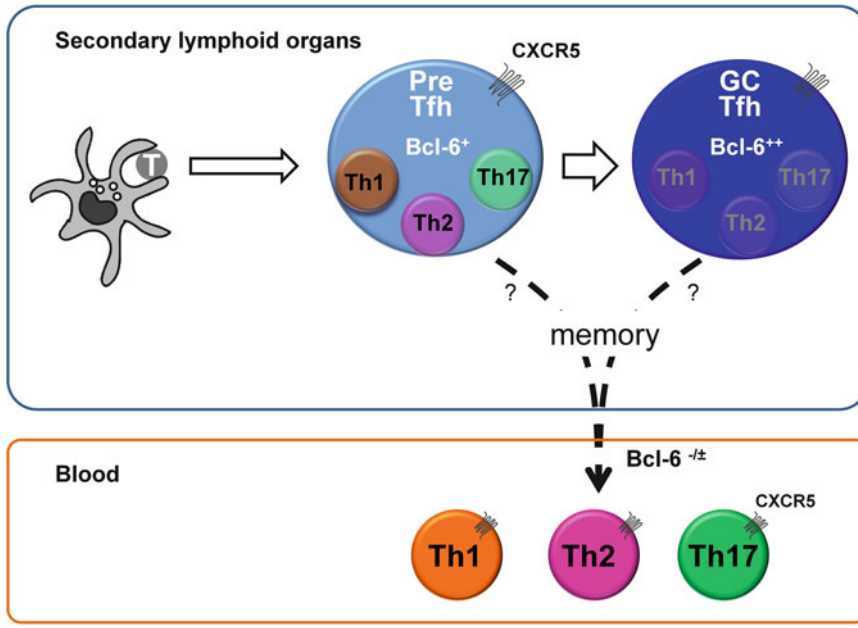

Fig. 10.2 Hypothetical model in the development of distinct Tfh subsets in humans. We surmise that Tfh-committed cells sharing properties with conventional Th1, Th2, and Th17 cells develop at the Pre-Tfh stage. During the maturation process towards GC-Tfh cells, transcription factor changes including Bcl-6 upregulation suppress the expression and/or function of transcription factors associated with conventional Th subsets. Once Tfh cells (or pre-Tfh cells) differentiate into memory cells, they decrease the expression of Bcl-6 and start to reveal the property of conventional Th subsets while maintaining the identity of the Tfh lineage

cells favoring protective mucosal antibody responses. Whether such Th1, Th2, and Th17-commited Tfh subsets are present in human secondary lymphoid organs is under investigation.

Importantly, alteration in the balance of blood CXCR5$^+$ CD4$^+$ T cell subsets, which likely reflects the type of Tfh cells in secondary lymphoid organs, was found to be associated with autoimmunity. Patients with juvenile dermatomyositis, a systemic autoimmune disease, display a profound skewing of blood CXCR5$^+$ CD4$^+$ T cell subsets towards Th2 and Th17 [63]. Significantly, the skewing of subsets correlates with disease activity and frequency of blood plasmablasts. Furthermore, a study on Sjogren's syndrome patient blood samples shows that CXCR5$^+$ Th17 cells are dominant in this disease, and the increase of these cells correlate with clinical characteristics including autoantibody titers and disease activity [64]. Thus, these studies provide a strong rationale that analysis on blood CXCR5$^+$ CD4$^+$ T cell subsets might provide diagnostic and/or prognostic biomarkers in human autoimmune diseases.

Whether blood CXCR5$^+$ CD4$^+$ T cells originate from cells that migrated out of GCs, Tfh precursors, or Tfh-committed extrafollicular helper cells [9, 65] is an important question, but will be challenging to address in humans.

10.5 Perspectives

Tfh cells in human secondary lymphoid organs and in blood are composed of functionally different subsets. Establishing the mechanisms whereby the DC system induces Tfh cells with different functions will facilitate the design of novel vaccines. In particular, establishing how DC system generates Tfh subsets associated with the induction of mucosal homing plasma cells will provide a significant insight in the development of novel mucosal vaccines.

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References

1. Crotty S. Follicular helper CD4 T cells (TFH). Annual review of immunology. 2011;29:621–63.
2. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annual review of immunology. 2008;26: 741–66.
3. Weinstein JS, Hernandez SG, Craft J. T cells that promote B-Cell maturation in systemic autoimmunity. Immunological reviews. 2012;247(1):160–71.
4. Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK. Visualization of specific B and T lymphocyte interactions in the lymph node. Science. 1998;281(5373):96–9.
5. Okada T, Miller MJ, Parker I, Krummel MF, Neighbors M, Hartley SB, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. PLoS biology. 2005;3(6): e150.
6. MacLennan IC, Gray D. Antigen-driven selection of virgin and memory B cells. Immunological reviews. 1986;91:61–85.
7. McHeyzer-Williams MG, McLean MJ, Lalor PA, Nossal GJ. Antigen-driven B cell differentiation in vivo. The Journal of experimental medicine. 1993; 178(1):295–307.
8. Jacob J, Kassir R, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. I. The architecture and dynamics of responding cell populations. The Journal of experimental medicine. 1991;173(5):1165–75.
9. MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, et al. Extrafollicular antibody responses. Immunological reviews. 2003;194:8–18.
10. Tarlinton DM. Evolution in miniature: selection, survival and distribution of antigen reactive cells in the germinal centre. Immunology and cell biology. 2008;86(2):133–8.
11. MacLennan IC. Germinal centers. Annual review of immunology. 1994;12:117–39.
12. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. The Journal of experimental medicine. 2000;192(11):1545–52.
13. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. The Journal of experimental medicine. 2000;192(11):1553–62.
14. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. The Journal of experimental medicine. 2001;193(12):1373–81.
15. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. European journal of immunology. 2006;36(7):1892–903.
16. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nature immunology. 2010;11(6):535–42.
17. Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol. 2005;175(4):2340–8.
18. Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, et al. ICOS Deficiency Is Associated with a Severe Reduction of CXCR5+CD4 Germinal Center Th Cells. J Immunol. 2006;177(7):4927–32.
19. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature. 1999;397(6716):263–6.
20. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity. 2008;29(1):127–37.
21. Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, et al. The CD40 antigen and its ligand. Annual review of immunology. 1994;12:881–922.
22. Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol. 2007;179(12):8180–90.
23. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. J Immunol. 2004;173(1):68–78.
24. Bentebibel S-E, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. Proceedings of the National Academy of Sciences. 2011;10.1073/pnas.1100898108.
25. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annual review of immunology. 2008;26:57–79.
26. Avery DT, Deenick EK, Ma CS, Suryani S, Simpson N, Chew GY, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. The Journal of experimental medicine. 2010;207(1):155–71, S1–5.

27. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245–52.
28. Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature. 2007;449(7161):419–26.
29. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl–6 directs T follicular helper cell lineage commitment. Immunity. 2009;31(3):457–68.
30. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. Science. 2009;325(5943):1001–5.
31. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 2009;325(5943):1006–10.
32. Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, et al. Bcl6 protein expression shapes pregerminal center B cell dynamics and follicular helper T cell heterogeneity. Immunity. 2011;34(6):961–72.
33. Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. Immunity. 2011;34(6):932–46.
34. Baumjohann D, Okada T, Ansel KM. Cutting Edge: Distinct Waves of BCL6 Expression during T Follicular Helper Cell Development. J Immunol. 2011.
35. Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, et al. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. Immunity. 2011;34(6):947–60.
36. Kroenke MA, Eto D, Locci M, Cho M, Davidson T, Haddad EK, et al. Bcl6 and Maf Cooperate To Instruct Human Follicular Helper CD4 T Cell Differentiation. J Immunol. 2012.
37. Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, et al. Dendritic cell subsets in health and disease. Immunological reviews. 2007;219:118–42.
38. Ueno H, Schmitt N, Klechevsky E, Pedroza-Gonzalez A, Matsui T, Zurawski G, et al. Harnessing human dendritic cell subsets for medicine. Immunological reviews. 2010;234(1):199–212.
39. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, et al. Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. Immunity. 2008;29(3):497–510.
40. Caux C, Massacrier C, Vanbervliet B, Dubois B, Durand I, Cella M, et al. CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis. Blood. 1997;90(4):1458–70.
41. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, et al. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity. 2005; 22(5):643–54.
42. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity. 2008;29(1):138–49.
43. Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. PLoS One. 2011;6(3):e17739.
44. Lu Kristina T, Kanno Y, Cannons Jennifer L, Handon R, Bible P, Elkahloun Abdel G, et al. Functional and Epigenetic Studies Reveal Multistep Differentiation and Plasticity of In Vitro-Generated and In Vivo-Derived Follicular T Helper Cells. Immunity. 2011;35(4):622–32.
45. Eddahri F, Denanglaire S, Bureau F, Spolski R, Leonard WJ, Leo O, et al. Interleukin-6/STAT3 signaling regulates the ability of naive T cells to acquire B-cell help capacities. Blood. 2009;113(11):2426–33.
46. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. Blood. 2012;119(17):3997–4008.
47. Schmitt N, Morita R, Bourdery L, Bentebibel SE, Zurawski SM, Banchereau J, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. Immunity. 2009;31(1):158–69.
48. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. Immunology and cell biology. 2009.
49. Ma CS, Suryani S, Avery DT, Chan A, Nanan R, Santner-Nanan B, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. Immunology and cell biology. 2009;87(8):590–600.
50. Lund R, Ahlfors H, Kainonen E, Lahesmaa AM, Dixon C, Lahesmaa R. Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. European journal of immunology. 2005;35(11): 3307–19.
51. Nakayamada S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, et al. Early Th1 Cell Differentiation Is Marked by a Tfh Cell-like Transition. Immunity. 2011;35(6):919–31.
52. Wei L, Vahedi G, Sun HW, Watford WT, Takatori H, Ramos HL, et al. Discrete Roles of STAT4 and STAT6 Transcription Factors in Tuning Epigenetic Modifications and Transcription during T Helper Cell Differentiation. Immunity. 2010;32(6):840–51.
53. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6): 655–69.
54. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. Blood. 2004;104(7):1952–60.

55. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. Nature immunology. 2009;10(4):385–93.
56. King IL, Mohrs M. IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. The Journal of experimental medicine. 2009;206(5):1001–7.
57. Zaretsky AG, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. The Journal of experimental medicine. 2009;206(5):991–9.
58. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nature immunology. 2009;10(2):167–75.
59. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. Immunity. 2009;30(3):324–35.
60. Tsuji M, Komatsu N, Kawamoto S, Suzuki K, Kanagawa O, Honjo T, et al. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science. 2009;323(5920):1488–92.
61. Forster R, Emrich T, Kremmer E, Lipp M. Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. Blood. 1994;84(3):830–40.
62. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis and rheumatism. 2010;62(1):234–44.
63. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity. 2011;34(1):108–21.
64. Li X-y, Wu Z-b, Ding J, Zheng Z-h, Li X-y, Chen L-n, et al. Role of the frequency of blood CD4+ CXCR5+ CCR6+ T cells in autoimmunity in patients with Sjögren's syndrome. Biochemical and Biophysical Research Communications. 2012.(http://dx.doi.org/10.1016/j.bbrc.2012.04.133).
65. Poholek AC, Hansen K, Hernandez SG, Eto D, Chandele A, Weinstein JS, et al. In Vivo Regulation of Bcl6 and T Follicular Helper Cell Development. J Immunol. 2010;doi:10.4049/jimmunol.0904023.

Differentiation and Activation of γδ T Lymphocytes: Focus on CD27 and CD28 Costimulatory Receptors

11

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Abstract

γδ T lymphocytes are major providers of the pro-inflammatory cytokines interferon-γ (IFN-γ) and interleukin-17 (IL-17) at early stages of (auto) immune responses. We and others have recently described the phenotype and differentiation requirements of two distinct murine γδ T cell subsets producing either IFN-γ or IL-17. Here we summarize our current understanding of the molecular mechanisms that control γδ T cell differentiation, which is programmed in the thymus, and peripheral activation upon infection. We focus on the costimulatory receptors CD27 and CD28, which play independent and non-redundant roles in the physiology of γδ T cells in mice and in humans.

Keywords

γδ T cells • T cell activation • Costimulation • CD27 • CD28

11.1 Biological Roles of γδ T Lymphocytes

Since their accidental discovery almost 30 years ago, T cells expressing a γδ T cell receptor (TCR) have been studied in multiple settings of immune responses [1]. As a result, it is now clear that γδ T cells comprise functionally specialized subsets that participate in host responses to many pathogens and tumors as well as in inflammation and immunopathology [2–4].

γδ T cells express clonally distributed antigen receptors, which is a hallmark of adaptive immunity. However, they are classically considered innate-like effectors, owing to their high frequency of preactivated cells displaying restricted antigen recognition repertoires associated with particular tissues. Moreover, γδ T cells respond in an MHC-independent manner to a variety of "stress-inducible" or pathogen-associated proteins or metabolites [1, 5]. Thus, the concept of γδ T cells as "first line of defense" has been proposed as a non-redundant system of responses based on

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an innate immunity program involved in systemic and specific responses depending on the inflammatory microenvironment, on microbes features, and on signals that are engaged [1, 5, 6].

The widespread implication of γδ T cells in immune responses is often attributed to the cells' rapid and abundant production of IFN-γ in mice and in humans [7, 8]. However, many recent studies have also described γδ T cells as critical sources of IL-17 in animal models of infection [9–11] or autoimmune disorders [12–14].

11.1.1 Infection

During infection with microorganisms as diverse as viruses, bacteria, and parasites, γδ T cells have been shown to expand, change their cell surface markers, and deploy effector functions [1]. In this context, murine γδ cells produce large amounts of IFN-γ and/or IL-17, depending on the type of microorganism. For example, we observed the accumulation of IFN-γ-producing, but not of IL-17-producing, γδ cells in the peritoneal cavity of mice infected with murid herpes virus 4 [15]. Of note, γδ lymphocytes have been repeatedly implicated in controlling infection with human [16] and murine [17] herpes viruses.

On the other hand, rapid IL-17 production in response to mycobacteria, *E. coli* or *Listeria*, is attributable to γδ cells rather than to TCRαβ$^+$ Th17 cells [10, 11, 18]. Moreover, γδ cells are also a critical provider of IL-17 in protective (neutrophil-mediated) responses against *Candida albicans* [19].

Finally, some microorganisms seem to elicit both IL-17 and IFN-γ responses by γδ cells. That is the case of *Plasmodium berghei*, a rodent parasite used in experimental malaria models. We have observed a marked expansion of both IL-17$^+$ and IFN-γ$^+$ γδ cells in peripheral lymphoid organs of *P. berghei*-infected C57Bl/6 mice [15, 20, 21]. Importantly, human γδ cells are also strongly activated by *P. falciparum* antigens [22], and malaria patients show striking expansions of γδ cells in the peripheral blood [23, 24].

In sum, γδ cells accumulate at sites of infection prior to αβ T cells and exert a rapid and potent pro-inflammatory role. Nonetheless, γδ T cell responses are not only restricted to early stages after infection. A later wave of γδ T cells can occur during or after pathogen clearance which interestingly seems to associate with anti-inflammatory properties [25]. In fact, it seems that γδ T cells are critical to prevent excessive (chronic) inflammation in targeted tissues following *Listeria* [26] or *Toxoplasma* infection [27].

11.1.2 Tissue Inflammation and Repair

The abundance of γδ cells in barrier tissues such as the skin, lung, and intestine makes them ideal candidates for participating in maintaining tissue homeostasis (even in the absence of infection). Namely, mice genetically deficient for γδ T cells (TCRδ$^{-/-}$) exhibited increased susceptibility to spontaneous or chemically induced skin inflammation [28]; and a delay in wound closure when administered punch biopsies [29]. This was attributed to the local absence of Vγ5$^+$ dendritic epidermal T cells (DETCs) producing anti-inflammatory mediators as well as keratinocyte growth factors required for epidermal remodeling. Similarly, human epidermal Vδ1$^+$ T cells from patients with healing wounds are activated and secrete growth factors (such as insulin-like growth factor 1), whereas patients with chronic, non-healing wounds have a defective epidermal T cell response [30, 31].

Tissue-resident γδ T cells have also been implicated in the repair of epithelia in other organs such as the intestine, where upon dextran sulfate sodium salt (DSS) treatment, TCRδ$^{-/-}$ mice display exacerbated inflammation and defects in the repair of intestinal damage [32, 33]. Similarly to the skin, epithelial cell proliferation in the intestine is known to restore barrier integrity and KGF seems to be an important growth factor in this process [32].

Contrasting with these immunoregulatory roles, γδ cells have also been implicated in the promotion of inflammatory diseases. For example, in a model of colitis, γδ intraepithelial lymphocytes (IELs) contributed to disease induction and aggravated the pathology [34]. More recently, a subset of IL-17$^+$ γδ cells was reported to promote

intestinal Th17 differentiation and thus exacerbated the inflammatory response in the colon [35]. Furthermore, γδ cells were also shown to play a key pro-inflammatory role in a murine model of cerebral ischemia, where γδ cells were the main providers of IL-17 [36]. The production of IL-17 by γδ cells has now also been strongly linked to autoimmune pathology.

11.1.3 Autoimmunity

The function of γδ T cells has been tested in a variety of animal models of induced autoimmunity, including experimental autoimmune encephalomyelitis (EAE), a model for human multiple sclerosis (MS), and collagen-induced arthritis (CIA), a model for human rheumatoid arthritis (RA). The majority of studies identified a disease-promoting effect of γδ T cells in EAE [13, 14, 37, 38] and CIA [12, 39]. In the case of EAE, induced by immunization with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA), γδ T cells expanded in secondary lymphoid tissues and migrated to the central nervous system (CNS), where they accumulated shortly before the peak of clinical signs of disease [13, 14, 40]. The pathogenic role of γδ T cells in EAE has been linked to their production of IFN-γ (and TNF-α) by some authors [40], while others have attributed it to IL-17 secretion [13, 14]. In the latter scenario, two distinct mechanisms of cross-talk with αβ T cells have been proposed: A priming effect on the induction of subsequent Th17 responses [13] and an inhibitory effect on the differentiation of Foxp3$^+$ regulatory T cells [14].

Very recently, skin-infiltrating IL-17$^+$ γδ T cells have also been shown to contribute to the development of skin lesions in a murine model of psoriasis [41], consistent with reports on the incidence of IL-17$^+$ γδ T cells in human psoriasis [42, 43].

11.1.4 Tumors

One of the most striking phenotypes of TCRδ$^{-/-}$ mice is their high susceptibility to the development of various types (transplantable, chemically induced, transgenic oncogene-driven or spontaneous) of tumors [5, 44]. Moreover, human γδ Tcells can use potent cytolytic mechanisms (perforin and granzymes; and death-inducing receptors like FAS and TRAIL-R) to kill leukemia, lymphoma, melanoma, and various carcinoma cell types [45] and are thus being targeted in cancer immunotherapy trials [46]. Furthermore, in vitro-activated human γδ T cells have been recently shown to target a small population of colon cancer stem cells, responsible for tumor resistance to conventional therapies [47], and to kill chemotherapy-resistant chronic myelogenous leukemia cell lines [48].

Another important function of γδ T cells derives from secreting large amounts of IFN-γ, a central cytokine in anti-tumor immune responses [7]. On the other hand, murine γδ T cells can be a major source of IL-17 upon tumor challenge [49, 50]. However, the role of IL-17 produced by γδ T cells within the tumor microenvironment remains controversial: It has been associated both with promotion of angiogenesis and tumor growth [49, 51] and with CD8$^+$ T cell recruitment and the therapeutic effects of chemotherapy against several transplanted tumor cell lines [50]. Thus, further studies are required to dissect the specific roles of IL-17- versus IFN-γ-producing γδ T cells in cancer immunity.

11.2 Differentiation of Functional γδ T Cell Subsets

In striking contrast to αβ Th1 and Th17 cells, which can take up to 7 days to differentiate and clonally expand under the influence of specific combinations of "polarizing" cytokines [52], γδ T cells from lymphoid organs and peripheral tissues of naïve C57Bl/6 mice express effector cytokines directly ex vivo [20, 53, 54]. This allows γδ T cells to be the main constitutive producers of IL-17 in tissues such as the lung and the skin [54] and presumably underlies their very rapid responses to various physiological challenges [5] (see above). Such responses are now being scored and interpreted based on the definition of functional (Th1-like and Th17-like) γδ T cell subsets.

11.2.1 Subsetting the Functions of γδ T Cells

One recent key advance in the study of the functional implications of γδ T cells was the definition of surface markers that allowed—at least in mice—the segregation of their IL-17- versus IFN-γ-producing activities. Previously, γδ T cells had been mostly subsetted according to TCR gene segment usage, since their functions in immune responses often associated with particular TCR(Vγ)-defined repertoires subsets. For example, Vγ4$^+$ and Vγ1$^+$ cells derived from the same tissue showed different, and sometimes opposing (pro- versus anti-inflammatory), effects in models of bacterial and viral infection and in allergic airway hyperresponsiveness (reviewed in ref. 6).

However, as reports describing distinct physiological contributions of IL-17- versus IFN-γ-producing γδ T cells (similarly to their αβ Th17 and Th1 counterparts) accumulated (see above), it became important to link the definition of γδ T cell subsets to these specific properties. In 2009, we identified the tumor necrosis factor receptor (TNFR) superfamily member CD27 as a surface marker that allowed the segregation of murine IL-17- versus IFN-γ-producing γδ T cells. Thus, CD27$^+$ γδ T cells isolated from spleen, lymph nodes, and various other tissues (such as lung or gut) make IFN-γ (but no IL-17) upon stimulation, whereas IL-17 production is restricted to the CD27$^-$ counterparts [20]. Both subsets responded to *Plasmodium berghei* infection by proliferating and producing the respective cytokines while maintaining their characteristic surface phenotypes [20].

Analogous studies by other groups segregated γδ T cells according to levels of CD122 or NK1.1 (expressed on the IFN-γ producers) and Scart-2 or CCR6 (on IL-17$^+$ γδ T cells) [53, 55–57]. Furthermore, constitutive expression of the IL-23R is selective to a subset of IL-17$^+$ γδ T cells that mostly overlaps with Scart-2$^+$ CCR6$^+$ Vγ4$^+$ cells in peripheral lymph nodes [14, 56, 58].

The definition of specific phenotypes of IFN-γ- versus IL-17-producing γδ T cells paved the way for the dissection of the cellular and molecular mechanisms underlying their differentiation and functional specialization.

11.2.2 Thymic Differentiation of Functional γδ T Cell Subsets

Pioneering work from our [20] and other laboratories [53, 55] demonstrated that the selective functional properties of γδ T cell subsets are acquired during their differentiation—or "developmental pre-programming" [5]—in the thymus. In fact, functional commitment of murine γδ T cell subsets was observed as early (in ontogeny) as in the fetal thymus (from E15 onwards) [20, 55]. Importantly, the distinct functional attributes "imprinted" in the thymus were preserved in the periphery [20, 53].

Various molecular mechanisms have been implicated in the functional differentiation of γδ T cell subsets in the murine thymus [59]. First, Chien and co-workers proposed that γδ T cells whose TCRδ chain junctional amino acid sequence includes a particular motif that enables them to recognize the nonclassical MHC class I molecule, T22 [60], commit to IFN-γ production upon TCR ligation in the thymus [53]. Conversely, IL-17 expression was restricted to the cells that had not met their TCR ligand during thymic development. Consistent with this model, a subsequent study on skin-homing, TCR-invariant (Vγ5Vδ1$^+$) DETCs demonstrated that the IFN-γ-producing fate required molecular interactions with Skint-1 [61], which is a key component of TCR-dependent positive selection events of thymic DETC progenitors [62, 63]. This notwithstanding, the role of TCR signaling in the "developmental pre-programming" of γδ cells is likely to be more complex than described for these particular monoclonal γδ cell subsets, as recently discussed by Pennington and Turchinovich [59].

On the other hand, our work showed that CD27 is more than simply a marker of IFN-γ$^+$ γδ cells, since CD70–CD27 signaling actively promoted their differentiation in vitro and in vivo [20]. Of note, we obtained evidence for a synergistic effect of TCR and CD27 signals in both the thymus [20] and in the periphery [15] (see ahead), consistent with the general view of CD27 as a "costimulatory receptor" (to the TCR).

As for IL-17$^+$ γδ cell development, it has been proposed that, instead of relying on TCR/CD27

signaling pathways, it requires input from other receptors such as TGFβR or Notch. Thus, *Tgfb*$^{-/-}$ and *Smad3*$^{-/-}$ mice [64], and likewise *Hes1*$^{-/-}$ animals [65], harbored strongly reduced numbers of IL-17^{+} γδ thymocytes. Furthermore, LTβR signaling also seems to be necessary for thymic development IL-17^{+} γδ cells [66], although paradoxically our previous data suggested that *Ltbr* expression on γδ thymocytes is downstream of CD27 signaling [20].

Following the engagement of these various receptors, signaling and transcriptional mediators are presumably the key to functional fates. While it is clear that γδ thymocytes express the transcription factors T-bet and RORγt [20], "master regulators" of *Ifng* and *Il17* expression, respectively [59], the dissection of the genetic program that controls functional commitment of γδ T cell subsets is an ongoing collective project [67]. The current state of the art regarding the differentiation of IL-17^{+} γδ cells, in particular, has been recently reviewed [68]. Briefly, the transcription factor RelB, acting downstream of LTβR, seems to be required for the expression of RORγt (and RORα) [66]; RORγt then acts in concert with Sox13 to drive γδ thymocytes toward an IL-17-producing fate, which must be suppressed by an Egr3-dependent gene regulatory network (downstream of TCR signaling) in order to divert γδ thymocytes to T-bet expression and IFN-γ production [61].

In summary, a current working model of murine γδ T cell differentiation proposes that signaling through TCRγδ [53] and CD27 [20] is required for the differentiation of IFN-γ^{+} γδ cells, whereas the IL-17-producing fate depends on cytokines such as lymphotoxin [66] or TGF-β [64]. It remains unknown if similar rules apply (or not) to human γδ thymocytes.

11.3 Peripheral Activation of γδ T Cell Subsets

While γδ T cell functions are programmed in the thymus, their response to challenge (such as infection) occurs in the periphery, where they need to be activated to proliferate and deploy effector functions. Thus, a critical step towards the development of more efficient therapeutic strategies based on γδ T cells is the improvement of our understanding of the mechanisms that control their peripheral activation and expansion.

11.3.1 With or Without the TCR?

Murine γδ T cell subsets seem to have very distinct requirements on TCR signaling for their activation and expansion: from obligatory for CD27^{+} γδ T cells to dispensable for their CD27^{-} (CCR6^{+}) counterparts. Recent data have raised the question of the relative importance of the somatically recombined TCR (and its coreceptors) versus innate immunity-associated receptors for the activation of γδ T cells in vivo [69, 70]. Thus, independent reports from Sutton et al. [13] and from our group [15] have shown that the innate cytokines IL-1β and IL-23, mostly secreted by macrophages or dendritic cells upon Toll-like receptor (TLR) stimulation, control the expansion of IL-17-producing (CD27^{-}) γδ T cells.

A controversial issue is whether CCR6^{+} γδ T cells themselves express TLRs, thus allowing a direct response to pathogen-associated molecular patterns. Martin et al. reported that a subset of peripheral CCR6^{+} CD44^{+} γδ T cells expressed TLR1, TLR2, as well as dectin-1, which provided responsiveness to the lipoprotein Pam_3CSK_4 (ligand for TLR1/2) and curdlan (ligand for TLR2 and dectin-1), leading to IL-17 production [71]. However, we have not observed any proliferation or IL-17 induction in highly purified CD27^{-} (CCR6^{+}) γδ T cells incubated with TLR agonists (ref. 15 and unpublished data). We therefore believe that the effect of TLR stimulation is indirect and mediated by myeloid cells secreting IL-1β and IL-23, which then induce the proliferation of CD27^{-} (IL-17-producing) γδ T cells.

By contrast, CD27^{+} γδ cells were refractory to IL-1β/IL-23 stimulation [15]; instead, a productive IFN-γ response by CD27^{+} γδ cells required triggering of both the TCR and the CD27 coreceptor [15]. Therefore, the dichotomous conditions for thymic generation of IL-17^{+} and IFN-γ^{+} γδ T cells seem to translate into differential

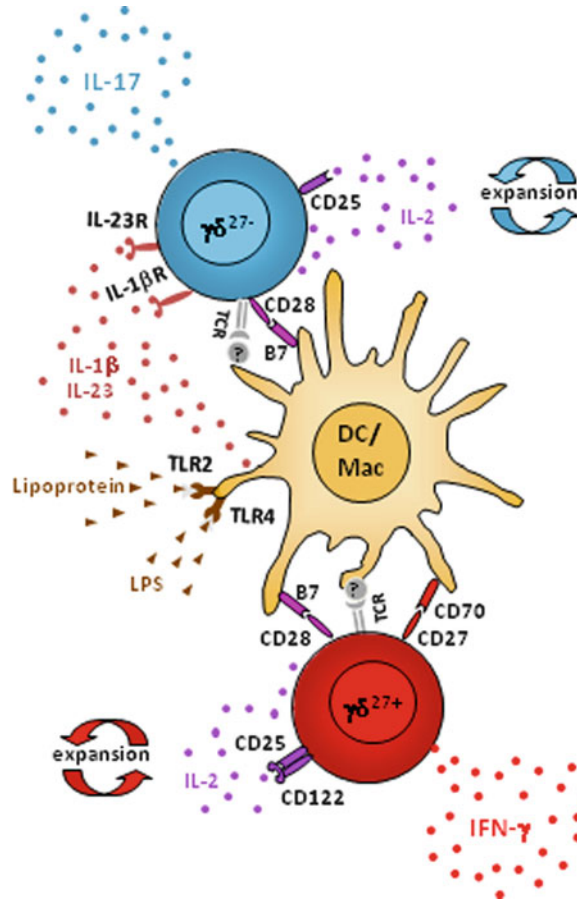

Fig. 11.1 Working model for mechanisms of peripheral activation of γδ T cell subsets. Thymic-derived γδ T cell subsets display distinct functional properties upon activation in peripheral lymphoid organs. IFN-γ-producing $CD27^+$ γδ ($γδ^{27+}$) cells require TCR signaling for expansion, and this process is greatly enhanced by both CD27 and CD28 costimulation. By contrast, $CD27^-$ γδ ($γδ^{27-}$) cells producing IL-17 can proliferate in response to innate signals, namely, IL-1β and IL-23 produced by TLR2/TLR4-activated dendritic cells (DC) and macrophages (Mac). However, $γδ^{27-}$ cell expansion also requires CD28 signaling, which is required for autocrine provision of IL-2

requirements for activation of these γδ T cell subsets in the periphery (Fig. 11.1).

When TCR-mediated activation is involved, costimulation through accessory receptors is also likely to be important. Extensive research on αβ T cells has clearly established that TCR stimulation ("signal 1") in the absence of additional activating signals results in a non-responsive state (also called "anergy") that is refractory to restimulation (reviewed in ref. 72). To avoid "anergy," co-ligation of other receptors, which provide "signal 2" (or costimulation), is usually required. We have therefore made significant efforts to dissect the costimulation requirements of γδ T cell subsets for peripheral activation and expansion in vivo [73].

11.3.2 The Role of CD27 Costimulation

Having identified CD27 as a thymic determinant of γδ T cell differentiation (see Sect. 2.2), we went on to explore its role in the peripheral activation of pre-programmed IFN-γ-producing γδ T cells. We observed that CD27 signals were absolutely required for the expansion of these cells upon infection with herpes viruses or malaria parasites in mice [15]. We further showed that CD70–CD27 interactions provide survival and proliferative signals that control TCRγδ-driven activation. Thus, CD27 signaling activated the non-canonical NF-kB pathway and enhanced the expression of anti-apoptotic (particularly *bcl2a1*)

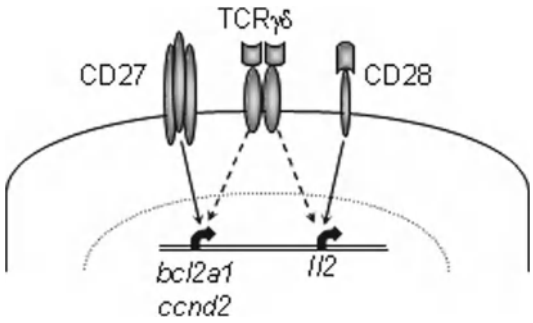

Fig. 11.2 CD27 and CD28 costimulatory pathways play non-redundant roles in γδ T cell activation. The two coreceptors act synergistically with TCRγδ to control distinct gene expression programs. CD28 signaling specifically induces *Il2* expression, whereas CD27 costimulation enhances the transcription of the genes encoding Bcl2A1 and Cyclin D2

and cell cycle-related (*cyclin d2*, *cdk4*, and *cdk6*) genes [15] (Fig. 11.2).

While these data were obtained with murine γδ T cells, we subsequently addressed the role of CD27 costimulation in human γδ T cell activation. Most γδ peripheral blood lymphocytes express a Vγ9Vδ2 TCR that selectively recognizes small non-peptidic prenyl pyrophosphates ("phosphoantigens") [74]. We detected CD27 expression on an average of 81% of Vγ9Vδ2 T cells from healthy donors [75]. Upon activation with PMA and ionomycin, the vast majority (>50%) of CD27$^+$ Vγ9Vδ2 T cells produced IFN-γ, whereas less than 1% made IL-17 [75]. Importantly, the proliferation of these cells was highly sensitive to CD70–CD27 modulation: administration of soluble recombinant CD70 enhanced, whereas blocking anti-CD27 (or anti-CD70) mAbs reduced, Vγ9Vδ2 T cell expansion in vitro. Moreover, CD27 signaling induced fluxes of intracellular calcium ions and, as in mice [15], upregulated the expression of *Bcl2a1* and *Cyclin D2* [75]. In fact, a major role of CD27 costimulation appeared to be the protection from activation-induced cell death (AICD) following phosphoantigen stimulation [75].

These data collectively suggest that the modulation of CD70–CD27 signals may be of great value for T cell-based immunotherapy, particularly in the context of cancer [46]. Consistent with this, work on CD8$^+$ T cells has demonstrated that the expansion of tumor-specific cytotoxic T lymphocytes (CTLs) is critically dependent on CD27 costimulation [76, 77].

11.3.3 The Role of CD28 Costimulation

Much of what is known about T cell costimulation has come from studies on CD28, an immunoglobulin superfamily receptor that binds to B7.1 (CD80) or B7.2 (CD86) [72]. By contrast to the well-established function of CD28 costimulation in αβ T cell activation, its relevance to γδ T cells has remained controversial due to various discrepant reports (reviewed in ref. 73). We therefore decided to revisit this issue and to further specify the potential contribution of CD28 signaling to the activation and expansion of CD27$^+$ and CD27$^-$ γδ T cell subsets in vivo.

We observed that CD28, constitutively expressed on freshly isolated lymphoid γδ T cells, promoted γδ T cell survival and proliferation in both mice and humans. Thus, γδ cell expansion was significantly enhanced by CD28 receptor agonists, but abrogated by B7 antibody-mediated blockade [22]. Mechanistically, we showed that the induction of IL-2 production is a major and specific function of CD28 (but not CD27) costimulation in γδ cells (Fig. 11.2), which are known to strongly benefit from IL-2 signals for their expansion [78, 79]. The fact that γδ cells themselves can produce high levels of IL-2 strictly upon CD28 costimulation defines important rules for their expansion in situ. Of note, CD28-deficient mice displayed reduced (relative to WT controls) numbers of total or activated γδ cells following *Plasmodium berghei* infection, which was not phenocopied in CD27-deficient animals. This demonstrates that the two costimulatory pathways play independent roles in γδ T cell activation in vivo [15, 22].

Most importantly, CD28-deficient mice failed to expand both IFN-γ$^+$ and IL-17$^+$ γδ cells in response to *Plasmodium* parasites [22], which contrasted with the selective effect of CD27 on IFN-γ-producing γδ cells [15]. Regarding the latter, we further showed that CD28 acts non-

redundantly and synergistically with CD27 in their activation and expansion following malaria infection [22].

Finally, the evidence that IL-17$^+$ γδ cells also rely on CD28 costimulatory signals for their expansion in vivo [22] is likely to reflect an underappreciated [69] dependence on TCR-mediated activation of CD27$^-$ γδ cells. These recent data have provoked a revision of our working model of γδ T cell activation (Fig. 11.1).

11.4 Conclusion and Future Perspectives

We have made significant progress in our understanding of the molecular mechanisms that control the differentiation and activation of γδ T cells in vivo. The costimulatory receptors CD27 and CD28 play central roles in these processes, thus creating a possibility for positive (in infection or cancer) or negative (in chronic inflammation or autoimmunity) modulation of γδ cell responses in the clinic. However, as most of this research has been performed on animal models, these findings should be further tested on human γδ T cells. On the other hand, the importance of additional coreceptors, and of the TCR itself, for the distinct γδ T cell subsets, should be further investigated and clarified. Finally, more insight is needed on the molecular mechanisms, downstream of receptors, responsible for functional commitment of γδ T cell subsets. In particular, the roles of epigenetics (chromatin modifications), on one hand, and post-transcriptional mechanisms (including microRNA-mediated regulation), on the other, are yet to be explored. Overall, these future lines of research should help us understand better the physiology of γδ T cell subsets and their contributions to immune responses.

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References

1. Hayday AC: **[gamma][delta] cells: a right time and a right place for a conserved third way of protection**. *Annu Rev Immunol* 2000, **18**:975–1026.
2. Hayday A, Tigelaar R: **Immunoregulation in the tissues by gammadelta T cells**. *Nat Rev Immunol* 2003, **3**:233–242.
3. Kabelitz D, Wesch D, He W: **Perspectives of gammadelta T cells in tumor immunology**. *Cancer Res* 2007, **67**:5–8.
4. O'Brien RL, Born WK: **gammadelta T cell subsets: a link between TCR and function?** *Semin Immunol* 2010, **22**:193–198.
5. Hayday AC: **Gammadelta T cells and the lymphoid stress-surveillance response**. *Immunity* 2009, **31**:184–196.
6. Born WK, Reardon CL, O'Brien RL: **The function of gammadelta T cells in innate immunity**. *Curr Opin Immunol* 2006, **18**:31–38.
7. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, Craft J, Yin Z: **Gamma delta T cells provide an early source of interferon gamma in tumor immunity**. *J Exp Med* 2003, **198**:433–442.
8. Gibbons DL, Haque SF, Silberzahn T, Hamilton K, Langford C, Ellis P, Carr R, Hayday AC: **Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants**. *Eur J Immunol* 2009, **39**:1794–1806.
9. Lockhart E, Green AM, Flynn JL: **IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection**. *J Immunol* 2006, **177**:4662–4669.
10. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y: **Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production**. *J Immunol* 2007, **178**:4466–4472.
11. Hamada S, Umemura M, Shiono T, Tanaka K, Yahagi A, Begum MD, Oshiro K, Okamoto Y, Watanabe H, Kawakami K, et al.: **IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver**. *J Immunol* 2008, **181**:3456–3463.
12. Roark CL, French JD, Taylor MA, Bendele AM, Born WK, O'Brien RL: **Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells**. *J Immunol* 2007, **179**:5576–5583.
13. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH: **Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity**. *Immunity* 2009, **31**:331–341.
14. Petermann F, Rothhammer V, Claussen MC, Haas JD, Blanco LR, Heink S, Prinz I, Hemmer B, Kuchroo VK, Oukka M, et al.: **gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism**. *Immunity* 2010, **33**:351–363.

15. Ribot JC, Chaves-Ferreira M, d'Orey F, Wencker M, Goncalves-Sousa N, Decalf J, Simas JP, Hayday AC, Silva-Santos B: **Cutting Edge: Adaptive Versus Innate Receptor Signals Selectively Control the Pool Sizes of Murine IFN-{gamma}- or IL-17-Producing {gamma}{delta} T Cells upon Infection**. *J Immunol* 2010, **185**:6421–6425.
16. Barcy S, De Rosa SC, Vieira J, Diem K, Ikoma M, Casper C, Corey L: **Gamma delta+ T cells involvement in viral immune control of chronic human herpesvirus 8 infection**. *J Immunol* 2008, **180**:3417–3425.
17. Sciammas R, Kodukula P, Tang Q, Hendricks RL, Bluestone JA: **T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis**. *J Exp Med* 1997, **185**:1969–1975.
18. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, Suda T, Sudo K, Nakae S, Iwakura Y, et al.: **IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection**. *J Immunol* 2007, **178**:3786–3796.
19. Dejima T, Shibata K, Yamada H, Hara H, Iwakura Y, Naito S, Yoshikai Y: **Protective role of naturally occurring interleukin-17A-producing gammadelta T cells in the lung at the early stage of systemic candidiasis in mice**. *Infect Immun* 2011, **79**:4503–4510.
20. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, Girardi M, Borst J, Hayday AC, Pennington DJ, et al.: **CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets**. *Nat Immunol* 2009, **10**:427–436.
21. Goncalves-Sousa N, Ribot JC, deBarros A, Correia DV, Caramalho I, Silva-Santos B: **Inhibition of murine gammadelta lymphocyte expansion and effector function by regulatory alphabeta T cells is cell-contact-dependent and sensitive to GITR modulation**. *Eur J Immunol* 2010, **40**:61–70.
22. Ribot JC, deBarros A, Mancio-Silva L, Pamplona A, Silva-Santos B: **B7-CD28 costimulatory signals control the survival and proliferation of murine and human gd T cells via interleukin-2 production.** *J Immunol* 2012, **189**(3):1202–1208.
23. Ho M, Webster HK, Tongtawe P, Pattanapanyasat K, Weidanz WP: **Increased gamma delta T cells in acute Plasmodium falciparum malaria**. *Immunol Lett* 1990, **25**:139–141.
24. Roussilhon C, Agrapart M, Ballet JJ, Bensussan A: **Tlymphocytes bearing the gamma delta T cell receptor in patients with acute Plasmodium falciparum malaria**. *J Infect Dis* 1990, **162**:283–285.
25. Andrew EM, Carding SR: **Murine gammadelta T cells in infections: beneficial or deleterious?** *Microbes Infect* 2005, **7**:529–536.
26. Fu YX, Roark CE, Kelly K, Drevets D, Campbell P, O'Brien R, Born W: **Immune protection and control of inflammatory tissue necrosis by gamma delta T cells**. *J Immunol* 1994, **153**:3101–3115.
27. Egan CE, Dalton JE, Andrew EM, Smith JE, Gubbels MJ, Striepen B, Carding SR: **A requirement for the Vgamma1+ subset of peripheral gammadelta T cells in the control of the systemic growth of Toxoplasma gondii and infection-induced pathology**. *J Immunol* 2005, **175**:8191–8199.
28. Girardi M, Lewis J, Glusac E, Filler RB, Geng L, Hayday AC, Tigelaar RE: **Resident skin-specific gammadelta T cells provide local, nonredundant regulation of cutaneous inflammation**. *J Exp Med* 2002, **195**:855–867.
29. Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, Havran WL: **A role for skin gammadelta T cells in wound repair**. *Science* 2002, **296**:747–749.
30. Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, Rudolph R, Jameson J, Havran WL: **A role for human skin-resident T cells in wound healing**. *J Exp Med* 2009, **206**:743–750.
31. Havran WL, Jameson JM: **Epidermal T cells and wound healing**. *J Immunol* 2010, **184**:5423–5428.
32. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R: **Protection of the intestinal mucosa by intraepithelial gamma delta T cells**. *Proc Natl Acad Sci U S A* 2002, **99**:14338–14343.
33. Tsuchiya T, Fukuda S, Hamada H, Nakamura A, Kohama Y, Ishikawa H, Tsujikawa K, Yamamoto H: **Role of gamma delta T cells in the inflammatory response of experimental colitis mice**. *J Immunol* 2003, **171**:5507–5513.
34. Simpson SJ, Hollander GA, Mizoguchi E, Allen D, Bhan AK, Wang B, Terhorst C: **Expression of proinflammatory cytokines by TCR alpha beta+ and TCR gamma delta+ T cells in an experimental model of colitis**. *Eur J Immunol* 1997, **27**:17–25.
35. Do JS, Visperas A, Dong C, Baldwin WM, 3rd, Min B: **Cutting edge: Generation of colitogenic Th17 CD4 T cells is enhanced by IL-17+ gammadelta T cells**. *J Immunol* 2011, **186**:4546–4550.
36. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, et al.: **Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury**. *Nat Med* 2009, **15**:946–950.
37. Rajan AJ, Gao YL, Raine CS, Brosnan CF: **A pathogenic role for gamma delta T cells in relapsing-remitting experimental allergic encephalomyelitis in the SJL mouse**. *J Immunol* 1996, **157**:941–949.
38. Odyniec A, Szczepanik M, Mycko MP, Stasiolek M, Raine CS, Selmaj KW: **Gammadelta T cells enhance the expression of experimental autoimmune encephalomyelitis by promoting antigen presentation and IL-12 production**. *J Immunol* 2004, **173**:682–694.
39. Ito Y, Usui T, Kobayashi S, Iguchi-Hashimoto M, Ito H, Yoshitomi H, Nakamura T, Shimizu M, Kawabata D, Yukawa N, et al.: **Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in**

rheumatoid arthritis. *Arthritis Rheum* 2009, **60**:2294–2303.
40. Wohler JE, Smith SS, Zinn KR, Bullard DC, Barnum SR: **Gammadelta T cells in EAE: early trafficking events and cytokine requirements**. *Eur J Immunol* 2009, **39**:1516–1526.
41. Pantelyushin S, Haak S, Ingold B, Kulig P, Heppner FL, Navarini AA, Becher B: **Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice**. *J Clin Invest* 2012.
42. Cai Y, Shen X, Ding C, Qi C, Li K, Li X, Jala VR, Zhang HG, Wang T, Zheng J, et al.: **Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation**. *Immunity* 2011, **35**:596–610.
43. Laggner U, Di Meglio P, Perera GK, Hundhausen C, Lacy KE, Ali N, Smith CH, Hayday AC, Nickoloff BJ, Nestle FO: **Identification of a novel proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a potential role in psoriasis**. *J Immunol* 2011, **187**:2783–2793.
44. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC: **Regulation of cutaneous malignancy by gammadelta T cells**. *Science* 2001, **294**: 605–609.
45. Bonneville M, O'Brien RL, Born WK: **Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity**. *Nat Rev Immunol* 2010, **10**:467–478.
46. Gomes AQ, Martins DS, Silva-Santos B: **Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application**. *Cancer Res* 2010, **70**:10024–10027.
47. Todaro M, D'Asaro M, Caccamo N, Iovino F, Francipane MG, Meraviglia S, Orlando V, La Mendola C, Gulotta G, Salerno A, et al.: **Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes**. *J Immunol* 2009, **182**:7287–7296.
48. D'Asaro M, La Mendola C, Di Liberto D, Orlando V, Todaro M, Spina M, Guggino G, Meraviglia S, Caccamo N, Messina A, et al.: **V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells**. *J Immunol* 2010, **184**:3260–3268.
49. Wakita D, Sumida K, Iwakura Y, Nishikawa H, Ohkuri T, Chamoto K, Kitamura H, Nishimura T: **Tumor-infiltrating IL-17-producing gammadelta T cells support the progression of tumor by promoting angiogenesis**. *Eur J Immunol* 2010, **40**:1927–1937.
50. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, Boucontet L, Apetoh L, Ghiringhelli F, Casares N, et al.: **Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy**. *J Exp Med* 2011, **208**:491–503.
51. Silva-Santos B: **Promoting angiogenesis within the tumor microenvironment: the secret life of murine lymphoid IL-17-producing gammadelta T cells**. *Eur J Immunol* 2010, **40**:1873–1876.
52. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM: **Th17: an effector CD4 T cell lineage with regulatory T cell ties**. *Immunity* 2006, **24**:677–688.
53. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, Steinman L, Saito T, Locksley RM, Davis MM, et al.: **Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma**. *Immunity* 2008, **29**:90–100.
54. Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, Riethmacher D, Si-Tahar M, Di Santo JP, Eberl G: **In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells**. *J Exp Med* 2008, **205**:1381–1393.
55. Shibata K, Yamada H, Nakamura R, Sun X, Itsumi M, Yoshikai Y: **Identification of CD25+ gamma delta T cells as fetal thymus-derived naturally occurring IL-17 producers**. *J Immunol* 2008, **181**:5940–5947.
56. Kisielow J, Kopf M, Karjalainen K: **SCART scavenger receptors identify a novel subset of adult gammadelta T cells**. *J Immunol* 2008, **181**:1710–1716.
57. Haas JD, Gonzalez FH, Schmitz S, Chennupati V, Fohse L, Kremmer E, Forster R, Prinz I: **CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells**. *Eur J Immunol* 2009, **39**:3488–3497.
58. Awasthi A, Riol-Blanco L, Jager A, Korn T, Pot C, Galileos G, Bettelli E, Kuchroo VK, Oukka M: **Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells**. *J Immunol* 2009, **182**:5904–5908.
59. Ciofani M, Zuniga-Pflucker JC: **Determining gammadelta versus alphass T cell development**. *Nat Rev Immunol* 2010, **10**:657–663.
60. Shin S, El-Diwany R, Schaffert S, Adams EJ, Garcia KC, Pereira P, Chien YH: **Antigen recognition determinants of gammadelta T cell receptors**. *Science* 2005, **308**:252–255.
61. Turchinovich G, Hayday AC: **Skint-1 identifies a common molecular mechanism for the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T cells**. *Immunity* 2011, **35**:59–68.
62. Lewis JM, Girardi M, Roberts SJ, Barbee SD, Hayday AC, Tigelaar RE: **Selection of the cutaneous intraepithelial gammadelta+ T cell repertoire by a thymic stromal determinant**. *Nat Immunol* 2006, **7**:843–850.
63. Boyden LM, Lewis JM, Barbee SD, Bas A, Girardi M, Hayday AC, Tigelaar RE, Lifton RP: **Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells**. *Nat Genet* 2008, **40**:656–662.
64. Do JS, Fink PJ, Li L, Spolski R, Robinson J, Leonard WJ, Letterio JJ, Min B: **Cutting edge: spontaneous development of IL-17-producing gamma delta T cells in the thymus occurs via a TGF-beta 1-dependent mechanism**. *J Immunol* 2010, **184**:1675–1679.

65. Shibata K, Yamada H, Sato T, Dejima T, Nakamura M, Ikawa T, Hara H, Yamasaki S, Kageyama R, Iwakura Y, et al.: **Notch-Hes1 pathway is required for the development of IL-17-producing gammadelta T cells**. *Blood* 2011, **118**:586–593.
66. Powolny-Budnicka I, Riemann M, Tanzer S, Schmid RM, Hehlgans T, Weih F: **RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in gammadelta T cells**. *Immunity* 2011, **34**:364–374.
67. Narayan K, Sylvia KE, Malhotra N, Yin CC, Martens G, Vallerskog T, Kornfeld H, Xiong N, Cohen NR, Brenner MB, et al.: **Intrathymic programming of effector fates in three molecularly distinct gammadelta T cell subtypes**. *Nat Immunol* 2012, **13**:511–518.
68. Hayes SM, Laird RM: **Genetic requirements for the development and differentiation of interleukin-17-producing gammadelta T cells**. *Crit Rev Immunol* 2012, **32**:81–95.
69. Kapsenberg ML: **Gammadelta T cell receptors without a job**. *Immunity* 2009, **31**:181–183.
70. O'Brien R, Jin N, Huang Y, Aydintug MK, Roark C, Born W: **Characteristics of IL-17-producing gammadelta T cells**. *Immunity* 2010, **32**:1; author reply 2–4.
71. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M: **Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals**. *Immunity* 2009, **31**:321–330.
72. Smith-Garvin JE, Koretzky GA, Jordan MS: **T cell activation**. *Annu Rev Immunol* 2009, **27**:591–619.
73. Ribot JC, debarros A, Silva-Santos B: **Searching for "signal 2": costimulation requirements of gammadelta T cells**. *Cell Mol Life Sci* 2011, **68**:2345–2355.
74. Morita CT, Jin C, Sarikonda G, Wang H: **Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens**. *Immunol Rev* 2007, **215**:59–76.
75. deBarros A, Chaves-Ferreira M, d'Orey F, Ribot JC, Silva-Santos B: **CD70-CD27 interactions provide survival and proliferative signals that regulate T-cell receptor-driven activation of human γδ peripheral blood lymphocytes**. *Eur J Immunol* 2011, **41**(1):195–201.
76. French RR, Taraban VY, Crowther GR, Rowley TF, Gray JC, Johnson PW, Tutt AL, Al-Shamkhani A, Glennie MJ: **Eradication of lymphoma by CD8 T cells following anti-CD40 monoclonal antibody therapy is critically dependent on CD27 costimulation**. *Blood* 2007, **109**:4810–4815.
77. Glouchkova L, Ackermann B, Zibert A, Meisel R, Siepermann M, Janka-Schaub GE, Goebel U, Troeger A, Dilloo D: **The CD70/CD27 pathway is critical for stimulation of an effective cytotoxic T cell response against B cell precursor acute lymphoblastic leukemia**. *J Immunol* 2009, **182**:718–725.
78. Casetti R, Perretta G, Taglioni A, Mattei M, Colizzi V, Dieli F, D'Offizi G, Malkovsky M, Poccia F: **Drug-induced expansion and differentiation of V gamma 9V delta 2 T cells invivo: the role of exogenous IL-2**. *J Immunol* 2005, **175**:1593–1598.
79. Correia DV, d'Orey F, Cardoso BA, Lanca T, Grosso AR, deBarros A, Martins LR, Barata JT, Silva-Santos B: **Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human gammadelta T-cells**. *PLoS One* 2009, **4**:e5657.

Decisions on the Road to Memory

12

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Abstract

A fundamental property of the adaptive immune system is the ability to generate antigen-specific memory, which protects against repeated infections with the same pathogens and determines the success of vaccination. Immune memory is built up alongside a response providing direct protection during the course of a primary immune response. For CD8 T cells, this involves the generation of two distinct types of effector cells. Short lived effector cells (SLECs) confer immediate protection, but contribute little to the memory repertoire. Memory precursor effector cells (MPECs) have the ability to respond to survival signals and develop into memory cells. These two types of cells can be distinguished on the basis of surface markers and express distinct genetic programs. A single naive CD8 T cell can give rise to both MPEC and SLEC daughter cells. This may involve an initial asymmetric division or depend on later instructive signals acting on equipotent daughter cells. Strong inflammatory signals favor the generation of SLECs and weaker inflammation favors the generation of MPECs. A distinguishing feature of MPECs is their ability to persist when most effector cells die. This survival depends on signals from the IL-7 receptor, which induce expression of anti-apoptotic factors. MPECs are therefore characterized by expression of the IL-7 receptor as well as the CCR7 chemokine receptor, which allows homing to areas in lymphoid organs where

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IL-7 is produced. Critical for persistence of MPECs is further their responsiveness to myeloid cell derived IL-15, which instructs these cells to switch their metabolic programs from glycolysis associated with rapid proliferation to fatty acid oxidation required during a more resting state. As the mechanisms determining generation of immunological memory are unraveled, opportunities will emerge for the improvement of vaccination strategies.

Keywords
CD8 T cell • Memory • Inflammation • MPEC • SLEC

12.1 Numbers

The adaptive immune response is a numbers game with ups and downs. Many different pathogens can potentially invade our tissues necessitating the existence of a large number of different antigen receptor specificities. As the frequency of individual antigen receptor specificities is inversely proportional to the total number of specificities, the number of naive cells carrying any individual specificity is low. Measurements among CD8 T cells in mice have shown that the pre-immune frequency of individual antigen specificities lies between 1 in 7×10^4 and 1 in 3×10^5 cells and a mouse thus contains around 80–1,200 naive CD8 T cells of each specificity [1]. This number stands in sharp contrast to the millions of cells that can be infected during a viral invasion. As killing by CD8 T cells requires direct cell–cell contact, tremendous expansion of the naive repertoire of CD8 T cells is thus necessary for effective removal of all these infected cells. Indeed, at the peak of a response, up to 10% of the CD8 T cell repertoire may consist of cells with a single antigen specificity, requiring around 10^4-fold or greater expansion of the naive repertoire [1]. Ten such responses would theoretically result in a doubling of the total number of CD8 T cells in a mouse. Clearly, maintaining all these excess effector CD8 T cells after clearance of the infections would be a great waste of resources and space and possibly even dangerous, given that these cytotoxic cells are armed to the teeth. After about a week into a primary CD8 T cells response, antigen-specific repertoires therefore contract again, ultimately leaving only about 5–10% of the newly generated cells to generate a stable pool of memory cells [2]. While only a minor fraction of the peak population, the number of memory CD8 T cells persisting is still several orders of magnitude greater than the naive repertoire, giving them a head start when the same microorganism reinfects the host and the repertoire must be expanded again. In combination with qualitative differences between memory and naive CD8 T cells, this greater precursor frequency causes memory responses to be both faster and more vigorous than primary responses, and thereby limit pathology from repeated infections [3]. Different types of memory CD8 T cells exist, which differ in their localization and functional abilities. Central memory (Tcm) cells express high levels of the lymph node homing receptor CD62L and correspondingly reside in lymph nodes and spleen. On the other hand, effector memory (Tem) CD8 T cells lack expression of CD62L and are preferentially found in tissues. Tem have limited ability to proliferate, but readily unleash their effector function, whereas Tcm proliferate vigorously and require a differentiation phase to acquire effector function [3].

As memory responses determine the success of vaccination, there is great interest in understanding the mechanisms controlling the generation of effective memory. The early events leading to the establishment of CD8 T cell memory are the subject of this chapter.

12.2 MPECs and SLECs: Cells with Different Potential

A key question is how it is decided which effector cells should die at the end of a primary response and which ones will live on to become memory

cells? Conceptually, two different models are possible: First, all effector cells could be equipotent and chance encounters with survival factors at and after the peak of the response could determine which cells survive and develop into memory cells. Alternatively, a decision could be made earlier, such that some effector cells are destined to die, while others are predisposed to seed the memory lineage. The available evidence clearly favors the latter possibility. Cell transfer experiments have shown that at the peak of the response two types of effector cells exist: short lived effector cells (SLECs), which die during contraction of the repertoire, and memory precursor effector cells (MPECs) [4]. These cells are found within two populations with distinct expression of the killer lectin-like receptor KLRG1 and the α-chain of the IL-7 receptor, CD127. KLRG1^{+}CD127^{-} cells are mostly SLECs, whereas MPECs are contained within a KLRG1^{-}CD127^{+} population [4, 5]. Further heterogeneity may exist within the MPEC population. A proportion of KLRG1^{-}CD127^{+} was recently shown to express CD62L, and it has been suggested that these cells may be the precursors of Tcm [6]. The relative distribution of cells in these populations varies between responses to different infections. For instance, over 60% of antigen-specific cell found at the peak of the response to *Listeria monocytogenes* or LCMV is KLRG1^{+}CD127^{-}, whereas this population makes up only 20% of the response to Influenza virus [6, 7]. A third population of KLRG1^{-}CD127^{-} cells is found at the peak of the response to some pathogens such as Influenza virus or vesicular stomatitis virus (VSV) [6]. Cells in this population have the capacity to give rise to all three populations, KLRG1^{-}CD127^{-}, KLRG1^{+}CD127^{-}, and KLRG1^{-}CD127^{+} [6]. These cells thus appear to be precursors to SLECs and MPECs and have been referred to as early effector cells (EECs) [6]. Commitment to the MPEC fate seems to already be made by cells in this population. It was shown that high expression of the transcriptional regulator Id3 among EEC marks those cells destined to develop into memory cells [8].

Both SLECs and MPECs are genuine effector cells, which produce effector cytokines such as γIFN as well as cytolytic effector molecules like Granzyme B and Perforin (Ronald A. Backer and Derk Amsen,Unpublished results). Nonetheless, significant differences exist between the two types of effector cells, apart from the ability to survive. SLECs produce higher levels of cytotoxic effector molecules than MPECs. This is true especially at later stages in the response, when MPECs gradually reduce production of these molecules [9]. In contrast, MPECs produce more IL-2. Differences are also found in the expression pattern of chemokine and homing receptors, with for instance preferential expression of the lymph node homing receptors CCR7 and CD62L on MPECs (see below) [6, 9, 10].

As would be expected from these phenotypic differences, the SLEC and MPEC fates are associated with distinct transcriptional programs. Dedicated transcription factors govern these gene expression programs. Several of these factors are preferentially expressed in one cell type or the other. For instance, the T-box transcription factor T-bet and the Zinc finger containing transcriptional repressor Blimp1 are both expressed at higher levels in SLECs than in MPECs and are necessary for the development of KLRG1^{+}CD127^{-} cells [5, 9, 11]. T-bet contributes to the establishment/maintenance of SLEC identity by promoting expression of the *Ifng* gene and repression of the gene encoding CD127. T-bet also induces expression of CD122, the β-chain of the receptor for IL-15, which helps maintain viability of SLECs at late stages in the response (see below) [12]. Blimp1 is necessary for high expression of cytotoxic molecules, such as Granzyme B, FasL, and Perforin [9, 11]. As Blimp1 is a transcriptional repressor, it seems likely that intermediate factors are involved in these positive effects. On the other hand, Blimp1 inhibits expression of multiple MPEC specific factors, such as CD127 and IL-2, as well as chemokine receptors and receptors involved in homing to lymphoid organs, such as CCR7 and CD62L [9, 11]. Blimp1 furthermore directly antagonizes the MPEC transcriptional program at a higher level by repressing expression of various MPEC promoting transcription factors. One of these is Bcl6, a factor necessary for development of stable MPEC cells, which itself inhibits

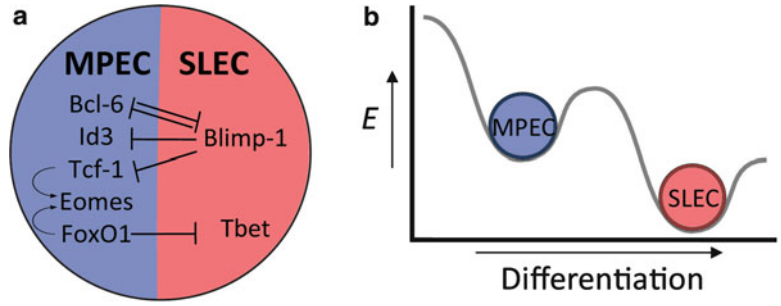

Fig. 12.1 (**a**) Transcriptional regulators dedicated to the establishment and maintenance of either MPEC or SLEC differentiation form cross-regulatory networks, which inhibit one another at multiple levels to generate stable identities. (**b**) The formation of MPECs and SLECs from single precursor cells represented as bistable differentiation states, allowing separation of SLECs and MPECs as distinct, relatively stable cell subsets. Both differentiated states are energetically more favorable than the transition state. Only after crossing a threshold do the cells transition, but intermediate phenotypes likely do not exist due to inherent instability. Because SLECS can be generated from MPECs, but probably not the other way around, the former are represented at a lower energetic state

expression of the Granzyme B gene as well as, in a mutually antagonistic fashion, the gene encoding Blimp1 [13]. Another MPEC factor inhibited by Blimp1 is Id3 [9], a helix-loop-helix factor which functions as dominant-negative regulator of DNA binding by E-proteins or other transcription factors [14]. Id3 is expressed at elevated levels in MPECs and is essential for their long-term survival through as yet unidentified mechanisms [8, 15]. Finally, the Wnt pathway transcription factor Tcf1 is preferentially expressed in MPECs and inhibited by Blimp1 [9, 16]. Like Id3, Tcf1 is not required for MPEC differentiation per se, but is essential for persistence of MPECs by transactivating the gene encoding the T-box factor Eomesodermin [16]. In its turn, Eomesodermin promotes expression of CD122, allowing MPECs and memory cells to respond to IL-15, which is necessary for initial survival and their later homeostatic turn over [12]. Finally, the FoxO1 transcription factor promotes the MPEC fate by inducing expression of CD127, CD62L, and Eomesodermin [17]. At the same time, FoxO1 inhibits expression of the SLEC factor T-bet [17].

A general picture that emerges is that dedicated transcription factors act both positively to promote one fate and negatively to inhibit the other (Fig. 12.1). This suggests that these two cell types exist in bistable states, such as found for instance also between T helper 1 and T helper 2 cells, reinforcing the notion that MPEC and SLEC truly represent distinct identities, rather than a continuum of phenotypes.

12.3 One Cell, Multiple Fates

How then is this heterogeneity of SLECs and MPECs generated? One possibility could be that different naive precursors develop into SLECs and MPECs. It has been proposed for instance that the timing of recruitment into the response affects the ultimate fate chosen by the CD8 naïve precursor. Indeed, not all antigen responsive cells are activated at the same time. For instance, 1 day after infection with VSV, only 35% of the naïve VSV specific CD8 T cells expressed the early activation marker CD69, whereas after 2 days nearly all VSV specific CD8 T cells did [1]. Furthermore, when naive precursors were transferred at different times into a mouse already making a response, those CD8 T cells transferred at a relatively late stage preferentially developed into MPEC [18]. It is therefore pos-

sible that the timing of activation does influence the choice between SLEC and MPEC differentiation. However, others have shown that one single Ag-specific CD8 T cell can give rise to a diverse population of effector and memory cells [19, 20]. When, for instance, a single naive antigen-specific T cell was transferred into a congenic host, it was found that its progeny consisted of both effector and memory cells [19]. In another experiment, naive CD8 T cells were engineered to carry unique genetic "barcodes," short random DNA sequences, which allow the tracing of their progeny. When mice with these CD8 T cells were infected with different model pathogens, the same barcodes were found back in both effector and memory cells [20]. If SLECs and MPECs were derived from different naive precursors, the prediction of this experiment would have been that a specific subset of barcodes should be found exclusively in the effector cells and not in the memory population. That such a population of barcodes was not found, suggests that the generation of SLEC and MPEC progeny from a single naive precursor is the rule rather than the exception.

12.4 Commitment at First Division or Later

Two models have been proposed to explain how a single cell gives rise to both of SLEC and MPEC progeny (Fig. 12.2) and each of these models has been supported by elegant experimental evidence. In the first model, the decision between SLEC and MPEC is taken already during a first asymmetric division of the naive CD8 T cell, in which one of the daughter cells adopts the SLEC and the other the MPEC fate [21]. It was shown that cognate interaction with peptide loaded antigen presenting cells (APC) results in a polarized organization of naive CD8 T cells with several molecules moving towards the site of interaction and others to the opposite side [21]. As cytokinesis occurs while the CD8 T cell is still in contact with the APC, this polarized organization of the original naive CD8 T cells results in unequal distribution of molecules between the two daughter cells. The cell closest to the APC (the proximal daughter) was found to receive greater levels of molecules associated with effec-

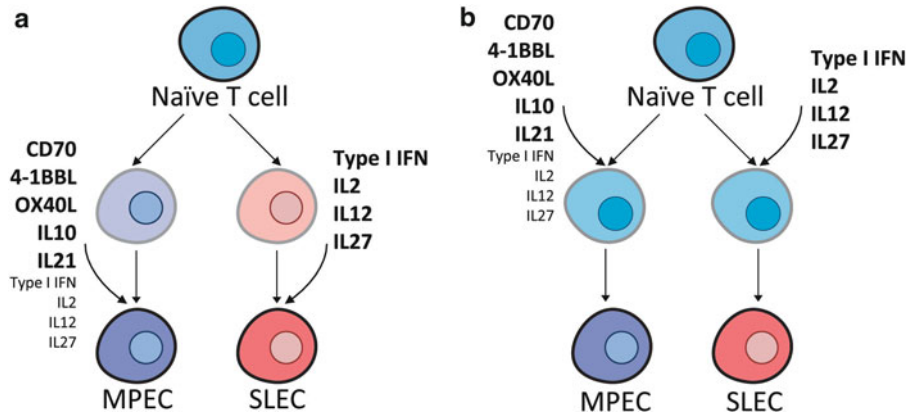

Fig. 12.2 Two models for the generation of both MPECs and SLECs from a single precursor. (**a**) $CD8^+$ T cell heterogeneity can be imparted during the first cell division after Ag encounter by asymmetric division of the naïve $CD8^+$ T cell. Asymmetrical segregation of cell fate determinants leads to the formation of phenotypically and functionally different daughter cells. Daughter cells do still require input from environmental signals to fully differentiate and/or to expand. (**b**) Equipotent daughter cells are formed, some of which will develop into SLEC and others into MPEC cells depending on signals they encounter. Commitment to SLEC or MPEC fates may occur after the first division as shown, or may happen after two or more divisions

tor fate, such as Granzyme B and T-bet [21, 22]. The distal daughter cell received greater levels of CD127, a marker associated with the MPEC fate. Upon transfer into recipient mice, distal daughter cells conferred greater protection to delayed infection than proximal daughter cells, consistent with the possibility that the distal cells possess greater potential to generate memory [21].

In the second model, naive CD8 T cells generate equipotent daughter cells that all develop effector function. Some of these daughters will subsequently develop into SLECs and others into MPECs, depending on signals encountered during the response. In support of this, it has been shown that memory T cells indeed derive from cells with effector cell characteristics. This conclusion was drawn from experiments in which transgenic mice expressed a tamoxifen controllable version of the Cre enzyme (ER-Cre) driven by the regulatory elements of the Granzyme B gene [23]. These mice additionally contained an expression construct encoding the yellow fluorescent protein (YFP) marker, which was inserted in the ubiquitously expressed *Rosa* locus, but was inactive until Cre mediated excision of a 5′ Stop sequence. This configuration allowed indelible marking of cells in which Cre had been expressed at some point as well as their progeny. It was found that addition of tamoxifen (to allow ER-Cre function) at early stages after primary infection led to the development of YFP^+ memory cells [23]. Therefore, ancestors of these memory cells had expressed the gene encoding the effector cell molecule Granzyme B at some stage. This experiment excludes an all or none model in which an early asymmetric division yields one daughter lineage with effector function and another daughter lineage without effector function, destined to develop into memory cells. The experiment does not, however, exclude a hybrid model, in which cells already committed to the MPEC fate still develop some effector function. Consistent with this possibility, $KLRG1^-CD127^+$ MPEC have indeed been shown to produce Granzyme B, especially early after infection [9]. At the same time, it should be noted that the evidence supporting a decisive role for asymmetric division in determining SLEC versus MPEC fate currently rests on a single type of experiment: adoptive transfer of cells, identified as distal or proximal daughter cells on the basis of small differences in expression of surface markers [21]. Although this approach was not wrong per se, independent confirmation of the importance of asymmetric division by additional experimental strategies still seems warranted.

12.5 Factors Promoting MPEC or SLEC Differentiation

Regardless of the point at which commitment to SLEC or MPEC is made, it is clear that external signals strongly affect the development of these cell types (Fig. 12.2). A general rule seems to be that signals associated with strong inflammation favor development of SLECs. Treatment of mice with antibiotics during infection with the intracellular bacterium *Listeria monocytogenes* results in diminished expansion of antigen-specific CD8 T cells [24]. This is due for the most part to an almost complete absence of SLEC generation. Numbers of MPECs generated under these conditions are close to those found in infections without antibiotics and these MPECs give rise to functional memory [24]. As treatment with antibiotics also reduced serum levels of inflammatory cytokines [24], a possible interpretation could be that such cytokines are required for SLEC formation. Likewise, SLEC differentiation was almost abrogated in CCR5 and CXCR3 double deficient mice infected with Influenza virus. These chemokine receptors are necessary for migration into the infected tissue and their absence presumably causes lower exposure of the activated CD8 T cells to inflammatory mediators [25]. Indeed, CD8 T cells lacking expression of functional receptors for IL-12 or type IFN generate fewer SLECs and proportionally more MPEC with the relative contribution of each cytokine varying depending on the infectious model studied [7, 26]. SLEC differentiation is not completely abrogated by the combined absence of both receptors, possibly due to a contribution of yet other inflammatory cytokines, such as IL-27 [7]. The finding that the numbers of $KLRG1^-CD127^+$ cells

generated are relatively unchanged when these inflammatory cytokines are absent, might seem to suggest that memory cell formation occurs by avoiding exposure to inflammatory cytokines. However, this idea is only partially correct. Whereas cells with the phenotypic characteristics of MPECs do indeed develop in normal numbers, combined deficiency for both IL-12 and type I IFN receptors results in poor memory development due to precipitous death of the MPECs at later stages in the response [26]. One possible explanation for the dual functions of inflammatory signals could be that strong or repeated exposure to inflammatory cytokines induces EECs to develop into SLECs, whereas weaker or shorter exposure induces critical aspects of a survival program in the developing MPECs.

Another factor promoting the generation of SLECs is IL-2. High concentrations of IL-2 promote acquisition of effector functions in tissue culture [27]. In vivo, expression of CD25, the α-chain of the high affinity IL-2 receptor, is uniformly high at early stages after infection, but at later stages $CD25^{high}$ and $CD25^{low}$ populations develop [28]. Of these, the $CD25^{high}$ population contains more proliferative cells, which eventually develop into SLECs. $CD25^{low}$ cells develop the phenotypic characteristics of MPECs and preferentially survive at later stages of the response. Expression of CD25 is elevated or maintained at high levels by IL-2 receptor and antigen receptor signaling as well as by costimulatory signals [28]. Help from CD4 T cells also promotes expression of CD25, possibly by providing a source of IL-2 [29]. CD25 expression is not an absolute requirement for the generation of SLECs. CD25-deficient CD8 T cells still generate such cells, but at reduced numbers [29]. On the other hand, the generation of MPECs by CD25-deficient CD8 T cells seems relatively normal, such that proportionally their frequency is increased [29]. Nonetheless, as is the case for inflammatory cytokines, IL-2 may not be dispensable for the generation of functional CD8 T cell memory: Although CD25-deficient CD8 T cells generate a memory population of normal size, such cells did not mount functional memory responses upon reinfection, although this was not found in all studies [29, 30]. It is not clear, however, whether IL-2 programs the development of functional memory cells or whether already differentiated memory cells require IL-2 receptor signals to mount an effective memory response [31].

A number of signals thus promotes development of SLECs. Does this mean that MPEC differentiation is a default fate or does induction of this fate require specific instructive signals as well? Although no conclusive evidence exists for this yet, several signals have shown to be necessary for CD8 memory T cell development. Two cytokines required for this are IL-10 and IL-21 [13]. IL-21 is produced predominantly by CD4 T cells and may be a component of CD4 T cell help to CD8 T cells [32]. IL-10 can be made by a variety of cells, including CD4 T cells and myeloid cells. Memory CD8 T cell responses are weak in mice lacking Stat3, a common effector of the IL-10 and IL-21 receptors [13]. However, this phenotype may not be caused by defective development of MPECs, but may rather be a consequence of an inability to maintain MPECs at later stages. MPEC cells gradually disappear in these mice and the same is true when both IL-10 and IL-21 are absent. As MPEC numbers dwindle, there is a gradual increase in a population of $KLRG1^{+}CD127^{-}$ cells under these conditions, suggesting that the loss of MPECs results from conversion into SLECs [13]. IL-10 and IL-21 were shown to induce expression of SOCS3, which in turn was shown to inhibit signaling via the IL-12 receptor [13]. It is possible, therefore, that IL-10 and IL-21 prevent differentiation of already formed MPECs into SLECs by insulating these cells from the influence of cytokines such as IL-12.

The TNF receptor family members CD27, 4-1BB, and OX40 have all been implicated in the generation of CD8 T cell memory [33]. Of these receptors, CD27 is found already on naive CD8 T cells, whereas the other two receptors are expressed sequentially after activation of CD8 T cells. Ligands for these receptors are expressed, among others, by activated dendritic cells and lymphocytes [33]. It was shown that CD27 signaling results in elevated expression of CD127 and thus augments the size of the MPEC pool

[34]. In addition, CD27 inhibits expression of critical components of the IL-2 and IL-12 receptors [34] and may thereby, like IL-10 and IL-21, insulate developing MPECs from signals promoting differentiation into SLEC-like cells. OX40 and 4-1BB contribute to the generation of CD8 T cell memory in part by promoting viability of the effector CD8 T cell pool [33, 35]. Interestingly, CD8 memory T cells generated in mice lacking expression of the ligands for OX40 or 4-1BB generated weaker recall responses even when transferred into wild-type mice [35]. This suggests that signaling through these receptors during primary responses induces a heritable program bestowing these cells with properties critical to memory cell expansion.

A final signal implicated in CD8 memory T cell differentiation is IL-15. This cytokine is produced both by dendritic cells and macrophages and binds to a receptor very similar to the one used by IL-2. Both receptors consist of the same β-chain (CD122) and γ-chain (CD132). Unique α-chains are used to create high affinity receptors. CD25 is the α-chain for the IL-2 receptor and CD215 is the α-chain for the IL-15 receptor [36]. This latter chain functions in an unusual manner, as it is not expressed on the same cell as the other two components of the receptor. Instead, it is found on the surface of macrophages and dendritic cells and functions by transpresenting IL-15. This transpresentation is essential for IL-15 function as evidenced by the identical phenotypes of mice lacking IL-15Rα or IL-15 itself [37, 38]. Despite the similarity between the receptors for IL-2 and IL-15, these cytokines induce quite different responses in CD8 T cells. As described above, IL-2 promotes terminal effector cell differentiation [27]. In contrast, addition of IL-15 to in vitro CD8 T cell cultures promotes the differentiation of cells expressing low levels of cytotoxic molecules, which behave as memory cells upon transfer into mice [39]. Although these findings suggest that IL-15 might serve as an inductive signal for MPEC differentiation, MPEC development is apparently normal in mice lacking IL-15 or its receptor, although the persistence of memory populations requires IL-15 for homeostatic self-renewal of already established memory cells [40, 41].

Both SLEC and MPEC generation are thus promoted by signals from the environment. Whether these signals instruct differentiation into either lineage, bolster programs already initiated or merely expand committed cells is not currently clear. It is not unlikely that all three occur. Thus, IL-2 presumably promotes rapid cycling of $CD25^{high}$ SLECs [28, 29]. However, IL-2 also contributes to the development of SLEC identity by inducing expression of Blimp and Perforin [9, 27]. Similarly, IL-12 induces expression of T-bet and thereby promotes acquisition of SLEC characteristics [5]. As for MPEC differentiation, limiting but not altogether avoiding exposure to inflammatory mediators seems key. The discrete differences in chemokine receptor expression between SLECs and MPECs likely play an important role in this, such that MPECs preferentially home to and remain in secondary lymphoid tissue, whereas SLECs go out to the inflamed tissues [9, 10]. Nonetheless, signals do seem to exist that positively affect MPEC differentiation as well. These may be especially important for the differentiation of memory cells with full proliferative and effector functions. Help from CD4 T cells is apparently essential for this and may depend on factors directly secreted by CD4 T cells, such as IL-2 and IL-21, or may indirectly depend on stimulating production of signals by APC, such as IL-12, IL-15, and ligands for CD27, OX40, and 4-1BB [30, 32, 35, 42].

12.6 Later Stages: Surviving Contraction

A critical property for MPECs is the ability to survive long term. After reaching peak numbers by about 7–12 days after infection, most of the CD8 effector T cells generated die, resulting in contraction of the antigen-specific repertoire. This contraction generally correlates with clearance of the pathogen, which would seem to suggest that the loss of effector cell viability is caused by disappearance of antigen. Somewhat surpris-

ingly, however, it has turned out that onset of the contraction phase can be separated from the clearance of the infection. For example, antibiotic treatment 2 days after infection with *L. monocytogenes* truncates the infection but has little impact on the onset or rate of $CD8^+$ T-cell contraction [3, 43]. Moreover, the onset and initial kinetics of antigen-specific $CD8^+$ T-cell contraction are similar after acute and chronic or persistent virus infection [43–45].

Inflammatory cues during the infection determine the degree of contraction. This seems causally related to the promotion of SLEC differentiation and expansion by inflammatory signals (as discussed above). Hardly any SLECs are generated in *L. monocytogenes* infected mice treated with antibiotics and correspondingly, contraction is minimal in these mice. Similarly, reduced contraction is observed in responses against Influenza virus when CD8 T cells lack CCR5 and CXCR3, necessary for migration into the inflamed tissues in the lungs. CD8 effector T cells lacking expression of these receptors do not develop into SLECs and generate an expanded memory pool [25].

Both SLECs and MPECs are kept alive by IL-15. In the absence of IL-15 receptor signaling, both cell types are lost precipitously after the peak of the response. On the other hand, repeated injection of recombinant IL-15 into mice prevents contraction of either cell type. Clearly, the ability of IL-15 to maintain effector cell viability is restricted under normal conditions as most cells die eventually. One possible explanation for this could be that the effector cells must interact with activated myeloid cells in the inflamed tissues, which transpresent IL-15 via IL15Ra on their surface [38, 46]. Numbers of such transpresenting cells may dwindle at later stages in the response and effector cell migration to these myeloid cells might diminish as inflammation subsides. As a consequence, effector cells would be decreasingly exposed to IL-15 resulting in loss of viability. Nonetheless, at later stages, the cells persisting as memory cells must still "see" IL-15, as this is required for their homeostatic turn over [40, 41]. Different APC may serve as a source of this IL-15 [38].

The superior ability of MPECs over SLECs to survive contraction depends to a large degree on their selective responsiveness to IL-7 due to expression of CD127. IL-7 is capable of maintaining the viability of memory cells for extended periods. Nonetheless, expression of CD127 is not sufficient for effector cell survival. Transgenic expression of CD127 could not rescue SLECs and not all $KLRG1^-CD127^+$ cells survive contraction [47]. It seems likely that responsiveness to IL-7 not only requires expression of CD127 but also the ability to home to locations where this cytokine is produced. Fibroblastic reticular cells in the T cell zones of secondary lymphoid organs are an important source of IL-7. These cells also secrete the CCL19 and CCL21 chemokines. Expression of the receptor for these chemokines, CCR7, is found on MPECs, but not on SLECs [10]. Correspondingly, MPECs are preferentially found in T cell zones in spleen, whereas SLECs localize to the red pulp [10]. An attractive model is therefore that only $CCR7^+$ MPECs survive contraction due to their ability to both home to the source of IL-7 and respond to it.

12.7 Mechanisms of Life and Death

Cell death during contraction of the antigen responsive repertoire is believed to occur through apoptosis. Two major apoptosis pathways exist: a cell-intrinsic pathway and a cell-extrinsic pathway (Fig. 12.3) [48, 49]. The *intrinsic pathway* integrates death signals from within a cell, such as DNA damage, oxidative stress, or starvation. It is characterized by the release of apoptogenic factors, such as Cytochrome c, from the mitochondrial intermembrane space into the cytosol. In the cytosol, Cytochrome c binds to a protein called Apaf-1, which ultimately results in activation of caspase 9 [49]. This caspase then activates downstream effector caspases, resulting in the demise of the cell. Permeabilization of the mitochondrial outer membrane is a critical step in the intrinsic apoptosis pathway, therefore. It is caused by the formation of membrane pores by pro-apoptotic Bcl-2 family members Bak and Bax. Anti-apoptotic Bcl-2 family members, such as

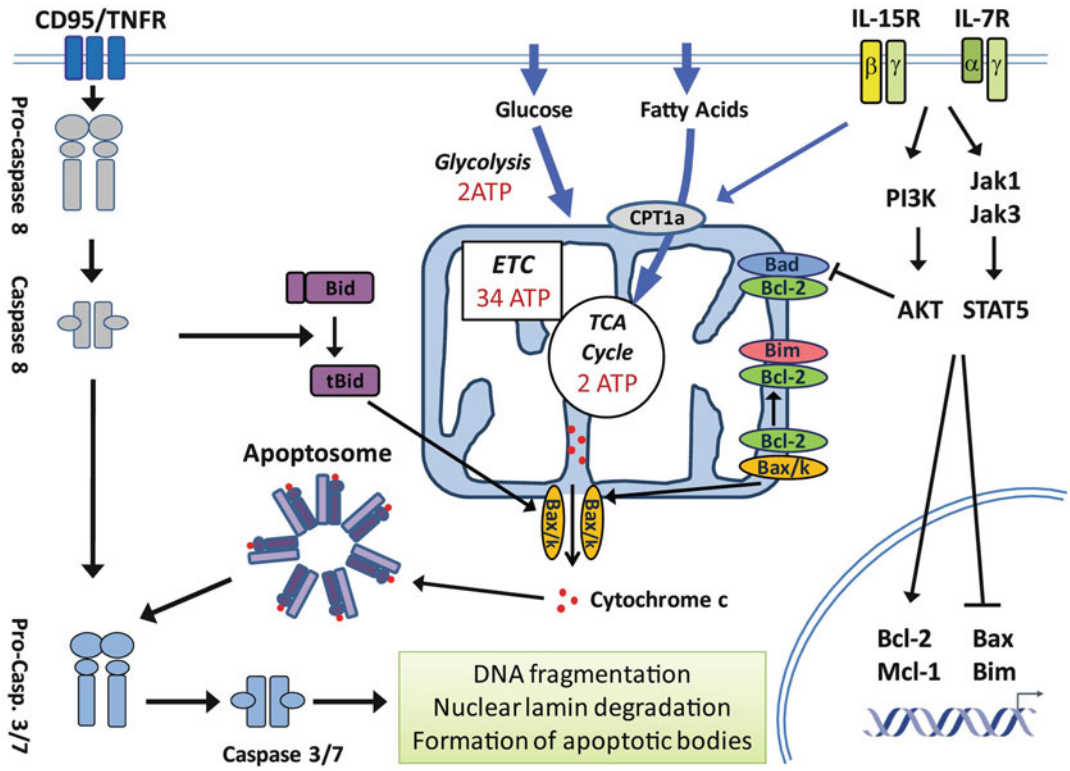

Fig. 12.3 Pathways controlling CD8 effector T cell survival and death. Shown is the extrinsic apoptosis pathway involving death receptor induced cleavage of Pro-caspase 8 to generate active caspase 8 followed by activation of effector caspases. Also shown is the intrinsic apoptosis pathway involving the generation of pores in the outer mitochondrial membrane by Bax/Bak and its inhibition by anti-apoptotic Bcl-2 family members. Finally, metabolic pathways are shown, depending on mitochondrial breakdown of fatty acids and cytosolic glycolysis. The Apoptosome consists of a multimer of Apaf-1, Cytochrome C, and Caspase 9. *Abbreviations*: *TCA* tricarboxylic acid, *ETC* electron transport chain. IL15Rβ = CD122, IL-7Rα = CD127

Bcl-2 and Bcl-XL, bind to Bax and Bak proteins and thereby prevent the assembly of the membrane pores [49]. An additional layer of control is formed by the pro-apoptotic BH3-only proteins Bim, Bid, Noxa, and Puma. These proteins bind to anti-apoptotic Bcl-2 family members and prevent them from inhibiting Bax and Bak multimerization [49].

The *extrinsic apoptosis pathway* is activated by engagement of death receptors such as Fas (CD95) and TNFR [48]. Ligand binding by these receptors results in activation of caspase 8, which in turn activates the effector caspases 3 and 7. These effector caspases cleave multiple substrates in the cell, leading to the death of the cell. Additionally, the extrinsic pathway can feed into the intrinsic pathway by caspase 8 mediated cleavage of Bid. After truncation, (t)Bid translocates to mitochondria where it acts in concert with pro-apoptotic Bcl-2 family members Bak and Bax to induce cell death [48].

A major regulator of cell death during contraction of the CD8 effector repertoire is Bim, a mediator in the intrinsic pathway. Deletion of Bim severely compromises the contraction of superantigen-activated T cells in vivo [50] and reduces contraction after herpes virus or LCMV infection [51, 52]. However, although the degree of contraction is reduced in BIM-deficient mice, some contraction does still take place, suggesting that additional pathways contribute to death of CD8 effector T cells [52–54]. Contraction is nor-

mal when CD8 T cells lack both CD95 (Fas) and TNF, two molecules required for the extrinsic apoptosis pathway [55, 56]. Nonetheless, the extrinsic apoptosis does contribute to contraction, as shown by the synergistic effect of Bim- and CD95-deficiency on contraction of antiviral immune responses [57]. CD95 may be especially important for contraction during chronic infections [58]. Likewise, the BH3-only protein Bid seems to be especially important for contraction during chronic infections. Mice lacking Bid display normal contraction of antigen-specific CD8 T cells after acute influenza virus infection, but not during chronic γ-herpes virus infections [51]. Combined loss of Bid and Bim synergistically enhanced the persistence of $CD8^+$ T cells during γ-herpesvirus infection [51]. Thus, both the extrinsic and the extrinsic apoptosis pathway contribute to induction of cell death during contraction of the antigen-specific repertoire of CD8 T cells.

How the survival cytokines IL-7 and IL-15 prevent induction of apoptosis is not fully clear. However, both cytokines induce expression of Bcl-2 and thus oppose the intrinsic apoptosis pathway (Fig. 12.3). Other survival signals, such as OX40L and 4-1BBL, upregulate expression of BCL-XL and downregulate expression of Bim [59]. IL-15 also prevents cell death through its effects on cellular metabolism (Fig. 12.3). During rapid proliferation, cells depend on cytoplasmic degradation of glucose to generate ATP, known as glycolysis. This process requires a constant influx of glucose, which is mediated by transmembrane glucose transporters. Resting cells or slowly proliferating cells, such as memory CD8 T cells, depend on oxidative phosphorylation in mitochondria to meet their energy demands. This process involves break down of glucose, amino acids, and fatty acids via the mitochondrial tricarboxylic acid cycle (TCA) and is much more efficient than glycolysis. CD8 effector T cells deficient in activating fatty acid metabolism display sharply accelerated contraction, whereas drugs that stimulate this process protect cells from contraction [60]. This demonstrates that the metabolic conversion from glycolysis to oxidative phosphorylation is critical for the survival of late stage effector cells, possibly because signals inducing expression of glucose transporters become limiting. IL-15 may have an important role in regulating this transition, by inducing expression of CPT1a, an enzyme responsible for the rate-limiting step in fatty acid metabolism. Indeed, ectopic expression of CPT1a resulted in greater persistence of antigen-specific CD8 T cells [61].

12.8 Concluding Remarks

The mechanisms involved in the generation of memory cells are starting to become clear. During the first 2 weeks after infection a number of decisions must be made. These include an apparently binary choice between a terminal effector cell fate with short lifespan or a long-lived self-renewing memory cell fate. Multiple signals influence this decision. For establishment of effective memory, cells must acquire the ability to survive the cellular massacre of repertoire contraction. In addition, the cells must develop as yet poorly defined traits that allow proliferation and development of full effector when called upon during reinfection with previously encountered pathogens. As the signals controlling these steps are identified, novel opportunities will likely arise to improve vaccination strategies, allowing expedited memory development when speed is required (such as for protection against bioterrorism) or even development of protective memory in vitro when patient health does not permit in vivo vaccination.

References

1. Obar JJ, Khanna KM, Lefrançois L. Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. Immunity. 2008 Jun;28(6):859–69.
2. D'Cruz LM, Rubinstein MP, Goldrath AW. Surviving the crash: transitioning from effector to memory CD8+ T cell. Seminars in immunology. 2009 Apr;21(2):92–8.
3. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nature reviews. Immunology. 2002 Apr;2(4):251–62.

4. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nature immunology. 2003 Dec;4(12):1191–8.
5. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity. 2007 Aug;27(2):281–95.
6. Obar JJ, Lefrançois L. Early signals during CD8 T cell priming regulate the generation of central memory cells. Journal of immunology . 2010 Jul 1;185(1):263–72.
7. Obar JJ, Jellison ER, Sheridan BS, Blair DA, Pham Q-M, Zickovich JM, et al. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. Journal of immunology . 2011 Nov 15;187(10):4967–78.
8. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. Nature immunology. 2011 Dec;12(12):1221–9.
9. Rutishauser RL, Martins G a, Kalachikov S, Chandele A, Parish I a, Meffre E, et al. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. Immunity. 2009 Aug 21;31(2):296–308.
10. Jung YW, Rutishauser RL, Joshi NS, Haberman AM, Kaech SM. Differential localization of effector and memory CD8 T cell subsets in lymphoid organs during acute viral infection. Journal of immunology . 2010 Nov 1;185(9):5315–25.
11. Kallies A, Xin A, Belz GT, Nutt SL. Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. Immunity. 2009 Aug 21;31(2):283–95.
12. Intlekofer AM, Takemoto N, Wherry EJ, Longworth S a, Northrup JT, Palanivel VR, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nature immunology. 2005 Dec;6(12): 1236–44.
13. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. Immunity. 2011 Nov 23;35(5):792–805.
14. Murre C. Helix-loop-helix proteins and lymphocyte development. Nature immunology. 2005 Nov;6(11):1079–86.
15. Ji Y, Pos Z, Rao M, Klebanoff C a, Yu Z, Sukumar M, et al. Repression of the DNA-binding inhibitor Id3 by Blimp-1 limits the formation of memory CD8+ T cells. Nature immunology. 2011 Dec;12(12):1230–7.
16. Zhou X, Yu S, Zhao D-M, Harty JT, Badovinac VP, Xue H-H. Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1. Immunity. 2010 Aug 27;33(2):229–40.
17. Rao RR, Li Q, Bupp MRG, Shrikant PA. Transcription Factor Foxo1 Represses T-bet-Mediated Effector Functions and Promotes Memory CD8(+) T Cell Differentiation. Immunity. 2012 Mar 14;36(3): 374–87.
18. D'Souza WN, Hedrick SM. Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. Journal of immunology . 2006 Jul 15;177(2):777–81.
19. Stemberger C, Huster KM, Koffler M, Anderl F, Schiemann M, Wagner H, et al. A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. Immunity. 2007 Dec;27(6):985–97.
20. Gerlach C, van Heijst JWJ, Swart E, Sie D, Armstrong N, Kerkhoven RM, et al. One naive T cell, multiple fates in CD8+ T cell differentiation. The Journal of experimental medicine. 2010 Jun 7;207(6):1235–46.
21. Chang JT, Palanivel VR, Kinjyo I, Schambach F, Intlekofer AM, Banerjee A, et al. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science. 2007 Mar 23;315(5819):1687–91.
22. Chang JT, Ciocca ML, Kinjyo I, Palanivel VR, McClurkin CE, Dejong CS, et al. Asymmetric proteasome segregation as a mechanism for unequal partitioning of the transcription factor T-bet during T lymphocyte division. Immunity. 2011 Apr 22;34(4): 492–504.
23. Bannard O, Kraman M, Fearon DT. Secondary replicative function of CD8+ T cells that had developed an effector phenotype. Science. 2009 Jan 23;323(5913):505–9.
24. Badovinac VP, Porter BB, Harty JT. CD8+ T cell contraction is controlled by early inflammation. Nature immunology. 2004 Aug;5(8):809–17.
25. Kohlmeier JE, Reiley WW, Perona-Wright G, Freeman ML, Yager EJ, Connor LM, et al. Inflammatory chemokine receptors regulate CD8(+) T cell contraction and memory generation following infection. The Journal of experimental medicine. 2011 Aug 1;208(8):1621–34.
26. Xiao Z, Casey KA, Jameson SC, Curtsinger JM, Mescher MF. Programming for CD8 T cell memory development requires IL-12 or type I IFN. Journal of immunology . 2009 Mar 1;182(5):2786–94.
27. Pipkin ME, Sacks J a, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. Immunity. 2010 Jan 29;32(1):79–90.
28. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith K a, Ahmed R. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. Immunity. 2010 Jan 29;32(1):91–103.
29. Obar JJ, Molloy MJ, Jellison ER, Stoklasek TA, Zhang W, Usherwood EJ, et al. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. Proceedings of the National

Academy of Sciences of the United States of America. 2010 Jan 5;107(1):193–8.
30. Williams M a, Tyznik AJ, Bevan MJ. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature. 2006 Jun 15;441(7095):890–3.
31. Feau S, Arens R, Togher S, Schoenberger SP. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. Nature immunology. 2011 Sep;12(9):908–13.
32. Novy P, Huang X, Leonard WJ, Yang Y. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection. Journal of immunology . 2011 Mar 1;186(5):2729–38.
33. Croft M. The role of TNF superfamily members in T-cell function and diseases. Nature reviews. Immunology. 2009 Apr;9(4):271–85.
34. Dong H, Franklin NA, Roberts DJ, Yagita H, Glennie MJ, Bullock TNJ. CD27 Stimulation Promotes the Frequency of IL-7 Receptor-Expressing Memory Precursors and Prevents IL-12-Mediated Loss of CD8+ T Cell Memory in the Absence of CD4+ T Cell Help. Journal of immunology. 2012 Apr 15;188(8):3829–38.
35. Hendriks J, Xiao Y, Rossen JWA, van der Sluijs KF, Sugamura K, Ishii N, et al. During viral infection of the respiratory tract, CD27, 4-1BB, and OX40 collectively determine formation of CD8+ memory T cells and their capacity for secondary expansion. Journal of immunology . 2005 Aug 1;175(3):1665–76.
36. Liao W, Lin J-X, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. Current opinion in immunology. 2011 Oct;23(5):598–604.
37. Mortier E, Woo T, Advincula R, Gozalo S, Ma A. IL-15Rα chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. The Journal of Experimental Medicine. 2008;205(5):1213–25.
38. Mortier E, Advincula R, Kim L, Chmura S, Barrera J, Reizis B, et al. Macrophage- and dendritic-cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8+ T cell subsets. Immunity. 2009 Nov 20;31(5):811–22.
39. Manjunath N, Shankar P, Wan J, Weninger W, Crowley MA, Hieshima K, et al. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. The Journal of clinical investigation. 2001 Sep;108(6):871–8.
40. Schluns KS, Williams K, Ma A, Zheng XX, Lefrançois L. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. Journal of immunology. 2002 May 15;168(10):4827–31.
41. Becker TC, Wherry EJ, Boone D, Murali-Krishna K, Antia R, Ma A, et al. Interleukin 15 Is Required for Proliferative Renewal of Virus-specific Memory CD8 T Cells. Journal of Experimental Medicine. 2002 Jun 17;195(12):1541–8.
42. Oh S, Perera LP, Terabe M, Ni L, Waldmann TA, Berzofsky JA. IL-15 as a mediator of CD4+ help for CD8+ T cell longevity and avoidance of TRAIL-mediated apoptosis. Proceedings of the National Academy of Sciences of the United States of America. 2008 May 1;105(13):5201–6.
43. Badovinac VP, Porter BB, Harty JT. Programmed contraction of CD8(+) T cells after infection. Nature Immunology. 2002;3(7):619–26.
44. Fuller MJ, Khanolkar A, Tebo AE, Zajac AJ. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. The Journal of Immunology. 2004;172(7):4204–14.
45. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(45):16004–9.
46. McGill J, Van Rooijen N, Legge KL. IL-15 trans-presentation by pulmonary dendritic cells promotes effector CD8 T cell survival during influenza virus infection. The Journal of experimental medicine. 2010 Mar 15;207(3):521–34.
47. Hand TW, Morre M, Kaech SM. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proceedings of the National Academy of Sciences of the United States of America. 2007 Jul 10;104(28):11730–5.
48. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. Nature reviews. Immunology. 2007 Jul;7(7):532–42.
49. Tait SWG, Green DR. Mitochondria and cell signalling. Journal of Cell Science. 2012 Mar 24;125(4):807–15.
50. Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, et al. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity. 2002;16(6):759–67.
51. Masson F, Kupresanin F, Mount A, Strasser A, Belz GT. Bid and Bim collaborate during induction of T cell death in persistent infection. The Journal of Immunology. 2011;186(7):4059–66.
52. Wojciechowski S, Jordan MB, Zhu Y, White J, Zajac AJ, Hildeman DA. Bim mediates apoptosis of CD127(lo) effector T cells and limits T cell memory. European Journal of Immunology. 2006;36(7):1694–706.
53. Grayson JM, Weant AE, Holbrook BC, Hildeman D. Role of Bim in Regulating CD8+ T-Cell Responses during Chronic Viral Infection. Journal of Virology. 2006;80(17):8627–38.
54. Wojciechowski S, Tripathi P, Bourdeau T, Acero L, Grimes HL, Katz JD, et al. Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. The Journal of Experimental Medicine. 2007; 204(7):1665–75.
55. Nguyen LT, McKall-Faienza K, Zakarian A, Speiser DE, Mak TW, Ohashi PS. TNF receptor 1 (TNFR1) and CD95 are not required for T cell deletion after virus infection but contribute to peptide-induced deletion under limited conditions. European Journal of Immunology. 2000;30(2):683–8.

56. Reich A, Körner H, Sedgwick JD, Pircher H. Immune down-regulation and peripheral deletion of CD8 T cells does not require TNF receptor-ligand interactions nor CD95 (Fas, APO-1). European Journal of Immunology. 2000;30(2):678–82.
57. Weant AE, Michalek RD, Khan IU, Holbrook BC, Willingham MC, Grayson JM. Apoptosis regulators Bim and Fas function concurrently to control autoimmunity and CD8+ T cell contraction. Immunity. 2008;28(2):218–30.
58. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. The EMBO journal. 1995 Dec 15;14(24):6136–47.
59. Sabbagh L, Pulle G, Liu Y, Tsitsikov EN, Watts TH. ERK-dependent Bim modulation downstream of the 4-1BB-TRAF1 signaling axis is a critical mediator of CD8 T cell survival in vivo. The Journal of Immunology. 2008;180(12):8093–101.
60. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang L-S, et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2009 Jul 2;460(7251):103–7.
61. van der Windt GJW, Everts B, Chang C-H, Curtis JD, Freitas TC, Amiel E, et al. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. Immunity. 2012 Jan 27;36(1):68–78.

Targeting the Skin for Microneedle Delivery of Influenza Vaccine

13

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Abstract

Influenza infection represents a major socioeconomic burden worldwide. Skin represents a new target that has gained much attention in recent years for delivery of influenza vaccine as an alternative to the conventional intramuscular route of immunization. In this review we describe different microneedle vaccination approaches used in vivo, including metal and dissolving microneedle patches that have demonstrated promising results. Additionally we analyze the immunological basis for microneedle skin immunization and targeting of the skin's dense population of antigen presenting cells, their role, characterization, and function. Additionally we analyze the importance of inflammatory signaling in the skin after microneedle delivery.

Keywords

Microneedles • Skin immunization • Influenza • Dendritic cells • Langerhans cells

13.1 Influenza Virus and Influenza Vaccination

13.1.1 Influenza Virus and Disease

Influenza virus represents one of the most common respiratory viral pathogens and is a major cause of morbidity and mortality worldwide [1, 2]. The virus is responsible for annual epidemics of influenza with seasonal outbreaks in the USA from October through April. The CDC estimates that more than 200,000 hospitalizations in the USA are attributed to influenza infection, annually [3, 4].

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In several cases, the magnitude of lung inflammation and respiratory distress can lead to serious complications and even death. It is estimated that more than 40,000 deaths in the USA alone are related to influenza infection or complications following the infection [5–7], while the number of deaths associated with influenza infection account up to 1.5 million worldwide [8–10]. The World Health Organization (WHO) estimates that each year 10–20% of the world's population is being infected by influenza virus [11]. Seasonal influenza infection can affect all age groups and genders [12]. The severity of influenza infection or complications associated with it are greater in certain high risk groups [13–15]. According to the CDC these groups include children younger than 5 years of age and particularly affected the ones younger than 2 years old [16–18], elderly individuals 65 years old and above [19–23], pregnant women [24, 25], and people with certain underlying medical conditions such as asthma [26, 27], chronic lung disease [28, 29], heart disease, diabetes [22, 30–32], immunocompromised individuals [33–35], and some others [36]. Additionally, people who live in nursing homes and long-term care facilities [30, 37, 38] as well as health care workers [39–42] are at high risk from influenza infection.

Influenza virus is a single-stranded negative sense RNA virus. There are three different serotypes of influenza viruses that can cause disease in humans, A, B, and C, distinguished by their antigenic differences in their nucleocapsid (NP) and matrix (M) protein. Influenza types A and B have eight separate segments encoding at least ten different proteins, they can spread easily among human population and are responsible for seasonal epidemics every year [43–58]. Influenza type C is very rare, it has seven separate different segments encoding nine proteins, and although it may cause mild respiratory disease it is not responsible for epidemics [59]. Influenza type A viruses have common internal antigens but can be divided into several different subtypes based on the antigenic properties of the two major proteins in their surface, the hemagglutinin (H) and neuraminidase (N) proteins. These two proteins also represent the two major surface antigens of influenza viruses. So far 17 different hemagglutinin and 10 neuraminidase proteins have been identified circulating in nature [60–63]. The two influenza A subtypes that cause seasonal influenza infections in humans are the H1N1 and H3N2 influenza viruses [63, 64]. Influenza B viruses have a limited host range (humans and seals) and are not divided into subtypes like influenza A subtypes but are classified based on their strain differences [65].

Influenza viruses exhibit a great ability to introduce minor or major changes in their two major surface proteins, the hemagglutinin and neuraminidase. Minor changes in the influenza virus genome are more common and are induced by the constant selective pressure caused by the host immune responses. These minor changes (antigenic drift) are characterized by point mutations in the HA and NA genes. Due to these changes, the host's preexisting immunity may only partially recognize the HA and NA proteins of a new strain resulting in decreased protection and subsequently higher infection rate [66–68]. Influenza A viruses circulate among humans as well as different animals, including ducks, chickens, pigs, horses, etc. This constant circulation of influenza viruses among different species results occasionally in genome recombination inside a reservoir host between different strains and in the appearance of an antigenically new influenza virus (antigenic shift) [69–72] that the human immune system has never encountered before and hence has no or little protection against it. Due to lack of preexisting immunity, the new virus spreads quickly causing pandemics and affecting millions worldwide. The five major pandemics of the twentieth century and the first pandemic of the twenty-first century, the swine origin A/California/07/09 strain, resulted from such antigenic shifts. Influenza represents a significant socioeconomic burden, leading to increased health care cost, high levels of work absenteeism, disruption in work, and productivity loss [73].

In the USA, annual influenza epidemics result in an average of 3.1 million hospitalization days and 31.4 million outpatient visits, while the total direct and indirect economic burden of annual influenza epidemics amounts to 87.1 billion

dollars [74]. The WHO places the number of infected individuals at high risk from influenza infection as more than 1.2 billion worldwide including 385 million elderly, 140 million infants, 700 million adults and children with underlying health conditions including pregnant women, and approximately 24 million health care workers [11].

13.1.2 Influenza Vaccination

Vaccination represents the best method of prevention and protection from influenza infection and its related complications, improving herd immunity, and reducing morbidity and mortality rates worldwide [2, 75–79]. Currently there are two different types of commercially available influenza vaccines on the market: (a) the trivalent inactivated influenza vaccine (TIV) administered intramuscularly with syringes, approved for use in infants older than 6 months and (b) the live attenuated influenza vaccine (LIV) given as nasal spray, approved for use only in healthy individuals from 2 to 49 years of age who are not pregnant [80]. The trivalent inactivated vaccine is the most widely utilized worldwide. There are three different types of inactivated influenza vaccine: whole virus vaccine, virus vaccine split after detergent treatment, and subunit vaccine consisting of purified HA and NA proteins. In the USA the current influenza vaccines are the split and subunit ones; both contain 15 μg of H1 and H3 hemagglutinins of the circulating seasonal influenza A subtypes and of type B influenza virus. These vaccine formulations were studied in the 1970s and proven to be safe, with reduced reactogenicity when compared to the whole inactivated influenza vaccine used until then [81–86]. Despite the excellent safety profile provided by the split and subunit influenza vaccines, the immune response following vaccination has been proven to be short-lived and not fully protective, especially in high risk groups such as the elderly, children, and immunocompromised individuals [14, 33, 87–90]. Thus studies have shown that the antibody titers to influenza wane within 7–8 months post-vaccination and that children unprimed to influenza require two vaccine doses to elicit protective immune responses. According to FDA guidelines an influenza vaccine is considered protective when the vaccinee develops anti-influenza hemagglutination inhibition titers above 40 [91]. In addition, the efficacy of these vaccines depends on how well matched the influenza strains in the vaccine are with the ones in circulation. According to the CDC, in randomized controlled trials conducted among healthy adults less than 65 years of age, the efficacy of inactivated influenza vaccines has been estimated to be between 50 and 70% during seasons in which the vaccine components were well matched to the circulating influenza viruses. Under conditions of suboptimal match, the efficacy of the inactivated vaccines fluctuates between 48% among high risk groups and 60% among healthy adults. In cases where the influenza vaccine and the circulating influenza viruses are poorly matched, the effectiveness of these vaccines is further reduced [92, 93]. All these facts strongly suggest the need for better vaccines or vaccine delivery approaches to improve protection, duration and breadth of immunity, as well as vaccine acceptance for worldwide coverage.

13.2 Skin as an Immunological Organ

13.2.1 Skin Structure, Functions, and Resident Cell Populations

A new vaccine delivery target that has gained more attention in recent years is the skin [94]. The skin is one of the most complex structures and the largest immunological organ of the human body [95]. Its main function is protective, serving as a physical barrier from numerous pathogens but also from injuries and UV radiation. It is also part of the body's homeostatic mechanism and an important sensory organ. It is composed of two primary layers, the epidermis and dermis [96]. The epidermis represents the most outer layer of the skin. It is 50–100 μm thick and it is divided into several sublayers; (1) *stratum corneum* which is the outer layer of epidermis, (2) *stratum germinativum*, (3) *stratum lucidum* that appears

in certain parts of the body, (4) *stratum granulosum* which contains squamous cells and filaggrin and prevents loss of nutrients, (5) *stratum spinosum* that further enhances structural support and prevents skin abrasion, and (6) *stratum basale* that contains epithelial cells which undergo rapid mitosis to replenish dead cells from upper layers. These layers are mostly consisting of keratinocytes, melanocytes, and Langerhans cells (LCs) [96]. Langerhans cells are present in all layers of the epidermis and are in close proximity to the *stratum corneum* [97]. These are immature APCs produced from bone marrow precursors that reach and populate the skin through the peripheral circulation [98]. The dermis lies beneath the epidermis and contains hair follicles, sweat and endocrine glands, lymphatic vessels, blood vessels, and several nerve endings. It is largely populated by dermal dendritic cells (DDCs) that are distinct from the epidermal Langerhans cells populations based on their surface markers. LCs express differential levels of CD11b, $CD205^{int/high}$, and more specifically CD207 (Langerin) while DCs express $CD11b^{high}$, and $CD205^{low/int}$ and CD207 negative [99, 100]. Additionally, these two populations are characterized by differences in chemokine receptor expression especially during the maturation and migration of LCs from tissues to draining lymph nodes [101–104]. The presence of two types of antigen presenting cells, LCs and DDCs, classify the skin as an immunological organ [105]. Additionally, the expression of Toll-like receptors [106, 107] (TLRs) on LCs, DDCs, and keratinocytes make it an ideal target for vaccine delivery [105]. These two types of APCs, in combination with other immunologically active cells residing in the skin including LC-like DCs, monocytes, and macrophages [108], recognize and take up the antigen upon delivery in the skin, and migrate while undergoing maturation to the proximal lymph nodes where they prime naïve T and B cells thus initiating and shaping the adaptive immune responses [97]. Both LCs and DDCs are involved in the process of T cell activation [97]. Studies have demonstrated that in the absence of a stimulus, epidermal LCs and dermal DCs express low levels of major histocompatibility molecules MHC class I and II and co-stimulatory or adhesion molecules [109]. For LCs it is possible that passive transfer and diffusion is involved in the process of antigen uptake or a more active mechanism has been proposed where LCs reach out and extend their arms in order to capture the antigen [110, 111]. Dermal DCs have also been shown to be actively involved in the antigen presenting process as well and to be immunologically highly active [105, 112]. Two subpopulations have been identified: dermal $langerin^{+}$ dendritic cells and dermal $langerin^{neg}$ dendritic cells [113]. Dermal DCs occur in higher numbers than epidermal LCs, they express high amounts of MHC class II molecules on their surface, and they are as potent in antigen presentation in naive T cells playing an important role in the regulation of skin immune response [105, 111, 113].

13.2.2 The Role of Inflammation During Skin Vaccination

The inflammatory environment and inflammatory response induced upon antigen entry into the skin seems to be very important and play a crucial role in the immune response. Several studies have demonstrated that LCs and DDCs can produce large amounts of IL-12, TNF-α, and type I interferons (IFNs) as well as attract and activate other innate lymphocytes such as NK cells, NKT cells, and γδ T cells that secrete large amounts of IFN-γ. A recent study by Martin et al. [114] demonstrated the importance of local responses induced after skin vaccine delivery. In this study, Martin et al. observed the upregulation of several important chemokines and cytokines after microneedle delivery and particularly interleukin 1β (IL-1β), macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein 2 (MIP-2), tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein 1 (MCP-1). These cytokines have been shown to contribute to the regulation and migration of LCs and DDCs in the draining lymph nodes. Furthermore other cytokines important to the proliferation, activation, and recruitment of neutrophils and monocytes such as

granulocyte colony-stimulation factor (G-CSF), interferon gamma induced protein 10 (IP-10), and cytokine-induced neutrophil chemoattractant (CXCL-1) were also increased after skin vaccination for influenza. These data demonstrate the numerous complex mechanisms activated upon delivery of the antigen into the skin that may be important for the improved immunological responses of the vaccine recipient. All these immunological advantages and mechanisms seem to favor skin delivery of influenza antigen compared to the conventional intramuscular immunization. Current inactivated influenza vaccines are administered intramuscularly in the deltoid muscle area. Several studies have demonstrated that myocytes contain low numbers of APCs and lack MHC class II expressing cells leading to poor antigen-dependent T cell activation and reduced humoral and cellular immune responses [115, 116]. All these limitations can potentially be overcome by skin immunization because of the many professional APCs populating the epidermis and the dermis, and thus achieving an improved quantitative and qualitative immune response when compared to intramuscular immunization.

13.3 Microneedle Vaccination

One of the most promising novel vaccine delivery platforms that takes advantage of the skin's immunological potential is microneedle technology [94, 117–120]. This technology relies on rapid delivery of the antigen into the skin epidermis and/or the dermis layers with high precision, and without causing any discomfort or irritation. The materials of choice used for fabrication are metals or polymers, both FDA approved and already applied in several other medical devices [116, 121–123]. Metal microneedle arrays coated with whole inactivated influenza virus (WIV) or monovalent subunit vaccine and polymer (PVP) microneedles encapsulating WIV have been successfully tested in vivo and have generated promising results for vaccine delivery methods of influenza antigen through the skin [116, 121–123].

13.3.1 Solid Metal Microneedle Arrays

Metal microneedle arrays are fabricated from stainless steel sheets by laser cutting. These are arrays of hundreds of microneedles projecting a few hundred microns from the base of the patch. To deburr and clean the microneedle edges and to make the tips sharp, microneedles are electropolished in an appropriate solution. Each needle is approximately up to 700 μm long. The microneedles are coated using a dip-coating process with different formulated coating solutions that ensure stability of the vaccine. The coating is performed using an appropriate apparatus and monitored by a video camera attached to a microscope. These metal microneedle arrays coated with the antigen, when applied onto the skin, pierce microscopic holes in the skin's epidermis with a thickness of 10–20 μm for antigen delivery [122, 124–126]. Several studies have demonstrated that by piercing the skin, transdermal permeability increases by as much as four orders of magnitude.

We have previously demonstrated that delivery of whole inactivated influenza vaccine using metal microneedles coated with the antigen can improve the duration of protective immune responses and lead to serological memory [116, 122]. In our latest studies using metal microneedle arrays we demonstrated successful delivery of influenza subunit vaccine in the mouse model in vivo [121], and we observed improved immune responses when compared to the conventional intramuscular administration of the vaccine. Microneedle immunized animals demonstrated enhanced humoral immune responses compared to intramuscularly immunized mice as shown by anti-influenza IgG titers, hemagglutination inhibition titers, and neutralizing antibody titers 9 months after a single dose of vaccine delivery [121] suggesting long-lived immune responses. Their functional antibody titers (HAI and NT) were maintained at levels that are indicative of protection (>40) even at 9 months post-immunization. These findings correlated well with the numbers of bone marrow influenza specific IgG secreting cells which were significantly higher in the microneedle immunized group. Furthermore in the same group the IgG1 and IgG2a isotype

profile showed a more balanced response when compared to the isotype profile induced after intramuscular vaccination, which predominantly induced IgG1 responses. The IgG2a isotype profile is indicative of cellular Th1 immune responses. A more balanced IgG1/IgG2a ratio observed after microneedle immunization could indicate the induction of cellular immune responses after vaccination [121]. Overall these data strongly suggest that delivery of subunit influenza vaccine through the skin can lead to improved humoral immune responses.

It is well established that split and subunit influenza vaccines are poor inducers of cellular immune responses [127]. Investigation of IFN-γ cells in the spleen of microneedle immunized animals revealed higher frequency of these cells indicating improved cellular immune responses [121]. Activation of both the humoral and cellular immune system can potentially provide improved protection when compared to the intramuscular route of vaccination. Indeed, studies have demonstrated a much more rapid clearance of the virus from the lungs of mice infected with 10×LD50 of homologous mouse adapted influenza virus after skin vaccine delivery as well as improved longevity of the immune response and improved protection [116, 121].

13.3.2 Dissolving Microneedle Patches

In contrast to coated metal microneedle arrays where the antigen is being coated on the surface of the needles, the polymer microneedles encapsulate the antigen [123, 128–130]. During delivery into the skin, the whole microneedle array (shaft and tip) dissolves delivering the vaccine cargo into the skin rapidly, eliminating biohazard sharps. This type of needle requires optimal geometry in order to achieve structural rigidity and stability during insertion into the skin [131]. Sullivan et al. designed and fabricated dissolving microneedle patches [123, 131]. The polymers used for microneedle manufacturing were FDA approved and used in several other medical applications. A slurry of vinylpyrollidone was mixed with lyophilized WIV rehydrated to the desired concentration and the mixture was polymerized at room temperature. This process was found to preserve vaccine antigenicity and prolonged shelf life while the microneedles were mechanically strong to ensure skin insertion, rapid dissolution of the needle into the skin, and successful vaccine delivery. Sullivan et al. showed successful dissolution of the microneedles up to 90% within the first 5 min of application into guinea pig skin [123]. This approach has several advantages, delivery of the vaccine to an easily accessible target such as the skin, elimination of biohazard sharps improving public safety and potential for self-administration [128], rendering influenza vaccination more attractive to the population thus ensuring better coverage, stability [124, 132], and rapid distribution of the vaccine. We demonstrated that dissolving microneedle patches induced robust protection in the mouse model after a single immunization with a low antigen dose, at least as good as the one observed after the systemic immunization. Skin delivery of influenza vaccine was followed by higher number of IFN-γ secreting cells in the spleen of microneedle immunized mice when compared to conventional intramuscular vaccine delivery and faster lung virus clearance after infection [123].

Overall, these studies demonstrate that a new platform technology for rapid and easy administration of influenza vaccine through the skin using metal microneedle arrays coated with the antigen or dissolving microneedle patches encapsulating influenza vaccine can be used for successful delivery of the antigen and improved immune responses and protection in vivo [121, 123].

13.3.3 Other Types of Skin Delivery Systems

There are other several types of devices in development from different groups that can be used for delivery of different antigens. One of these designs involves hollow microneedles [120, 131, 133–136]. In this case after delivery and insertion of the microneedles into the target organ the drug/antigen is delivered by a continuous flow into the skin after which the hollow microneedle

patch is being removed. Recent studies have demonstrated that a skin penetration depth of 750 μm to 1.5 mm is ideal for intradermal delivery of drugs and antigens including insulin, anthrax vaccine, and even influenza vaccine [120, 121, 131, 134, 137–143]. Another microneedle design that has been successfully tested in vivo is the Nanopatch [144, 145]. This is an array of densely packed projections that are dry-coated with influenza vaccine formulation and applied to the skin for 2 min. In this case delivery of influenza antigen through the skin induced improved immune responses when compared to the conventional intramuscular route of delivery with the additional advantage of a dose sparing effect [145]. Currently there is one FDA approved influenza vaccine in the market for intradermal delivery, Fluzone manufactured by Sanofi Pasteur. Intradermal delivery relies on the same principles of targeting similar populations of antigen presenting cells in the skin that microneedle delivery is based on. Early results from clinical trials demonstrate that intradermal delivery of the Fluzone vaccine through the skin induced similar seroconvertion rates as that induced after intramuscular delivery but with a dose sparing effect; from 15 μg HA per strain (45 μg HA total) used for the conventional intramuscular injection the dose was reduced to 9 μg HA (27 μg HA total) [146]. These results confirm the hypothesis that targeting skin APCs improves immune responses and support the promise of skin vaccination for various drugs and vaccines.

13.4 Conclusions

The complex structure of the skin and the quantity and quality of immunologically active cells that it contains [96, 105] establishes this organ as an ideal target for vaccine delivery. After several years of investigation, significant advances have been made indicating the importance of innate cell populations residing in the skin and the mechanisms behind antigen uptake. Numerous microneedle devices are in development exploiting the unique features of skin. The selection of the best design relies mostly on the type of drug to be delivered and on the type of antigen presenting cells that must be targeted. In the case of influenza vaccination very simple designs have been successfully tested in vivo and show promising results that are in the process to be advanced in clinical trials. Several important advantages make this method ideal for large-scale immunization programs. The simplicity of the method makes it ideal for self-administration. Since the skin is an easily accessed organ, and the method can eliminate biohazardous sharps, it can be completed without the need for highly trained personnel. Additionally preliminary data in humans demonstrate dose sparing further reducing the cost of this vaccination route [146]. Taking under consideration the immunological advantages achieved after microneedle delivery, the data suggest that this method could be an alternative to the conventional intramuscular route of immunization. The logistical advantages such as the ease and the simplicity of administration, the high safety profile, the better acceptance by the public [119, 120, 131, 147], and the immunological advantages [121] make this approach an important future direction in influenza vaccination.

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References

1. Fiore, A.E., et al. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2008. *MMWR Recomm Rep* **57**, 1–60 (2008).
2. Monto, A.S. Seasonal influenza and vaccination coverage. *Vaccine* **28 Suppl 4**, D33–44 (2010).
3. Osterholm, M.T. Preparing for the next pandemic. *N Engl J Med* **352**, 1839–1842 (2005).
4. Thompson, W.W., et al. Influenza-associated hospitalizations in the United States. *JAMA* **292**, 1333–1340 (2004).
5. Beigel, J.H. Influenza. *Crit Care Med* **36**, 2660–2666 (2008).

6. Dushoff, J., Plotkin, J.B., Viboud, C., Earn, D.J. & Simonsen, L. Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. *Am J Epidemiol* **163**, 181–187 (2006).
7. Heron, M. Deaths: leading causes for 2007. *Natl Vital Stat Rep* **59**, 1–95 (2011).
8. Simonsen, L. The global impact of influenza on morbidity and mortality. *Vaccine* **17 Suppl 1**, S3–10 (1999).
9. Palese, P. Influenza: old and new threats. *Nat Med* **10**, S82–87 (2004).
10. Cox, N.J. & Subbarao, K. Influenza. *Lancet* **354**, 1277–1282 (1999).
11. WHO. Immunization, Vaccines and Biologicals-Influenza. (2008).
12. Zhou, H., et al. Hospitalizations associated with influenza and respiratory syncytial virus in the United States, 1993–2008. *Clin Infect Dis* **54**, 1427–1436 (2012).
13. Longini, I.M., Jr. & Halloran, M.E. Strategy for distribution of influenza vaccine to high-risk groups and children. *Am J Epidemiol* **161**, 303–306 (2005).
14. Ompad, D.C., Galea, S. & Vlahov, D. Distribution of influenza vaccine to high-risk groups. *Epidemiol Rev* **28**, 54–70 (2006).
15. Loerbroks, A., Stock, C., Bosch, J.A., Litaker, D.G. & Apfelbacher, C.J. Influenza vaccination coverage among high-risk groups in 11 European countries. *Eur J Public Health* (2011).
16. O'Brien, M.A., et al. Incidence of outpatient visits and hospitalizations related to influenza in infants and young children. *Pediatrics* **113**, 585–593 (2004).
17. Izurieta, H.S., et al. Influenza and the rates of hospitalization for respiratory disease among infants and young children. *N Engl J Med* **342**, 232–239 (2000).
18. Nolan, T., et al. Immunogenicity of a monovalent 2009 influenza A(H1N1) vaccine in infants and children: a randomized trial. *JAMA* **303**, 37–46 (2010).
19. Avelino-Silva, V.I., et al. Campaign, counseling and compliance with influenza vaccine among older persons. *Clinics (Sao Paulo)* **66**, 2031–2035 (2011).
20. Miraglia, J.L., et al. Immunogenicity and reactogenicity of 2009 influenza A (H1N1) inactivated monovalent non-adjuvanted vaccine in elderly and immunocompromised patients. *PloS one* **6**, e27214 (2011).
21. Mullooly, J.P., et al. Influenza vaccination programs for elderly persons: cost-effectiveness in a health maintenance organization. *Ann Intern Med* **121**, 947–952 (1994).
22. Nichol, K.L., et al. Influenza vaccination and reduction in hospitalizations for cardiac disease and stroke among the elderly. *N Engl J Med* **348**, 1322–1332 (2003).
23. Reber, A.J., et al. Immunosenescence and Challenges of Vaccination against Influenza in the Aging Population. *Aging Dis* **3**, 68–90 (2012).
24. Myers, E.R., Misurski, D.A. & Swamy, G.K. Influence of timing of seasonal influenza vaccination on effectiveness and cost-effectiveness in pregnancy. *Am J Obstet Gynecol* **204**, S128–140 (2011).
25. Blanchard-Rohner, G. & Siegrist, C.A. Vaccination during pregnancy to protect infants against influenza: why and why not? *Vaccine* **29**, 7542–7550 (2011).
26. Ford, E.S., Mannino, D.M. & Williams, S.G. Asthma and influenza vaccination: findings from the 1999–2001 National Health Interview Surveys. *Chest* **124**, 783–789 (2003).
27. Ford, E.S., Williams, S.G., Mannino, D.M. & Redd, S.C. Influenza vaccination coverage among adults with asthma: findings from the 2000 Behavioral Risk Factor Surveillance System. *Am J Med* **116**, 555–558 (2004).
28. Plans-Rubio, P. Prevention and control of influenza in persons with chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* **2**, 41–53 (2007).
29. Hsu, A.C., See, H.V. & Wark, P.A. Innate immunity to influenza in chronic airways diseases. *Respirology* (2012).
30. Iorio, A.M., et al. Influenza vaccination in patients on long-term anticoagulant therapy. *Vaccine* **24**, 6624–6628 (2006).
31. Shaw, S.M., Williams, S.G., Yonan, N. & Fildes, J.E. Decreased immune responses to influenza vaccination in patients with heart failure. *J Card Fail* **15**, 549; author reply 549–551 (2009).
32. Ott, S.R., et al. [The impact of viruses in lower respiratory tract infections of the adult. Part II: acute bronchitis, acute exacerbated COPD, pneumonia, and influenza]. *Pneumologie* **64**, 18–27 (2010).
33. Hakim, H., et al. Immunogenicity and safety of inactivated monovalent 2009 H1N1 influenza A vaccine in immunocompromised children and young adults. *Vaccine* **30**, 879–885 (2012).
34. Bickel, M., et al. Low rate of seroconversion after vaccination with a split virion, adjuvanted pandemic H1N1 influenza vaccine in HIV-1-infected patients. *AIDS* **24**, F31–35 (2010).
35. Cooper, C., et al. Immunogenicity is not improved by increased antigen dose or booster dosing of seasonal influenza vaccine in a randomized trial of HIV infected adults. *PloS one* **6**, e17758 (2011).
36. Jain, S. & Chaves, S.S. Obesity and influenza. *Clin Infect Dis* **53**, 422–424 (2011).
37. Camilloni, B., et al. An influenza B outbreak during the 2007/2008 winter among appropriately immunized elderly people living in a nursing home. *Vaccine* **28**, 7536–7541 (2010).
38. Potter, J.M., O'Donnel, B., Carman, W.F., Roberts, M.A. & Stott, D.J. Serological response to influenza vaccination and nutritional and functional status of patients in geriatric medical long-term care. *Age Ageing* **28**, 141–145 (1999).
39. Clarke, C.E. & McComas, K. Seeking and processing influenza vaccine information: a study of health care workers at a large urban hospital. *Health Commun* **27**, 244–256 (2012).

40. Potter, J., et al. Influenza vaccination of health care workers in long-term-care hospitals reduces the mortality of elderly patients. *J Infect Dis* **175**, 1–6 (1997).
41. Carman, W.F., et al. Effects of influenza vaccination of health-care workers on mortality of elderly people in long-term care: a randomised controlled trial. *Lancet* **355**, 93–97 (2000).
42. Music, T. Protecting patients, protecting healthcare workers: a review of the role of influenza vaccination. *Int Nurs Rev* **59**, 161–167 (2012).
43. Medina, R.A. & Garcia-Sastre, A. Influenza A viruses: new research developments. *Nat Rev Microbiol* **9**, 590–603 (2011).
44. Guan, R., et al. Structural basis for the sequence-specific recognition of human ISG15 by the NS1 protein of influenza B virus. *Proc Natl Acad Sci USA* **108**, 13468–13473 (2011).
45. Sridharan, H., Zhao, C. & Krug, R.M. Species specificity of the NS1 protein of influenza B virus: NS1 binds only human and non-human primate ubiquitin-like ISG15 proteins. *J Biol Chem* **285**, 7852–7856 (2010).
46. Higgins, R.R., et al. Recovery of Influenza B Virus with the H273Y Point Mutation in the neuraminidase Active site from a Human Patient. *J Clin Microbiol* (2012).
47. Ak, O., et al. Influenza B-associated encephalopathy in two adults. *J Infect Chemother* (2012).
48. Rath, B., et al. Virus Load Kinetics and Resistance Development during Oseltamivir Treatment in Infants and Children Infected with Influenza A (H1N1) 2009 and Influenza B Viruses. *Pediatr Infect Dis J* (2012).
49. Steininger, C., et al. Acute encephalopathy associated with influenza A virus infection. *Clin Infect Dis* **36**, 567–574 (2003).
50. Baccam, P., Beauchemin, C., Macken, C.A., Hayden, F.G. & Perelson, A.S. Kinetics of influenza A virus infection in humans. *J Virol* **80**, 7590–7599 (2006).
51. Canini, L. & Carrat, F. Population modeling of influenza A/H1N1 virus kinetics and symptom dynamics. *J Virol* **85**, 2764–2770 (2011).
52. Russell, C.A. The global circulation of seasonal influenza A (H3N2) viruses. *Science* **320**, 340–346 (2008).
53. Russell, R.J., Stevens, D.J., Haire, L.F., Gamblin, S.J. & Skehel, J.J. Avian and human receptor binding by hemagglutinins of influenza A viruses. *Glycoconj. J.* **23**, 85–92 (2006).
54. Skountzou, I. Immunity to pre-1950 H1N1 influenza viruses confers cross-protection against the pandemic swine-origin 2009 A (H1N1) influenza virus. *J. Immunol.* **185**, 1642–1649 (2010).
55. Xu, R. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science* **328**, 357–360 (2010).
56. Perez, D.R. Fitness of pandemic H1N1 and seasonal influenza A viruses during co-infection: evidence of competitive advantage of pandemic H1N1 influenza versus seasonal influenza. *PLoS Curr.* **1**, RRN1011 (2009).
57. Chen, R. & Holmes, E.C. The evolutionary dynamics of human influenza B virus. *J Mol Evol* **66**, 655–663 (2008).
58. Hampson, A.W. & Mackenzie, J.S. The influenza viruses. *Med J Aust* **185**, S39–43 (2006).
59. Racaniello, V.R. & Palese, P. Isolation of influenza C virus recombinants. *J Virol* **32**, 1006–1014 (1979).
60. Fouchier, R.A. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**, 2814–2822 (2005).
61. Tong, S., et al. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci USA* **109**, 4269–4274 (2012).
62. Krauss, S., Walker, D. & Webster, R.G. Influenza virus isolation. *Methods Mol Biol* **865**, 11–24 (2012).
63. CDC. Influenza Type A Viruses and Subtypes. (2011).
64. Khiabanian, H., Farrell, G.M., St George, K. & Rabadan, R. Differences in patient age distribution between influenza A subtypes. *PloS one* **4**, e6832 (2009).
65. Pica, N., Chou, Y.Y., Bouvier, N.M. & Palese, P. Transmission of influenza B viruses in the guinea pig. *J Virol* **86**, 4279–4287 (2012).
66. Sandbulte, M.R., et al. Discordant antigenic drift of neuraminidase and hemagglutinin in H1N1 and H3N2 influenza viruses. *Proc Natl Acad Sci USA* **108**, 20748–20753 (2011).
67. Stray, S.J. & Pittman, L.B. Subtype- and antigenic site-specific differences in biophysical influences on evolution of influenza virus hemagglutinin. *Virol J* **9**, 91 (2012).
68. Yewdell, J.W. Viva la revolucion: rethinking influenza a virus antigenic drift. *Curr Opin Virol* **1**, 177–183 (2011).
69. Salazar, M.I., Lopez-Ortega, O., Leon-Avila, G., Ramirez-Gonzalez, J.E. & Castro-Mussot, M.E. [The origin of the genetic variability of influenza viruses]. *Gac Med Mex* **146**, 199–206 (2010).
70. Epstein, S.L. & Price, G.E. Cross-protective immunity to influenza A viruses. *Expert Rev Vaccines* **9**, 1325–1341 (2010).
71. Bouvier, N.M. & Palese, P. The biology of influenza viruses. *Vaccine* **26 Suppl 4**, D49–53 (2008).
72. Grebe, K.M., Yewdell, J.W. & Bennink, J.R. Heterosubtypic immunity to influenza A virus: where do we stand? *Microbes Infect* **10**, 1024–1029 (2008).
73. Szucs, T. The socio-economic burden of influenza. *J Antimicrob Chemother* **44 Suppl B**, 11–15 (1999).
74. Molinari, N.A. The annual impact of seasonal influenza in the US: measuring disease burden and costs. *Vaccine* **25**, 5086–5096 (2007).
75. Monto, A.S. Seasonal influenza vaccinations: specialized products for different target groups. *Vaccine* **28 Suppl 4**, D14–23 (2010).

76. Hannoun, C., Megas, F. & Piercy, J. Immunogenicity and protective efficacy of influenza vaccination. *Virus Res* **103**, 133–138 (2004).
77. Fiore, A.E., et al. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. *MMWR Recomm Rep* **59**, 1–62 (2010).
78. Hanon, E. Vaccination strategies against influenza. *Bull Mem Acad R Med Belg* **164**, 283–287 (2009).
79. Nichol, K.L. & Treanor, J.J. Vaccines for seasonal and pandemic influenza. *J Infect Dis* **194 Suppl 2**, S111–118 (2006).
80. CDC. Seasonal Influenza (Flu). (2011).
81. Beyer, W.E., Nauta, J.J., Palache, A.M., Giezeman, K.M. & Osterhaus, A.D. Immunogenicity and safety of inactivated influenza vaccines in primed populations: a systematic literature review and meta-analysis. *Vaccine* **29**, 5785–5792 (2011).
82. Gross, P.A. & Ennis, F.A. Influenza vaccine: split-product versus whole-virus types--How do they differ. *N Engl J Med* **296**, 567–568 (1977).
83. Gross, P.A., et al. A controlled double-blind comparison of reactogenicity, immunogenicity, and protective efficacy of whole-virus and split-product influenza vaccines in children. *J Infect Dis* **136**, 623–632 (1977).
84. Johansson, B.E. & Brett, I.C. Changing perspective on immunization against influenza. *Vaccine* **25**, 3062–3065 (2007).
85. Cook, I.F., Barr, I., Hartel, G., Pond, D. & Hampson, A.W. Reactogenicity and immunogenicity of an inactivated influenza vaccine administered by intramuscular or subcutaneous injection in elderly adults. *Vaccine* **24**, 2395–2402 (2006).
86. Schwartz, B. & Wortley, P. Mass vaccination for annual and pandemic influenza. *Current topics in microbiology and immunology* **304**, 131–152 (2006).
87. Fredrickson, K., et al. Influenza vaccination coverage among children aged 6-23 months - six immunization information system sentinel sites, United States, 2005-06 influenza season. *MMWR Morb Mortal Wkly Rep* **55**, 1329–1330 (2006).
88. McElhaney, J.E. Influenza vaccine responses in older adults. *Ageing Res Rev* **10**, 379–388 (2011).
89. Ellebedy, A.H. & Webby, R.J. Influenza vaccines. *Vaccine* **27 Suppl 4**, D65–68 (2009).
90. Marcelin, G., et al. Inactivated seasonal influenza vaccines increase serum antibodies to the neuraminidase of pandemic influenza A(H1N1) 2009 virus in an age-dependent manner. *J Infect Dis* **202**, 1634–1638 (2010).
91. Coudeville, L., et al. Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a bayesian random-effects model. *BMC Med Res Methodol* **10**, 18 (2010).
92. CDC. Vaccine Effectiveness - How Well Does the Flu Vaccine Work? (2011).
93. CDC. Flu Vaccine Effectiveness: Questions and Answers for Health Professionals. (2011).
94. Kim, Y.C. & Prausnitz, M.R. Enabling skin vaccination using new delivery technologies. *Drug Deliv Transl Res* **1**, 7–12 (2011).
95. Jepps, O.G., Dancik, Y., Anissimov, Y.G. & Roberts, M.S. Modeling the human skin barrier - Towards a better understanding of dermal absorption. *Adv Drug Deliv Rev* (2012).
96. Skountzou, I. & Kang, S.M. Transcutaneous immunization with influenza vaccines. *Current topics in microbiology and immunology* **333**, 347–368 (2009).
97. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
98. Romani, N., et al. Morphological and phenotypical characterization of bone marrow-derived dendritic Thy-1-positive epidermal cells of the mouse. *J Invest Dermatol* **85**, 91 s-95 s (1985).
99. Itano, A.A., et al. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* **19**, 47–57 (2003).
100. Valladeau, J., et al. Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* **12**, 71–81 (2000).
101. Allavena, P., et al. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. *Immunol Rev* **177**, 141–149 (2000).
102. Sozzani, S., Allavena, P., Vecchi, A. & Mantovani, A. Chemokines and dendritic cell traffic. *J Clin Immunol* **20**, 151–160 (2000).
103. Locati, M., Allavena, P., Sozzani, S. & Mantovanii, A. Shaping and tuning of the chemokine system by regulation of receptor expression and signaling: dendritic cells as a paradigm. *J Neuroimmunol* **107**, 174–177 (2000).
104. Sozzani, S., et al. In vitro and in vivo regulation of chemokine receptors. *Eur Cytokine Netw* **11**, 502–503 (2000).
105. Romani, N., et al. Targeting skin dendritic cells to improve intradermal vaccination. *Current topics in microbiology and immunology* **351**, 113–138 (2012).
106. Miller, L.S. Toll-like receptors in skin. *Adv Dermatol* **24**, 71–87 (2008).
107. Abdelsadik, A. & Trad, A. Toll-like receptors on the fork roads between innate and adaptive immunity. *Hum Immunol* **72**, 1188–1193 (2011).
108. Dupasquier, M., Stoitzner, P., van Oudenaren, A., Romani, N. & Leenen, P.J. Macrophages and dendritic cells constitute a major subpopulation of cells in the mouse dermis. *J Invest Dermatol* **123**, 876–879 (2004).

109. Debenedictis, C., Joubeh, S., Zhang, G., Barria, M. & Ghohestani, R.F. Immune functions of the skin. *Clin Dermatol* **19**, 573–585 (2001).
110. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H. & Amagai, M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J Exp Med* **206**, 2937–2946 (2009).
111. Flacher, V., et al. Epidermal Langerhans cells rapidly capture and present antigens from C-type lectin-targeting antibodies deposited in the dermis. *J Invest Dermatol* **130**, 755–762 (2010).
112. Romani, N., Brunner, P.M. & Stingl, G. Changing views of the role of Langerhans cells. *J Invest Dermatol* **132**, 872–881 (2012).
113. Flacher, V., et al. Skin Langerin(+) dendritic cells transport intradermally injected anti-DEC-205 antibodies but are not essential for subsequent cytotoxic CD8(+) T cell responses. *J Immunol* **188**, 2146–2155 (2012).
114. del Pilar Martin, M., et al. Local response to microneedle-based influenza immunization in the skin. *MBio* **3**, e00012–00012 (2012).
115. Raz, E., et al. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc Natl Acad Sci USA* **91**, 9519–9523 (1994).
116. Koutsonanos, D.G., et al. Serological memory and long-term protection to novel H1N1 influenza virus after skin vaccination. *J Infect Dis* **204**, 582–591 (2011).
117. Prausnitz, M.R. Microneedles for transdermal drug delivery. *Adv Drug Deliv Rev* **56**, 581–587 (2004).
118. Prausnitz, M.R., Mitragotri, S. & Langer, R. Current status and future potential of transdermal drug delivery. *Nat Rev Drug Discov* **3**, 115–124 (2004).
119. Kim, Y.C., Park, J.H. & Prausnitz, M.R. Microneedles for drug and vaccine delivery. *Adv Drug Deliv Rev* (2012).
120. Kim, Y.C., Jarrahian, C., Zehrung, D., Mitragotri, S. & Prausnitz, M.R. Delivery systems for intradermal vaccination. *Current topics in microbiology and immunology* **351**, 77–112 (2012).
121. Koutsonanos, D.G., et al. Delivery of subunit influenza vaccine to skin with microneedles improves immunogenicity and long-lived protection. *Sci Rep* **2**, 357 (2012).
122. Koutsonanos, D.G., et al. Transdermal influenza immunization with vaccine-coated microneedle arrays. *PloS one* **4**, e4773 (2009).
123. Sullivan, S.P., et al. Dissolving polymer microneedle patches for influenza vaccination. *Nat Med* **16**, 915v920 (2010).
124. Kim, Y.C., Quan, F.S., Compans, R.W., Kang, S.M. & Prausnitz, M.R. Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity. *Journal of controlled release : official journal of the Controlled Release Society* **142**, 187v195 (2010).
125. Gill, H.S. & Prausnitz, M.R. Coating formulations for microneedles. *Pharm Res* **24**, 1369–1380 (2007).
126. Gill, H.S. & Prausnitz, M.R. Coated microneedles for transdermal delivery. *Journal of controlled release : official journal of the Controlled Release Society* **117**, 227–237 (2007).
127. Koyama, S., et al. Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Sci Transl Med* **2**, 25ra24 (2010).
128. Chu, L.Y., Choi, S.O. & Prausnitz, M.R. Fabrication of dissolving polymer microneedles for controlled drug encapsulation and delivery: Bubble and pedestal microneedle designs. *J Pharm Sci* **99**, 4228–4238 (2010).
129. Lee, J.W., Park, J.H. & Prausnitz, M.R. Dissolving microneedles for transdermal drug delivery. *Biomaterials* **29**, 2113–2124 (2008).
130. Chu, L.Y. & Prausnitz, M.R. Separable arrowhead microneedles. *Journal of controlled release : official journal of the Controlled Release Society* **149**, 242–249 (2011).
131. Prausnitz, M.R., Mikszta, J.A., Cormier, M. & Andrianov, A.K. Microneedle-based vaccines. *Current topics in microbiology and immunology* **333**, 369–393 (2009).
132. Choi, H.J., et al. Stability of influenza vaccine coated onto microneedles. *Biomaterials* **33**, 3756–3769 (2012).
133. Patel, S.R., Lin, A.S., Edelhauser, H.F. & Prausnitz, M.R. Suprachoroidal drug delivery to the back of the eye using hollow microneedles. *Pharm Res* **28**, 166–176 (2011).
134. Gupta, J., Denson, D.D., Felner, E.I. & Prausnitz, M.R. Rapid local anesthesia in humans using minimally invasive microneedles. *Clin J Pain* **28**, 129–135 (2012).
135. Wang, P.M., Cornwell, M., Hill, J. & Prausnitz, M.R. Precise microinjection into skin using hollow microneedles. *J Invest Dermatol* **126**, 1080–1087 (2006).
136. Gupta, J., Felner, E.I. & Prausnitz, M.R. Minimally invasive insulin delivery in subjects with type 1 diabetes using hollow microneedles. *Diabetes Technol Ther* **11**, 329–337 (2009).
137. Banks, S.L., et al. Transdermal delivery of naltrexol and skin permeability lifetime after microneedle treatment in hairless guinea pigs. *J Pharm Sci* **99**, 3072–3080 (2010).
138. Gupta, J., Felner, E.I. & Prausnitz, M.R. Rapid pharmacokinetics of intradermal insulin administered using microneedles in type 1 diabetes subjects. *Diabetes Technol Ther* **13**, 451–456 (2011).
139. Lee, J.W., Choi, S.O., Felner, E.I. & Prausnitz, M.R. Dissolving microneedle patch for transdermal delivery of human growth hormone. *Small* **7**, 531–539 (2011).

140. Andrews, S., Lee, J.W., Choi, S.O. & Prausnitz, M.R. Transdermal insulin delivery using microdermabrasion. *Pharm Res* **28**, 2110–2118 (2011).
141. Tas, C., et al. Delivery of salmon calcitonin using a microneedle patch. *Int J Pharm* **423**, 257–263 (2012).
142. Song, J.M., et al. DNA Vaccination in the Skin Using Microneedles Improves Protection Against Influenza. *Mol Ther* (2012).
143. Kim, Y.C., et al. Increased immunogenicity of avian influenza DNA vaccine delivered to the skin using a microneedle patch. *European journal of pharmaceutics and biopharmaceutics: official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V* (2012).
144. Prow, T.W., et al. Nanopatch-targeted skin vaccination against West Nile Virus and Chikungunya virus in mice. *Small* **6**, 1776–1784 (2010).
145. Fernando, G.J., et al. Nanopatch targeted delivery of both antigen and adjuvant to skin synergistically drives enhanced antibody responses. *Journal of controlled release : official journal of the Controlled Release Society* **159**, 215–221 (2012).
146. FDA. Influenza Virus Vaccine, Trivalent, Types A and B. (2012).
147. Norman, J.J. & Prausnitz, M.R. Improving patient acceptance of insulin therapy by improving needle design. *J Diabetes Sci Technol* **6**, 336–338 (2012).

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