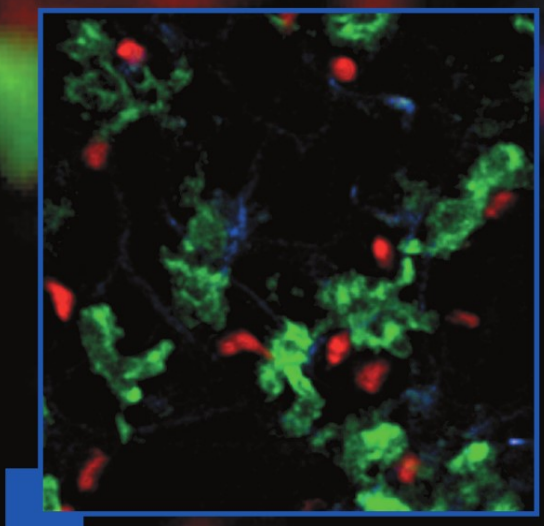
B. Pulendran R. Ahmed (Eds.)

From Innate Immunity to Immunological Memory



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B. Pulendran and R. Ahmed (Eds.)

From Innate Immunity to Immunological Memory

With 13 Figures and 6 Tables

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Cover Illustration:
The two intravital multi-photon micrographs show Ag-presenting dendritic cells (DCs) interacting with TCR transgenic T cells in a popliteal lymph node (LN) of an anesthetized mouse. In the background image the DCs (which were injected in the animal's footpad one day earlier) are shown in red and CD4 and CD8 T cells are labeled in blue and green, respectively. In the smaller foreground image, a meshwork of LN-resident endogenous DCs are shown, which express chimeric MHC class II linked to GFP (green). CD4 T cells were labeled in red and interstitial collagen fibers are blue. Both images were generated in Dr. Ulrich H. von Andrian's lab by Dr. Thorsten Mempel.

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From Innate Immunity to Immunological Memory

The ability to remember an antigenic encounter for several decades, even for a life time, is one of the fundamental properties of the immune system. This phenomenon known as "immunological memory," is the foundation upon which the concept of vaccination rests. Therefore, understanding the mechanisms by which immunological memory is regulated is of paramount importance. Recent advances in immunology, particularly in the field of innate immunity, suggest that the innate immune system plays fundamental roles in influencing the generation and maintenance of immunological memory. Indeed, emerging evidence suggests that events that occur early, within hours if not minutes of pathogen or vaccine entry profoundly shape the quantity, quality and duration of immunological memory. The present volume assembles a collection of essays from leading experts that span the entire spectrum research from understanding the molecular mechanisms of innate immune recognition, to dendritic cell function, to the generation and maintenance of antigen-specific B and T-cell responses, and memory.

The first two reviews focus on aspects of innate immunity, specifically on how the innate immune system 'senses' pathogens and modulates the adaptive immunity against them. The article by Shizuo Akira [1] discusses pathogen recognition by Toll-like receptors [TLRs]. In the eight years since the demonstration of the critical roles they play in microbe sensing in the mammalian immune system, it has become clear that each TLR senses a different set of microbial stimuli, and that individual TLRs activate distinct but overlapping signaling pathways and transcription factors that drive specific biological responses against the pathogens. Following on this theme, the article by Steinman and Hemmi reviews the important roles played by dendritic cells (DCs) in sensing microbes or vaccines, and in modulating the quality and quantity of adaptive immune responses. DCs appear to play a unique role in integrating signals from various sources, including microbial stimuli that signal directly via TLRs and other pathogen recognition receptors (PRRs), but also from other cells of the innate immune system, including NK cells and NKT cells.

The next three chapters focus on aspects of adaptive immunity and immunological memory. McHeyzer-Williams and McHeyzer-Williams discuss the generation and maintenance of antigen-specific T-helper and B cell responses. Recent studies provide insights into the dynamics of interaction between antigen-bearing DCs and antigen-specific T-helper cells, and how this programs the quality of T-helper and B cell responses. Following along this theme, Tan and Surh discuss the development of antigen-specific memory T cells, guided by a progressive set of environmental and cell intrinsic cues, which include the type of antigen-bearing DCs, as well as inflammatory mediators. An interesting aspect of this is that inflammatory mediators play important roles, not only in the genesis of antigen-specific T cell responses, but also in the maintenance and homeostasis of such cells. Finally, the chapter by Welsh and colleagues discusses how the T-cell receptor repertoire of cells in the memory T cell pool is generated and modulated by the innate immune cytokines, and how this is modulated in response to other infections.

The emerging evidence that TLR signaling controls critical aspects of innate and adaptive immunity offers novel strategies for enhancing the efficacy of vaccines against infectious diseases. In this context, the final article by McCluskie and Krieg discuss how CpG DNA, which signals through TLR9 induce potent stimulation of plasmacytoid DCs, and B cells and promote strong Th1 responses. The addition of CpG DNA to most vaccines appear to induce faster seroconversion, with fewer vaccinations, which might facilitate reduced antigen doses. Thus, in the short period since the discovery of TLRs, they have already begun to enter several clinical trials. The hope is specific triggering of particular combinations of TLRs or PRRs, would yield immune responses and immunological memory, which are as good, or even better, that that induced by the best of our empirically derived vaccines.

Atlanta, June 2006 *Bali Pulendran, Rafi Ahmed*

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TLR Signaling

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Abstract Mammalian Toll-like receptors (TLRs) play a critical role in detection of invading pathogens as well as triggering of subsequent inflammatory and immune responses. Each TLR recognizes distinct microbial components and activates different signaling pathways by selective utilization of adaptor molecules. The signaling via TLRs is delivered from the cell surface and/or the endosome. Recently, the intracytoplasmic detection system of microbes has been identified in mammals as well. Peptidoglycan breakdown products and double-stranded RNA are sensed by NOD family and RNA helicase domain containing proteins, respectively. Thus, mammals make use of both receptor-type and intracellular proteins as detectors of invading pathogens.

1 Introduction

Toll-like receptors (TLRs) play a critical role in innate immunity as well as acquired immunity (Beutler 2004; Medzhitov 2001; Takeda et al. 2003). TLRs are vertebrate counterparts of Toll in *Drosophila*. *Drosophila* Toll, originally identified as a receptor critical for dorsoventral axis formation in fly embryonic development, has been shown to be essential to antifungal responses in flies (Lemaitre et al. 1996). This finding led to identification of TLRs in mammals (Medzhitov et al. 1997). The TLR family is now composed of 11 members, but only these members may be enough to detect all pathogens from bacteria to viruses (Table 1). One reason is that TLR targets the com-

Table 1 Toll-like receptor ligands

Receptor	Ligands	Synthetic analogues
TLR1	Bacterial lipoproteins	Triacyl lipopeptides
TLR2	Lipoproteins/lipopeptides Peptidoglycan Lipoteichoic acid Zymosan (Heat-shock protein 70)?	Diacyl and triacyl lipopeptides
TLR3	Viral double-stranded RNA	Poly I:C
TLR4	Lipopolysaccharide Taxol RS virus F protein (Heat-shock proteins)? Tamm-Horsfall glycoprotein	Synthetic lipid A
TLR5	Bacterial flagellin	
TLR6	Mycoplasma lipopeptides Zymosan	Diacyl lipopeptides
TLR7	Single-stranded RNA Viral RNA	Imidazoquinolines siRNA
TLR8	Single-stranded RNA Viral RNA	Imidazoquinolines
TLR9	Bacterial DNA Hemozoin (malaria pigment) Viral DNA	CpG-containing oligonucleotides
TLR10	ND	ND
TLR11	ND	Uropathogenic bacteria

ND, not determined

ponents common to pathogens as well as essential for life of pathogens, and therefore it is difficult for them to change the structure. A second reason is that a single TLR recognizes several pathogen components that are structurally unrelated to each other. For example, besides lipopolysaccharide (LPS), TLR recognizes Taxol and several viral envelop glycoproteins. TLR9, a CpG DNA receptor, also recognizes hemozoin, a malarial pigment (Coban et al. 2005). At present the mechanism underlying how a single TLR recognizes entirely different molecular structure is an enigma. We must await the crystallography of the extracellular leucine-rich domain for elucidation.

TLRs are type 1 transmembrane receptors and consist of an extracellular leucine-rich repeat domain and an intracellular domain [Toll-interleukin 1 receptor (TIR) domain] that is homologous to that of interleukin (IL)-1Rs. Thus, The signaling of these two receptor families is similar, and the molecules involved in TLR signaling are shared by IL-1R signaling. However, as shown below, recent studies have shown that the signaling pathways of individual TLRs are more complex than previously thought.

2
TLR Signaling Pathways

2.1
MyD88-Dependent and Independent Pathways

Engagement of IL-1R triggers a cell signaling cascade involving sequentially myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor activated kinase (IRAK), tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), and activation of nuclear factor (NF)-κB (Fig. 1). The cytoplasmic portion of IL-1R is highly homologous to that of TLR. Therefore, these two receptors utilize similar signaling molecules. Indeed, the analysis of MyD88-deficient mice revealed that MyD88 plays a crucial role in TLR and IL-1R signaling pathways (Adachi et al. 1998). In addition, MyD88-deficient macrophages are completely defective in response to LPS and other microbial components in terms of cytokine production and B cell proliferation (Kawai et al. 1999; Takeuchi et al. 2000). These findings demonstrate that MyD88 is a key adaptor molecule in both IL-1R and TLR signaling. Until ligands of TLRs have been identified, all the responses to TLR ligands were considered to be identical and entirely dependent on MyD88. However, stimulation of cells with TLR ligands revealed different patterns of gene expression, indicating that the signaling pathway through individual TLRs differs in each case (Akira and Takeda 2004).

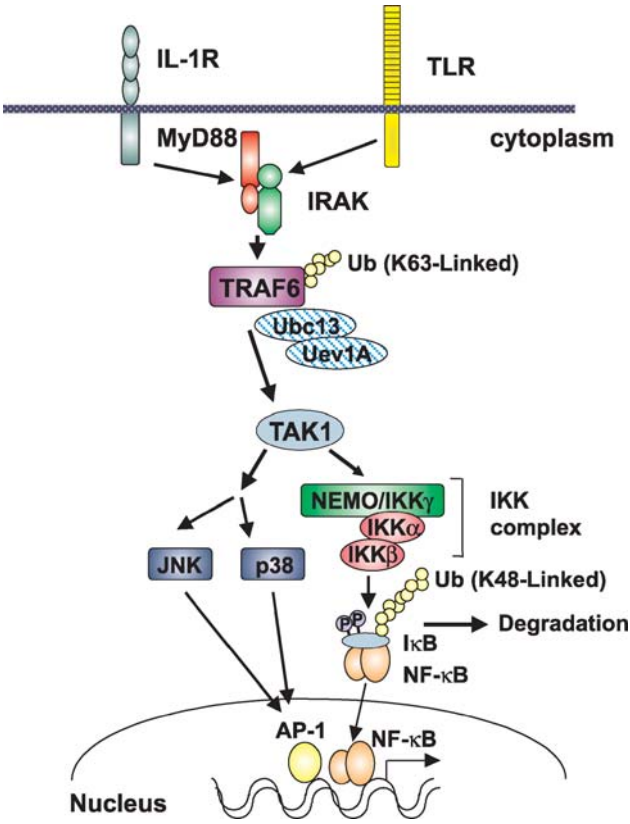

Fig. 1 MyD88-dependent pathway in IL-1R/TLR signaling. IL-1R and TLR signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAKs (IRAK-1 and IRAK-4) to the receptor upon ligand binding. IRAKs in turn recruit TRAF6, which activates two ubiquitination proteins, Ubc13 and Uev1A. Ubc13 and Uev1A form a complex that catalyzes the formation of a lysine 63-linked polyubiquitin chain. Ubiquitinated TRAF6 activates TAK1, then leading to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates IκB. Phosphorylated IκB proteins are rapidly degraded by the proteasome, resulting in nuclear translocation of NF-κB. TAK1 activates MAP kinases such as c-Jun N-terminal kinase (JNK) and p38 as well

Evidence supporting this speculation came from the analysis of MyD88-deficient macrophages (Kawai et al. 1999). Whereas activation of signaling molecules such as NF-κB and MAP kinases was not observed in MyD88-deficient cells in response to TLR2 (lipoprotein), TLR7 (imidazoquinolines),

or TLR9 (CpG DNA) ligands, LPS (TLR4 ligand) activation of NF-κB and MAP kinases was detected with delayed kinetics in MyD88-deficient cells. This finding indicated the existence of a MyD88-independent pathway in LPS signaling. The MyD88-independent pathway activates interferon regulatory factor-3 (IRF3) (Kawai et al. 2001). IRF3 is present ubiquitously in many cells, undergoes phosphorylation, forms a dimer, and translocates to the nucleus in viral infection or in response to LPS. In addition to the TLR4 ligand, the TLR3 ligand—double-stranded RNA (dsRNA)—activates IRF-3 as well as interferon (IFN)-β and IFN-inducible genes in MyD88-deficient cells (Alexopoulou et al. 2001). These data show that TLR3- and TLR4-mediated signaling possesses MyD88-independent pathways.

In addition to MyD88, several TIR domain-containing adaptor proteins were later identified. They include TIR domain-containing adapter protein (TIRAP)/MyD88-adapter-like (Mal), TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF) and TRIF-related adaptor molecule (TRAM). The knockout experiment revealed that individual adaptors play a critical role in the TLR signaling (Fig. 2).

2.2 Roles of Adaptors in TLR Signaling

2.2.1 TIRAP/Mal

TIRAP/Mal was originally cloned as a candidate adaptor involved in the MyD88-independent pathway of TLR4 signaling (Horng et al. 2001; Fitzgerald et al. 2001). Unlike MyD88, TIRAP does not harbor a death domain. Despite in vitro supporting evidence, TIRAP knockout mice did not show any defects in the MyD88-independent pathway (Horng et al. 2002; Yamamoto et al. 2002). Induction of IFN-β and IFN-inducible genes as well as IRF3 activation was normally observed in TIRAP-deficient cells in response to LPS (TLR4 ligand) or poly I-C (TLR3 ligand). Delayed NF-κB activation was also detected in LPS-stimulated TIRAP-deficient cells. Furthermore, IFN-β and IFN-inducible genes were induced in MyD88/TIRAP double-deficient cells in response to LPS, demonstrating that TIRAP is not involved in the MyD88-independent pathway of TLR4 signaling. However, TIRAP-deficient macrophages did not produce inflammatory cytokines after LPS stimulation. LPS-stimulated splenocyte proliferation was also abolished in TIRAP knockout mice, showing that TIRAP is essential for the MyD88-dependent pathway of TLR4 signaling. Unexpectedly, TIRAP-deficient cells showed impaired cytokine production in response to TLR2 ligands, peptidoglycan, and mycoplasmal lipopeptides, although the response to TLR3, TLR5, TLR7, and TLR9 lig-

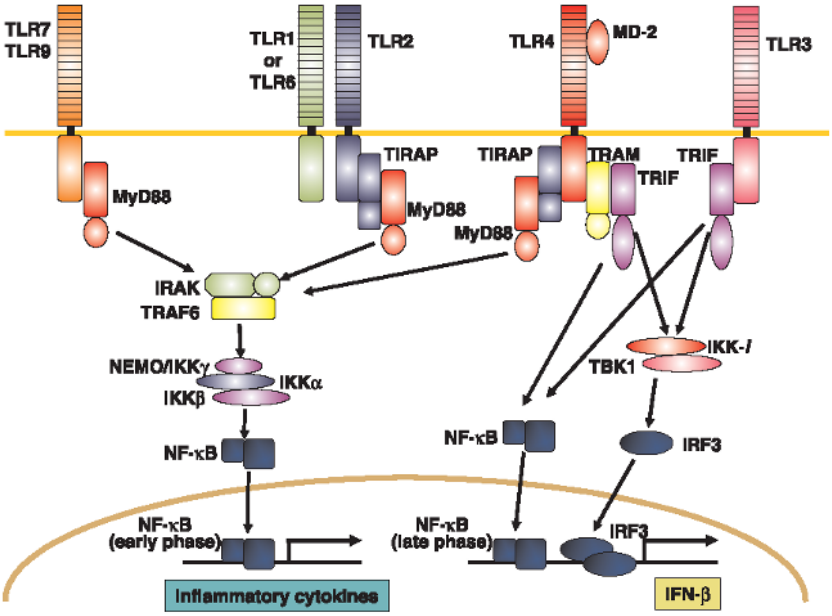

Fig. 2 TLR signaling pathways. MyD88 is involved in the signaling pathways of all TLRs except for TLR3. The MyD88-dependent pathway is responsible for induction of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF3 and induction of IFN-β are regulated by the third TIR domain-containing adaptor, TRIF. Nontypical IKKs, IKK-*i* and TBK1, phosphorylate and activate IRF3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway

ands were comparable to those observed in wild-type cells. Taken together, these findings demonstrate that TIRAP is essential for MyD88-dependent signaling through TLR2 and TLR4.

2.2.2 TRIF

TRIF (also known as TICAM1) is the third TIR domain-containing adaptor that was identified independently by database search and acts as an interacting partner with TLR3 by yeast two-hybrid system (Yamamoto et al. 2002; Oshiumi et al. 2003). TRIF is a larger protein (712 amino acids, a.a.) compared with other TIR-domain containing adaptors (MyD88 296 a.a., TRAP 235 a.a.,

TRAM 235 a.a.). In vitro overexpression of TRIF activated the IFN-β promoter much more strongly than the NF-κB-dependent promoter. TRIF-deficient mice were severely impaired in the induction of IFN-β and IFN-inducible genes mediated by TLR3 and TLR4 ligands, demonstrating that TRIF is an essential signal transducer in TLR3- and TLR4-mediated MyD88-independent pathways (Yamamoto et al. 2003). Analysis of mutant mice designated *lps2*, which were generated by *N*-ethyl-*N*-nitrosourea-induced random mutations, shows the similar conclusion (Hoebe et al. 2003). It is of note that the production of inflammatory cytokines induced by LPS is severely impaired in TRIF-deficient cells as is the case of MyD88-deficient cells, revealing the critical role of TRIF in inflammatory cytokine production as well. In contrast to MyD88-deficient cells, LPS-induced activation of IRAK1 and the early phase activation of NF-κB and mitogen-activated protein (MAP) kinases were normal in TRIF-deficient cells, although the late-phase activation of NF-κB and MAP kinases was impaired. These findings strongly suggest that activation of both MyD88-dependent and TRIF-dependent pathways are necessary for induction of inflammatory cytokines, while the TRIF-dependent pathway is sufficient for induction of IFN-β and IFN-inducible genes

2.2.3 TRAM

The fourth adaptor was identified by database search and termed TRAM or TICAM2 (Oshiumi et al. 2003; Yamamoto et al. 2003). TRAM-deficient mice showed normal responses to TLR2, TLR7, and TLR9 ligands (Yamamoto et al. 2003). However, LPS-induced production of inflammatory cytokines as well as induction of IFβ and IFN-inducible genes was severely impaired in TRAM-deficient cells, which is very similar to the phenotype of TRIF-deficient cells. However, TRAM-deficient cells responded normally to TLR3 ligands. These findings show that TRAM is essential for the MyD88-independent pathway of TLR4 signaling but not of TLR3 signaling. Thus, the difference among TLR responses is in part due to differential utilization of adaptor molecules.

2.3 Mechanisms of Type 1 Interferon Induction by TRIF-Dependent Pathway

2.3.1 IRF3 Kinases, TBK1 and IKK-*i*

Two IκB kinase (IKK)-related kinases are involved in the activation of IRF3 in the TRIF-dependent signaling pathway as well as viral infection. They are called TA NK-binding kinase-1 (TBK1) and IKK-*i* (also called IKKε). These

two kinases share homology with IKKα/β. Deficiency of TBK1 in mice resulted in embryonic lethality caused by massive liver degeneration. Although these two kinases were initially thought to be implicated in NF-κB activation, and indeed a report showed reduced expression of certain genes regulated by NF-κB in TBK1-deficient embryonic fibroblasts (EF) (Bonnard et al. 2000), our analysis of TBK1-deficient EF did not confirm the later finding. Recently, two groups independently showed that IKK-*i* and TBK1 phosphorylate and activate IRF3 and IRF7, leading to the transcriptional activation of genes for IFN-β, RANTES, and ISG54 in viral infection as well as in the TRIF-dependent signaling pathway (Sharma et al. 2003; Fitzgerald et al. 2003; Fig. 2). The role of these IKK-related kinases in the TRIF-dependent pathway and during viral infection were analyzed using embryonic fibroblasts lacking TBK1, IKK-*i*, or both (Hemmi et al. 2004; McWhirter et al. 2004; Perry et al. 2004). TBK1-deficient mouse embryo fibroblasts (MEFs) showed marked decrease in the induction of IFN-β and IFN-inducible genes in response to LPS as well as viral infection. On the other hand, IKK-*i*-deficient cells did not show impaired response to LPS, polyI-C, or viral infection. However, in the case of TBK1/IKK-*i* double-deficient EFs, the marginal IFN-β induction and diminished IRF3 activation observed in poly I-C stimulated TBK1-deficient cells were completely abolished, indicating that IKK-*i* plays some role in poly I-C signaling.

2.3.2
Signaling Pathways Downstream of TRIF

The signaling pathways downstream of TRIF have been clarified. After stimulation of TLR3 or TLR4, TRIF recruits IRF3 kinase, TBK1. This association leads to IRF3 phosphorylation, which results in dimer formation of IRF3, its translocation from the cytoplasm to the nucleus, and finally induction of IFN-β. NF-κB can be activated in the absence of MyD88 in response to LPS, but the NF-κB activation is delayed compared with normal cells. It is well known that the gene induction of IFN-β requires activation of both IRF3 and NF-κB. Recently, the molecular mechanism of the late NF-κB activation became evident. In addition to TBK1 recruitment, TRIF also recruits TRAF6 and RIP-1 (Sato et al. 2003; Meylan et al. 2004). These associations are responsible for MyD88-independent NF-κB activation. This pathway is coupled with the recognition of viral components, and considered to be involved in antiviral action.

2.4
Type 1 IFN Induction in PDC

Dendritic cells (DCs) are categorized into myeloid DC (MDC) and plasmacytoid DC (PDC) (Colonna et al. 2004). PDC express TLR9 and TLR7. PDC

express IRF7 constitutively, unlike other cell types where IRF7 is induced by type 1 IFNs. PDC produce a large amount of IFN-α in response to viral infection or TLR7 or TLR9 ligands. Different from the case of TRIF-dependent type 1 IFN production in TLR3 and TLR4 signaling, IFN production via TLR7 and TLR9 receptors in PDC depends on MyD88. We have shown that a direct association of MyD88 and IRF7 could be involved in induction of type 1 IFN (Kawai et al. 2004). The search for the molecule interacting with MyD88 by the yeast two-hybrid system led to identification of IRF7 as a partner of MyD88. These molecules associate directly via interaction of the death domain of MyD88 and the inhibitory domain (amino acid 238 and 285) of IRF7. Cotransfection of MyD88 and IRF7 synergistically augments the IFN-α but not IFN-β promoter activity. MyD88 induces phosphorylation of IRF7. IRF7 nuclear translocation in response to CpG DNA is abolished in MyD88-deficient cells. TRAF6 also associates with IRF7 at the same region where MyD88 interacts. These results indicate that TLR-dependent IFN-α induction in PDC requires the formation of a complex consisting of MyD88, TRAF6, and IRF7 (Fig. 3). The similar finding is reported by Honda et al. (2004). Furthermore, our recent result has shown that IRAK-1 may be the kinase involved in IRF7 activation in PDC (Uematsu et al. 2005).

3
Intracytoplasmic Detection of Pathogens

3.1
Detection of Peptidoglycan by NOD Family

The nucleotide-binding oligomerization domain (NOD) family, particularly NOD1 and NOD2, is involved in the intracytoplasmic sensing system of bacterial components in mammals (Girardin et al. 2003a, b; Chamaillard et al. 2003; Inohara et al. 2003). NOD2 is a cytoplasmic protein composed of two N-terminal caspase activating and recruitment domains (CARDs), a central NOD, and a C-terminal leucine-rich repeat (LRR) domain. NOD1 contains a single CARD. NOD1 and NOD2 both activate NF-κB through interaction with the downstream CARD domain-containing kinases known as kinase receptor interacting protein 2 (RIP2) or RICK. The LRR domains of both proteins are involved in recognition of distinct peptidoglycan breakdown products. The minimal moiety in PGN recognized by NOD2 is muramyl dipeptide MurNAc-L-Ala-D-isoGln (MDP), whereas NOD1 detects γ-D-glutamyl-meso-DAP (iE-DAP), a dipeptide primarily found in PGN from gram-negative bacteria (Fig. 4). NOD2 was identified as the first susceptibility gene involved

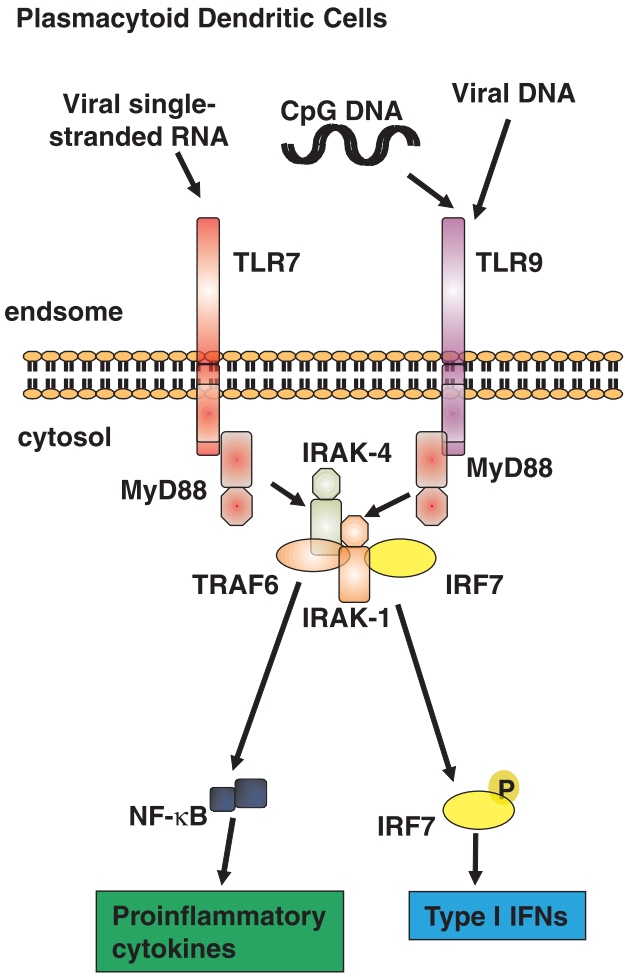

Fig. 3 Type 1 IFN production in PDC. Type 1 IFN induction in PDC requires the formation of a complex consisting of MyD88, IRAKs, TRAF6, and IRF7. IRAK-1 is dispensable for the NF-κB activation but specifically regulates type 1 IFN production through activation of IRF7. IRAK-4 may locate upstream of IRAK-1 and be involved in the activation of IRAK-1

in the etiology of Crohn's disease (Ogura et al. 2001; Hugot et al. 2001). Although in vitro overexpression of NOD2 strongly activates NF-κB, Crohn's disease-associated mutations of NOD2 lose the activity. This finding may be contradictory because Crohn's disease patients show increased NF-κB activity. However, a recent report has shown that knockin mice harboring the NOD2

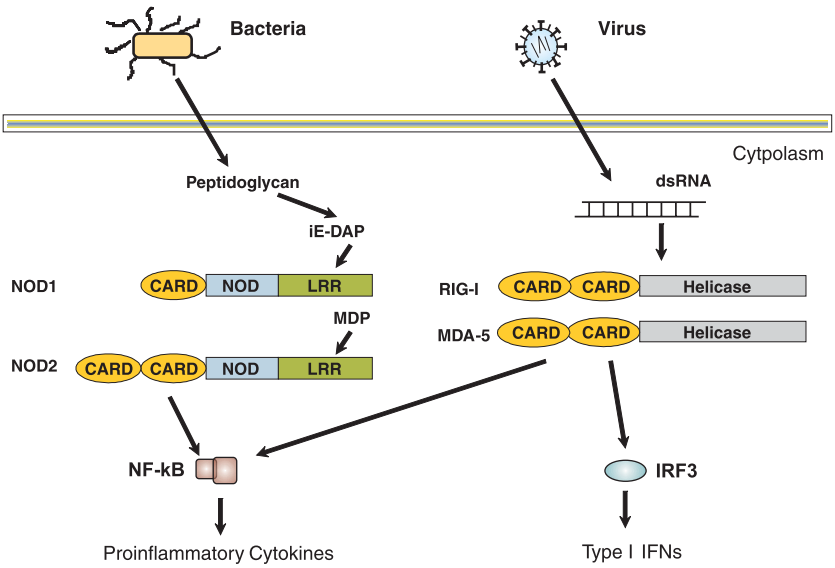

Fig. 4 Intracytoplasmic recognition of pathogens. Bacteria are recognized intracellularly. NOD1 and NOD2 recognize different motifs found in the layer of PGN, namely iE-DAP and MDP, respectively. NOD1 and NOD2 are composed of C-terminal LRR, central NOD domain, and N-terminal CARD domain. They detect bacterial components via the LRR domain, and signal through the CARD domain to activate NF-κB. Viruses are also recognized intracellularly in a TLR-independent manner. dsRNA, which is generated for viral replication, is recognized by RIG-I via its helicase domain. The CARD domain of RIG-I is required for activation of IRF3 and NF-κB

mutation in Crohn's disease exhibit elevated NF-κB activation in response to MDP, linking the exaggerated NF-κB activation to intestinal inflammation (Maeda et al. 2005). Mutations in NOD2 are also responsible for another autoinflammatory disease affecting the eye and joints called Blau syndrome (Miceli-Richard et al. 2001).

3.2 Detection of Intracellular Double-Stranded RNA by RIG-1 and MDA-5

Induction of type 1 IFN by intracellular dsRNA does not require TLR3, but depends on TBK1, suggesting that TBK1 is involved in TRIF-dependent as well as TRIF-independent type 1 IFN induction. This suggests that intracellular dsRNA does not require TLR3. Recently, retinoic acid-inducible gene-I (RIG-I) was implied as the candidate for detector of intracellular dsRNA (Yoneyama et al. 2004; Fig. 4). RIG-I is a member of the DexH box family protein and

regarded as a putative RNA helicase. This molecule also contains two CARDs at its N terminus. RIG-I protein is located in the cytoplasm of cells. The CARD of RIG-I transduces signals that lead to the activation of IRF3 and NF-κB. Full-length RIG-I interacts with dsRNA and augments interferon production in response to viral infection in an ATPase-dependent manner. Melanoma-differentiation associated gene-5 (MDA-5) is highly homologous to RIG-I and induced during differentiation of a melanoma cell line as well as in response to type 1 interferon (Kang et al. 2002). MDA-5 has similar properties to RIG-I in dsRNA-induced production of IFN-β. A recent study has shown that the C-terminal domain of the V protein of SV5, and of other paramyxoviruses, binds specifically to MDA-5, and inhibits the activation of the IFN-β promoter by dsRNA (Kang et al. 2004). The functional difference between RIG-I and MDA-5 remains unknown.

4 Conclusion

Although each TLR signaling pathway was formally thought to be identical, identification of TLR ligands clearly revealed the difference in gene expression patterns exerted by individual TLR ligands. The differences among TLR responses primarily depend on the differential utilization of adaptor molecules, which then activate distinct downstream signaling molecules. There are two main pathways in TLR signaling, MyD88-dependent and TRIF-dependent pathways. The former pathway is essential for inflammatory and immune responses including inflammatory cytokine production whereas the latter is involved in antiviral responses such as type 1 IFN induction. Recent data show that type 1 IFN production in response to TLR7 or TLR9 ligand depends on MyD88, and requires formation of a complex consisting of MyD88, TRAF6, IRAK1, and IRF7. This mechanism explains production of a large amount of type 1 IFN in PDC during viral infection. Evidence supporting the presence of intracytoplasmic sensors of pathogens is now emerging in mammals, as was the case with plants. NOD1 and NOD2 recognize peptidoglycan breakdown products, and the mutation of NOD2 is responsible for development of Crohn's diseases. RNA-helicases including RIG-I and MDA5 recognize dsRNA and are suggested to be involved in type 1 IFN induction during viral infection. Future investigation will reveal how much TLRs, intracytoplasmic sensors, or a combination of these molecules contribute to the host response to pathogens.

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Dendritic Cells: Translating Innate to Adaptive Immunity

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Abstract The innate immune system provides many ways to quickly resist infection. The two best-studied defenses in dendritic cells (DCs) are the production of protective cytokines—like interleukin (IL)-12 and type I interferons—and the activation and expansion of innate lymphocytes. IL-12 and type I interferons influence distinct steps in the adaptive immune response of lymphocytes, including the polarization of T-helper type 1 (Th1) $CD4^+$ T cells, the development of cytolytic T cells and memory, and the antibody response. DCs have many other innate features that do not by themselves provide innate resistance but are critical for the induction of adaptive immunity. We have emphasized three intricate and innate properties of DCs that account for their sentinel and sensor roles in the immune system: (1) special mechanisms for antigen capture and processing, (2) the capacity to migrate to defined sites in lymphoid organs, especially the T cell areas, to initiate immunity, and (3) their rapid differentiation or maturation in response to a variety of stimuli ranging from Toll-like receptor (TLR) ligands to many other nonmicrobial factors such as cytokines, innate lymphocytes, and immune complexes. The combination of innate defenses and innate physiological properties allows DCs to serve as a major link between innate and adaptive immunity. DCs and their subsets contribute to many subjects that are ripe for study including memory, B cell responses, mucosal immunity, tolerance, and vaccine design. DC biology should continue to be helpful in understanding pathogenesis and protection in the setting of prevalent clinical problems.

1
Innate Defenses Provided by DCs

1.1
Introduction

The innate and adaptive arms of the immune system deal with a remarkable diversity of microbial agents. Many defense mechanisms have evolved. Some of the innate defenses (Table 1) are known to be well developed in dendritic cells (DCs), particularly the production of immune-enhancing cytokines and the mobilization of innate lymphocytes (NK, NKT, γδT). In addition, DCs have a number of innate or built in properties that lead to strong, adaptive immunity including memory. DCs are sentinels. They are able to capture and process antigens effectively and to migrate to lymphoid tissues, where the immune system is alerted to make an appropriate response. At the same time, DCs are sensors, undergoing extensive, stimulus-dependent and typically irreversible differentiation, which is called "maturation." The maturation stimulus influences the type of the ensuing immune response, e.g., T-helper type 1 (Th1) vs Th2 types of $CD4^+$ T cell responses [1–3]. DCs undergo maturation in response to microbial and nonmicrobial stimuli and initiate adaptive immunity not only to infection but also to transplants, tumors, autoantigens, and allergens. Following a brief summary of the innate defenses that are provided by DCs, we will concentrate on the properties that allow these cells to link innate and adaptive immunity, particularly antigen-specific, T cell-mediated immunity.

Table 1 Mechanisms of innate resistance to infection

Phagocytosis
Secretion of proteins and peptides
Microbial binding lectins and pentraxins
Defensins and other antimicrobial peptides
Complement
Interferons and other cytokines
Innate lymphocytes

1.2
Definition

The term "innate immunity" has two equally rich meanings. One refers to innate "defenses," processes that provide rapid resistance to microbial infection (Table 1). There are many such defenses, but as a group they do not have the

degree of specificity and the memory that are characteristic of adaptive resistance. Innate defenses are expressed by many different bone marrow-derived cells and in some cases by non-hematopoietic ones. A second meaning of innate immunity is "built in." These are properties that often do not directly contain a pathogen but instead allow for the transition to adaptive immunity. Several innate features are most developed in DCs including antigen capture and presentation, homing to lymphoid tissues, and maturation in response to a plethora of stimuli. These features of DCs allow an early response to be translated into adaptive resistance and memory.

1.3 Phagocytosis

The classical innate defense mechanism is phagocytosis, followed by intracellular killing and digestion. This is the form of host resistance discovered by Metchnikoff, the father of innate immunity. Granulocytes and macrophages are the principal phagocytes. DCs have phagocytic properties but, typically, uptake is not extensive and limited to one or a few particles per cell. Moreover, phagocytosis by DCs is not yet known to contribute to microbial clearance and killing, i.e., innate defenses, although it is clearly valuable for efficient antigen processing and presentation on MHC class I and II products [4, 5].

1.4 Cytokines

Several innate defenses involve the secretion of proteins and peptides (Table 1). For DCs, cytokines have been the main products studied to date. For example, DCs are known to produce large amounts of interleukin (IL)-12 and type I interferons. In some instances where it has been studied, DCs are the major source of these immune-enhancing cytokines upon microbial exposure in vivo [6, 7]. These cytokines provide innate resistance, e.g., IL-12 mobilizes natural killer (NK) cells while interferons are anti-viral. In addition, innate lymphocytes and interferons act back on the DCs to drive maturation, and on T and B lymphocytes to enhance adaptive immunity (reviewed in [8, 9]).

1.5 Innate Lymphocytes

DCs are distinct in being able to expand the numbers and function of different types of innate lymphocytes, and then to respond through maturation to induce adaptive immunity. Innate lymphocytes are able to produce large amounts of protective cytokines within hours of stimulation, particularly

type II or γ-interferon, but the lymphocytes do not seem to develop memory. NK cells are the prototype [10–12], but NKT cells [13, 14] and γδ T cells [15] might be included. The interaction of DCs with these innate lymphocyte leads to tumor necrosis factor (TNF)-α and IL-12 production by the DCs, as well as further DC differentiation or maturation to elicit adaptive immunity to captured antigens. This topic has been reviewed elsewhere [16], including the fact that some innate lymphocytes are able to sense both infected and transformed or "stressed" cells.

1.6 Summary

DCs exhibit innate responses that can lead quickly to resistance or defense against infection. The two best-characterized responses are the production of large amounts of immune-enhancing cytokines and the mobilization of innate lymphocytes. However, as we shall now discuss at length, DCs also have innate properties that allow for the induction of adaptive immunity. Three sets of innate features are vital to the initiation of adaptive immunity. These are: (1) the distribution of DCs in vivo as sentinels for antigen capture and clonal selection of T cells, (2) the repertoire of antigen receptors and processing capacities expressed by DCs, and (3) the ability of DCs to sense microbial and other stimuli to undergo maturation.

2 The Tissue Distribution and Migration of DCs In Vivo

2.1 Definition

DCs were discovered as a distinct type of leukocyte with distinct functions, particularly potent stimulation for responses by naïve and resting T lymphocytes. The DCs could be identified and enriched based on several properties: their expression of high levels of antigen-presenting MHC class II molecules (originally termed "Ia antigens" [17, 18]), a lack of typical properties of macrophages and lymphocytes, a distinct morphology including a stellate cell shape and unusual motility involving the continual extension of cell processes in many directions, and the 33D1 [19, 20] and NLDC-145 antigens [21]. Many of these properties were used to identify DCs in different tissues [22–26], including their localization in tissue sections [27]. DCs were found at body surfaces, in the interstitial spaces of most organs except the brain parenchyma, in afferent lymph, and in the T cell areas of spleen, lymph

nodes, and Peyer's patch. The identification of DCs in lymph [28–32] was critical to the idea that DCs could leave peripheral organs and migrate to the T cell areas to initiate cell-mediated immunity. The migration of DCs has recently been reviewed [33].

2.2 DCs at Body Surfaces

The translation of innate to adaptive immunity requires that antigens, which are typically deposited in peripheral tissues during infection, gain access to lymphoid organs. The latter are designed to facilitate the selection of rare clones of antigen-reactive lymphocytes from the recirculating pool. DCs fulfill this requirement, serving as sentinels to pick up antigens and then move to the lymphoid organs to initiate immunity. DCs form networks of cells along body surfaces, often intimately associated with the epithelium. In skin, the DCs or Langerhans cells are found in the suprabasal layer [34]. In respiratory epithelium, DCs are found within and just below the epithelium [35, 36]. In the intestine, DCs lie just below the epithelium [37], but in some instances—as in the ileum—they send processes between the occluding junctions into the lumen [38, 39]. In mucosal-associated lymphoid organs, like the Peyer's patch, but also oral-associated lymphoid organs like the tonsils and nasal-associated lymphoid tissue, DCs lie beneath a specialized epithelium containing antigen-transporting M cells [40–42]. A new network of DCs has just been identified in the muscularis layer of the mouse intestine [43]. In order for DCs to home to inflamed epithelia, an important interaction is the chemokine CCL20/MIP-1α made by the epithelium and the chemokine receptor CCR6 on the DCs [40, 44]. Fractalkine and CXCR3 additionally are responsible for the extension of DC processes through epithelia [38]. In the steady state, i.e., in the ostensible absence of infection and inflammation, peripheral DCs continuously capture environmental proteins, e.g., from the airway and intestinal lumen [45, 46], as well as self constituents [47]. In sum, DCs are positioned for antigen capture, but what is interesting is that this seems to go on during the steady state, a feature that may allow DCs to induce tolerance to harmless self and environmental antigens, as we will stress (Sect. 6.4).

2.3 DCs in Afferent Lymph

When afferent lymphatic vessels are cannulated, DCs are always found in the effluent, so that DCs seem to be migrating continuously in the lymph, from tissues to lymphoid organs [48]. The protein within the lymph then leaves the

lymphoid tissue in efferent lymphatics to enter the thoracic duct and return to the blood stream, whereas the DCs remain within the local lymphoid organ and are typically not found in efferent or thoracic duct lymph. Even in the steady state, DCs are carrying cargo from the periphery. For example, in the mesenteric lymph, a subset of DCs contain apoptotic bodies, earlier termed DNA positive inclusions [31]; these cells also can be marked for the keratins and nonspecific esterases of the intestinal epithelium [47]. The DCs in the lymph in the steady state may arise from DCs that are trafficking from the blood into tissues prior to entry into the lymph, but a subset of monocytes may also be a source [49–51]. DC migration into the afferent lymph can also be increased markedly in response to many different stimuli. This likely involves an increased expression of CCR7 on the DCs [52] as well as the corresponding chemokines, CCL19 and 21 on lymphatics and in the lymph node [53, 54]. Recently, Pasare and Medzhitov used lipopolysaccharide to stimulate DC migration during protein immunization [55]. They found that a lack of the Toll-like receptor (TLR) adaptor protein MyD88 blocked immunization but did not alter the increase in DC migration to the lymph nodes in response to lipopolysaccharide (LPS), indicating that these components of DC function—immunization and migration—are separately controlled [55].

2.4 DCs in Blood

DCs have been studied in blood from humans and monkeys, where they comprise at least two subsets termed myeloid and plasmacytoid (PDCs) [56], expressing high and low levels of the CD11c integrin in humans. In mice, PDCs do express some CD11c. Myeloid and PDCs are distinguished as well on the basis of commercially available antibodies to blood DC antigens or "BDCAs," e.g., BDCA-1 for myeloid DCs and BDCA-2 for PDCs. Circulating myeloid DCs and PDCs may derive directly from the marrow in the steady state, and these outputs can increase in infection [57, 58]. During perturbation it is additionally possible that DCs can be mobilized from tissues and move into the blood and then to the spleen. The latter seems to occur during transplantation [59] and would allow DCs from the graft to stimulate the direct pathway of rejection, as occurs in the primary mixed leukocyte reaction, a classical assay for the immunostimulating function of DCs [60].

2.5 DCs in the T Cell Areas of Peripheral Lymphoid Tissues

Once in the lymphoid organ, most DCs are found in the T cell areas. Traffic to the B cell areas may well occur but needs further definition. It is often

assumed that DCs in the T cell areas are continually derived from the lymph, but precursors to some subsets of DCs may enter from the blood. Also, proliferating DCs and DC precursors have been identified in mouse spleen [61] and skin [62], allowing for local regeneration and expansion of DC numbers. Identification of DCs in tissue sections is achieved by a combination of criteria in addition to their large irregular cell shape. These include high expression of MHC class II and the CD11c integrin, a lack of lymphocyte and macrophage markers, and expression of various receptors for antigen uptake (see Sect. 3). The distribution of DCs in the lymph node has now been visualized by two-photon microscopy of living tissue. Migrating mature DCs arrive in the T cell area where they efficiently select T cells specific for the presented antigen [63–65]. In the T cell area, these DCs join a network that is present in the steady state [66]. Stable contacts develop when antigen-bearing DCs encounter their cognate T cells, and these contacts persist for a day or more, both in the steady state when DCs can be tolerogenic, or upon maturation when immunity develops [67, 68]. This fits the observation that the commitment of T cells to proliferation, which can occur prior to either tolerance or immunity [69–71], requires sustained triggering by antigen or mitogen for about a day [72]. In the steady state, one also can observe some DCs in the subcapsular sinus, quite distinct from the highly endocytic and more numerous macrophages there [66]. These DCs seem to be in transit from lymph to the deeper T cell areas. In contrast, the network of DCs in the T cell area continually form and retract processes but do not move translationally [66], much as was observed ex vivo when DCs were discovered more than 30 years ago [73]. In sum, while DCs constitute just a few percent of the cells in a lymph node, their size and pervasive cell shape puts them in a position to scan T cells circulating through lymphoid tissues and then to select clones appropriate for the presented antigens.

2.6
DCs at Mucosal Surfaces and in Mucosal-Associated Lymphoid Tissues

As mentioned, DCs are positioned for antigen capture at internal or mucosal body surfaces. Their subsequent traffic can include movement to the draining lymph nodes, e.g., the mesenteric node in the case of the intestine, as well as movement to the T cell areas ("interfollicular zones") of the mucosal-associated lymphoid tissues, like the Peyer's patch. It is possible that DCs in the epithelium and lamina propria home to the mesenteric lymph node, whereas DCs beneath the antigen-transporting epithelium of the Peyer's patch home to the local T cell area. Injection of TNF-α or TLR ligands leads to a marked mobilization of DCs from the intestine into the mesenteric afferent

lymph [74]. DCs at mucosal surfaces are likely to be important for studying the generation of protective immunity as well as the maintenance of tolerance against chronic inflammatory disease and allergy.

2.7 Summary

A significant innate property of DCs, which facilitates their function in the initiation of adaptive immunity, is their ready access to antigens and their capacity to move to the peripheral lymphoid tissues. Antigen uptake can be enhanced by numerous potential endocytosis receptors (next section), while migration to the T cell areas facilitates the selection of T cell clones and determines T cell fate decisions involved in peripheral tolerance and immunity. These features of DCs are deployed in the steady state, where they can be used to induce peripheral tolerance to self and harmless environmental antigens (reviewed in [75]). There are still substantial gaps in our knowledge of several topics, e.g., the control mechanisms for DC migration and homing in the steady state, movement into the B cell areas, and traffic at mucosal surfaces.

3 Antigen Uptake Receptors and the Endocytic System of DCs

3.1 Definition

The translation of innate to adaptive immunity requires that antigens be captured and processed intracellularly prior to the formation of ligands ("presented antigen") for the T cell receptor. Generally these ligands are complexes of peptides with MHC products, but others are being identified, such as complexes of glycolipids with CD1 molecules. The term antigen presentation is best restricted to the uptake, processing, and presentation of processed antigens, e.g., as peptide–MHC complexes. All cells with MHC products present antigens, but as we will summarize here, DCs are specialized in each of the uptake, processing, and presentation steps. In subsequent sections, we will consider the additional accessory or costimulating functions of DCs that by definition work together with presented antigen to stimulate adaptive immunity.

3.2 A Spectrum of Potential Antigen Receptors on DCs

Early studies with cells from mice [76–79] indicated that many DCs were in an immature state and needed to acquire their immune-inducing capacities. In

some instances this only required a short period in culture. The term "immature" described the lack of strong T cell-stimulating activity, but the capture of antigens is often selectively expressed by immature forms of DCs [80, 81]. Immature DCs express an array of receptors that are able to mediate endocytosis (Fig. 1). Uptake function has been studied in three ways. In some cases ligands have been followed into the cell. In others, antibodies to the lectin have been used as surrogate ligands to document uptake. Third, endocytosis has been predicted by characteristic coated pit localization sequences in the cytosolic domains and additional motifs for targeting within the cell.

Many uptake receptors on DCs, and the list seems to be expanding, are calcium dependent or C-type lectins (Fig. 1). These may form multimers but not by covalent means, in contrast to the numerous dimeric C-type lectins that are expressed on NK and other cells. C-type lectins on DCs can either be type II transmembrane proteins with a single, carboxy terminal lectin domain, or type I proteins with multiple lectin domains. The sugar recognition properties of some of these lectins have been defined, but in most instances, relatively little is known about the natural ligands for these lectins. DC-SIGN/CD209 is a well-studied exception, since it is able to recognize mannose and fucosyl residues on the surface of a variety of pathogens including human immunodeficiency virus (HIV), cytomegalovirus (CMV), Ebola virus, dengue virus, Candida, and certain Leishmania [82], while the macrophage mannose receptor/CD206 recognizes mannose residues on certain self constituents, lysosomal hydrolases [83]. DCs also express several potential receptors for dying cells, although these are still poorly defined in vivo (Sect. 3.5). Additionally, DCs express FcγRs (and FcεRs), which mediate presentation of immune complexes and antibody coated tumor cells on both MHC class I and II. FcγRs also influence the state of DC maturation. Immunoreceptor tyrosine-based acti-

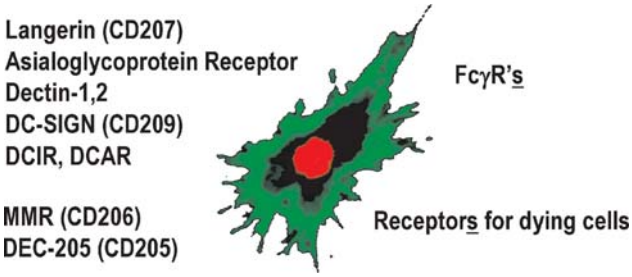

Fig. 1 Potential receptors for antigen uptake by dendritic cells (DCs). Some of the lectins that are expressed by DCs, or DC subsets, are shown. There are several Fcγ receptors for uptake of immune complexes and receptors for dying cells. However, relatively little information on DC receptors for dying cells is available in vivo

vation motif (ITAM)-associated receptors stimulate maturation, while FcγRs with inhibitory immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences block it [84, 85]. For most of the potential uptake receptors on DCs, much of the research has been on isolated cells and not in vivo.

3.3 Functions of Endocytic Receptors

There is considerable potential to receptor function beyond ligand binding and uptake (Table 2). We will cite four examples. First, receptors can associate with other signaling molecules. Dectin-1, which is expressed on macrophages and DCs, binds yeast or zymosan particles but, in addition, dectin-1 associates with TLR-2 and thereby signals TNF-α and IL-12 production [86, 87]. Dectin-1 also has an ITAM motif that, following phosphorylation, attracts the src kinase, syk, and mediates production of two other cytokines, IL-2 and IL-10 [88]. Second, receptors can follow distinct intracellular trafficking pathways dictated by sequences in the cytosolic domain. For DEC-205/CD205, a stretch of three acidic amino acids, allows this receptor (uniquely at this time) to slowly recycle through MHC class II positive late endosomal compartments [89]. The MMR/CD206, in contrast, behaves in a more typical way for a coated pit localized receptor, i.e., it enters the cell and rapidly recycles through early endosomal compartments. The trafficking of DEC-205 through MHC II compartments may explain its greatly increased capacity for class II presentation relative to the macrophage mannose receptor (MMR) [89]. A third enigmatic consequence of antigen uptake is cross presentation on MHC class I by DCs, which is evident for proteins captured within dying cells, immune complexes, and DEC-205-associated antigens. DCs are a major cell type for cross presentation in vivo [5, 70, 90–92], but it is not clear how this cross presentation comes about. One proposal is that fusion of the endocytic vesicle with the rough endoplasmic reticulum (ER) is required [93,

Table 2 The endocytic system of dendritic cells

Many potential uptake receptors, sometimes expressed in a DC subset-restricted fashion
Uptake receptors can associate with other signaling molecules like TLRs.
Uptake receptors can route to distinct antigen-processing compartments.
Regulation at many levels, e.g., uptake, intravacuolar pH, protease content
Processing of antigens towards the formation of ligands for T cell receptors, e.g., MHC-peptide, seems efficient, occurring with relatively low doses of administered antigen.

94], but this pathway has been called into question [95]. For some proteins, cross presentation onto MHC class I is clearly dependent on transporters of antigenic peptides (TAP), implying that proteins or their fragments exit the endocytic system for potential proteolysis and TAP transport. A conserved tyrosine in the cytosolic domain of MHC class I molecules may be required for the exogenous pathway, by controlling traffic to a special intracellular compartment [96]. An important part of the cross presentation equation is that in mice, the CD8α^+ subset of DCs is the principal cross-presenting cell in spleen and lymph nodes [5, 91, 97]. This probably reflects two functions: the capacity of CD8α^+ DCs to internalize certain ligands, like dying cells and anti-DEC-205 associated antigens [5, 70], and also to efficiently cross-present the internalized ligands following uptake [97, 98]. Fourth, distinct receptors can be expressed on distinct subsets of DCs, which in turn may influence the consequence of antigen uptake and processing (Sect. 3.6). Altogether, the presence of numerous uptake receptors enables DCs to efficiently take up many different ligands, but also, the receptors mediate distinct "post uptake" outcomes.

3.4
The Endocytic System of DCs and Its Regulation

The endocytic system, and not just the repertoire of uptake receptors (Fig. 1), is proving to be a distinctive innate feature of DCs (Table 2). At this point, much of the research involves studies of DCs generated from mouse bone marrow progenitors, or from human monocytes, i.e., there is relatively little information on the bulk of the DCs that occupy lymph and lymphoid tissues. What is emerging from the ex vivo studies is a significant and perhaps unique regulation of the DC endocytic system at several levels (reviewed in [99]). To begin, pinocytosis and phagocytosis can be curtailed when DCs undergo maturation, through inactivation of a required rhoGTPase [100]. This seems to limit the presentation of peptides to antigens that are captured in the periphery, when the DCs are immature and responding to innate stimuli, and not to self antigens that would be taken up following arrival of the mature DCs in the T cell areas. The lysosomes of certain immature DCs are also unusual relative to other cells, particularly macrophages, in that proteins are degraded slowly by DCs. This reflects two features: a relatively high intravacuolar pH [101] and a relative lack of proteases (rather than other lysosomal acid hydrolases) [102]. When DCs receive a maturation stimulus, a proton pump assembles on the vacuolar membrane, the pH falls to 4.5–5.0, and proteolysis begins (presumably at a limited rate relative to scavenger macrophages). As a result, invariant chain and antigen catabolism are enhanced, thus freeing the MHC class II peptide-binding groove for binding of antigenic peptides. Following the for-

mation of peptide-MHC II complexes within maturing DCs, the complexes move within distinct nonlysosomal vesicles to the cell surface [103, 104]. In the case of bone marrow-derived DCs, these transport organelles also contain the costimulatory molecule CD86, which then remains clustered with the peptide–MHC complexes at the cell surface [103]. Possibly this clustering in maturing DCs translates into efficient and prolonged costimulation of the TCR and CD28 on T cells.

3.5 Uptake of Dying Cells by DCs

The uptake and processing of dying cells provides DCs with the means to present cell-associated antigens to both $CD4^+$ and $CD8^+$ T cells. When it comes to identifying receptors used by DCs to capture dying cells in vivo, there is a striking finding, which is that the $CD8\alpha^+$ subset of mouse DCs selectively captures autologous dying splenocytes, allogeneic cells killed by NK cells, tumor cells, and virus-infected cells [5, 105]. In contrast, for latex particles, both $CD8\alpha^+$ and $CD8\alpha^-$ DCs show comparable phagocytic activity. To date, the mouse is the primary species in which the expression of the CD8αα homodimer serves as a DC subset marker, but a $CD4^-$ subset of rat DCs seems to be an analogous subset that handles dying cells in vivo [47].

In macrophages, there are many receptors that can contribute to the uptake of dying cells, but again, much of the evidence has come from in vitro approaches. Some receptors that have been implicated in uptake include scavenger receptors [106], the phosphatidyl serine receptor [107], certain integrins [108, 109], CD91/calreticulin [110], CD14 [111], C1qR [112], CD93 or C1qRp [113], and CD36 [114]. For some of these, a role for uptake in vivo has been obtained, i.e., for the Mer family of tyrosine kinases [115], the phosphatidyl serine receptor [116], and MFG-E8 [117]. However, the vivo functions that have been studied have not included to antigen presentation.

In DCs, one has a reciprocal situation to the macrophage. There is good evidence for the presentation of dying cells to T cells in vivo, but the responsible receptors remain to be identified. Some dying cell receptors in macrophages can be expressed by immature DCs [118–122], but this research has not been extended in vivo. As mentioned, a perplexing feature is the selective uptake of certain dying cells by $CD8\alpha^+$ DCs. CD36, DEC-205, and Langerin are all selectively expressed, but to date, none of these molecules seems essential for the uptake of dying cells [123, 124]. An understudied topic is the death and reprocessing of DCs themselves, which has been documented when DCs arrive in a lymph node and are processed by resident DCs there [4]. The uptake of dying cells in vivo is a major area of DC biology and extends to many

clinically relevant topics, since cell death takes place in self tissues, tumors, transplants, and infected cells, allowing DCs to capture antigens for purposes of tolerance and immunity.

3.6
Distinct Endocytic Receptors on DC Subsets

Another feature of uptake receptors is that they can be restricted to subsets of DCs. DEC-205/CD205 and Langerin/CD207 (Fig. 1) are expressed primarily on the CD8α$^+$ subset of DCs in spleen and lymph node (although in skin-draining lymph nodes, the LCs that migrate from skin to the node are CD205/207 high but CD8α low [125]). The CD8α$^+$ subset of DCs in mice is also specialized to take up dying cells, and this is postulated to involve distinct uptake receptors. DC-SIGN/CD209 is enigmatic because in mice there is a lack of antibodies to this lectin, but the messenger RNA (mRNA) is primarily found in CD8α low DCs (H. Hemmi, unpublished) [126]. This area of DC biology is just emerging, but the suggestion is that DC subsets are predetermined to capture distinct ligands through distinct receptors.

3.7
Processing of Glycolipids and Presentation on CD1 Family Molecules

The CD1 family of nonclassical MHC class I-like molecules recognizes various glycolipids, both microbial and self derived. DCs are a major site for the expression of CD1a (Langerhans cells), CD1b and CD1c (dermal DCs, other interstitial DCs, and myeloid DCs) and CD1d (most DCs). The different CD1s seem to capture glycolipids from different endocytic compartments, e.g., CD1a is primarily found in early endosomes, whereas CD1b and CD1c localize to late endosomes [127, 128]. The research on CD1a, b, and c is for the most part limited to ex vivo studies, since these molecules are found in humans, not mice.

Presentation on CD1d is well developed in mice and is increasingly studied in vivo. CD1d presents glycolipids to the invariant TCR on NKT lymphocytes. The glycolipids can derive from endogenous [129], microbial [130, 131], and pharmacologic or synthetic [132–134] sources. An important feature of CD1d presentation by DCs to NKT cells is that it leads to changes in DC function. A single dose of the synthetic glycolipid α-galactosyl ceramide leads to DC maturation and to Th1 CD4$^+$ and CD8$^+$ T cell responses to protein antigens that are simultaneously captured [13, 14]. On the other hand, multiple doses of the glycolipid dampen immunity, and this can involve the formation of regulatory IL-10-producing DCs [135]. Since NKT cells can differentiate along

several functionally distinct pathways (Th1 and Th2, T reg), the capacity of DCs to handle CD1 binding glycolipids provides another dimension to the control of immunity.

3.8 Summary

A distinct innate feature of DCs is their endocytic system (Table 2), which helps to explain the efficiency with which these cells translate innate to adaptive immunity. Relatively low doses of antigen often suffice for DCs to present antigens to T cells [69, 70, 92, 136]. DCs express a large number of potential uptake receptors. Some of these already are known to lead to presentation on both MHC class I (the cross-presentation or exogenous pathway) and MHC class II products, and very likely to other presenting molecules like the CD1 family. The endocytic system of DCs is peculiar in its capacity for regulation at many levels during maturation, including the expression of uptake receptors, formation of endocytic vacuoles, and the acidity and therefore activity of the DC vacuolar system. A good deal of cell biology needs to be deciphered. For example, what is the nature of the cross-presentation pathway to MHC class I where DCs seem so adept? Are there specializations for antigen presentation to B cells, since DCs can have direct effects on B cells? What are the consequences of selective expression of specific pattern-recognition receptors on subsets of DCs? How do DCs capture many types of dying cells in vivo, and do the dying cells further influence (increase or decrease) DC maturation? Overall, the endocytic system of DCs seems specialized for antigen presentation rather than clearance and scavenging, as is the case of professional phagocytes like macrophages and granulocytes.

4 Costimulatory Molecules of Dendritic Cells

4.1 Definition

The term costimulation embraces many concepts and molecular players. The classical meaning is that costimulation provides a “second signal,” in addition to peptide-MHC or “signal one,” and this second signal leads directly to immunity. The B7 family of molecules is the best studied, but this family also includes negative regulators of T cells, such as PD-L1 or B7-H1. DCs can express high levels of these B7 second signals, but as we will discuss, high expression of B7s by itself may not lead directly to the initiation of immunity.

A further concept is that the absence of costimulation leads to tolerance by anergy or deletion, rather than clonal expansion. DCs are specialized inducers of peripheral tolerance in the steady state, which means that targeting of antigens to DCs greatly increases the efficiency with which tolerance can be induced [69, 70, 92, 137]. Interestingly, tolerance via DCs can require more than the expression of peptide–MHC or "signal one" on DCs. Additional B7 family molecules contribute, such as PD-L1 (a ligand for PD-1, programmed death-1, on T cells) and even CD80/86 (which interacts with the negative regulator CTLA-4 on T cells) [138]. The functional consequences of costimulatory molecules, even if one only considers the B7 family, are therefore intricate and can be both immune enhancing and regulatory.

4.2 The B7 Family

As emphasized by Janeway and Medzhitov, a critical link between innate and adaptive immunity is the presence of pathogen recognition receptors that signal the upregulation of T cell costimulators, and thereby the initiation of adaptive immunity [139]. A lack of two B7 molecules, CD80 and CD86, greatly reduces the immunizing capacity of antigen presenting DCs in vivo [140]. However, the situation is proving to be more complicated than the hypothesis that the expression of CD80/86 is sufficient to allow DCs to initiate adaptive immunity.

First, many DCs in lymphoid organs seem to express costimulatory molecules in the steady state, particularly the B7 family as well as CD40. However, the targeting of antigen to these DCs does not lead to a primary immune response, as is evident from studies with dying cells [92] and antigens targeted to the DEC-205 receptor [69, 70]. Therefore, in the steady state, $CD86^+$ DCs can be functionally immature, i.e., weak at directing T cell differentiation toward interferon-ɣ production and cytolysis, and in establishing memory. One possibility is that CD86 becomes immunostimulatory on DCs when it is expressed at much higher levels or is physically aggregated with the presented MHC–peptide complexes. DCs express relatively high levels of CD86 and other B7 family molecules relative to other cells [141, 142], and as mentioned, maturing DCs discharge clusters of CD86 with MHC II-peptide complexes, which then persist for many hours on the DC surface [103]. This clustering may allow costimulation to begin.

A second feature is that DCs express "negative costimulators" like PD-L1 or B7-H1. PD-L1 ligates PD-1, which allows DCs in the steady state to mediate peripheral T cell tolerance rather than immunity [138]. Also, DCs upregulate both positive and negative costimulators when they are induced to mature.

Likewise, PD-1 and CTLA-4 typically are upregulated on T cells later in the response to antigen, whereas the positive costimulator CD28 is expressed by naïve T cells.

A third perplexing point relates to the consequence of the upregulation of B7 molecules on DCs during maturation. DCs are striking in the rapidity with which increased expression of B7 family members takes place (4–8 h) and the magnitude of expression, e.g., mature DCs can express ten times the levels of CD86 relative to activated B cells and macrophages [141]. However, new evidence indicates that the heightened expression of CD86 and CD80 is not itself sufficient for the induction of immunity, beginning with the paper by Fujii et al. on DC maturation induced by NKT cells [140]. When DCs took up antigen and simultaneously were induced to mature by NKT cells activated by α-Gal Cer, the DCs induced $CD4^+$ and $CD8^+$ T cell immunity. Immunization was ablated when the DCs lacked CD40, but it was shown that these CD40-negative DCs nonetheless presented antigen well to $CD4^+$ and $CD8^+$ T cells and expressed very high levels of CD80 and CD86. Thus, a CD40-dependent maturation event was required beyond signals one (MHC-peptide) and two (high levels of CD80 and CD86), and this was not the production of IL-12 [140]. Likewise, Sporri and Reis e Sousa showed that all DCs in mouse spleen upregulate CD86 in response to in vivo administration of TLR ligands, but, in an elegant experiment, they found that only the DCs that responded directly to the TLR ligands—as opposed to bystander DCs—were capable of inducing antibody responses with a Th1 isotype profile [143]. In sum, high levels of B7 costimulators represent a characteristic feature of maturing DCs, but there are additional costimulatory features that control the quality and quantity of the primary immune response and very likely memory.

4.3 The TNF-TNF-Receptor (TNF-R) Family Including CD40

Immature DCs, such as some DC subsets in lymphoid tissues, express the TNF-R family member CD40, but expression is enhanced further with maturation. CD40 ligation mediates many important steps of DC biology, including their development from progenitors [144], migration to and survival within the T cell areas [145, 146], improved presentation on MHC class I molecules [147], production of IL-12 [148], and, as mentioned above, the maturation of DCs to induce combined Th1 $CD4^+$ and $CD8^+$ immunity [140]. DCs can express many different TNF and TNF-R family members. More research on the functions of these molecules in vivo is needed.

4.4
The Notch Family

It is newly recognized that DCs can express members of the notch family including jagged-2 and delta-1. The experiments are, for the time being, in culture, where T cell fate decisions can be determined by the type of notch protein expressed on the DC, e.g., delta-1 for Th1 and jagged-2 for Th2 [149].

4.5
Costimulatory Cytokines

Cytokines are increasingly on the center stage of immunology, including costimulation of adaptive immunity. IL-12 and type I interferons are the best-characterized mediators for such adaptive responses as the development of Th1 type T cells [150], antibodies [151, 152], and cytotoxic T cells [153]. These cytokines also feed back to induce or sustain DC maturation, e.g., IL-12 can recruit NK cells that mature DCs and type I interferons do likewise. IL-6 production by DCs allows effector T cells to overcome suppression by $CD4^+$ $CD25^+$ T cells [154]. An interesting new subset of DCs is termed "Tip DCs" (for *T*NF and *i*nducible nitric oxide synthase *p*roducers) [155, 156]. Tip DCs have the potential to protect against an infection like listeriosis [155], and to cause pathology, as in psoriasis [156]. Therefore, cytokines are likely to be a major contributor to the innate protective functions of DCs and in the translation of innate to adaptive immunity.

4.6
Other Costimulatory Molecules

DCs can express high levels of ICAM-1/CD54 and LFA-3/CD58, which are recognized by LFA-1/CD11a and CD2 on T cells respectively. The reciprocal is also true, i.e., DCs can express CD11a and CD2. DCs express different semaphorins, e.g., semaphorin 4A and 6C, which can increase T cell differentiation toward Th1 [157]. DCs produce chemokines, which not only attract specific subsets of lymphocytes but also may contribute to costimulation [158]. An intriguing finding relates to the T-bet transcription factor, originally discovered as a key to Th1 differentiation in T cells [159]. T-bet is also expressed in maturing DCs, and T-bet knockout DCs are strikingly deficient in inducing Th1-type T cell immunity [160].

4.7
Summary

DCs are powerful accessory or costimulatory cells for T cell responses including the initiation of immunity in vivo. DCs can express the highest levels of

the traditional costimulator CD86, as well as a wide range of other accessory molecules, especially TNF family members and cytokines, which influence T cell differentiation and possibly memory. This section has mentioned a few of the enigmas in this field. What is the precise role of heightened CD80 and CD86 expression that occurs during DC maturation? What are the consequences for T cells of individual TNF family members (e.g., OX40L, 4-1BBL, GITR) on DCs? How does the transcription factor T-bet alter DC function? Is the production of inflammatory cytokines (e.g., TNF-α, IL-6, IL-12, interferons) one of the most critical means whereby DCs translate innate to different forms of adaptive immunity? We suspect that the key to the function of DCs in adaptive immunity is not so much the expression of qualitatively distinct costimulators. There are other possibilities, such as quantity and speed of expression, which are in turn coupled to special cell homing and antigen capture/processing functions (above).

5
DC Maturation: The Link Between Innate and Adaptive Immunity

5.1
Definition

The term maturation was first used to describe the development of DCs that is required for the induction of immunity [76, 77]. The initial experiments involved Langerhans cells (LCs), which were found to be weak stimulators of the mixed leukocyte reaction and other T cell proliferative responses to mitogens. The LCs only became strong stimulators after culture in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), and this maturation of T cell stimulatory function was accompanied by extensive differentiation, i.e., the appearance and loss of many DC markers and the development of a highly "dendritic" morphology. Strikingly, freshly isolated LCs could capture antigens for presentation to activated T cells, while the mature LCs did not process proteins [80, 81]. These observations, which antedated current ideas about costimulation, clearly distinguished two broad requirements for immunity: an antigen capture step that was carried out by immature LCs and an accessory (later "costimulatory") function that was carried out by DCs that were surprisingly no longer capable of antigen capture. Shortly thereafter, when monoclonal antibodies to CD86 became available, it was recognized that maturing DCs could upregulate the expression of B7-2/CD86 rapidly and to much higher levels than other cells, e.g., LPS stimulated macrophages and B cells [141, 142]. Many scientists have considered

Table 3 Stimuli that induce features of dendritic cell maturation

Ligands for Toll-like receptors
Other microbial products like cholera toxin, filamentous hemagglutin
CD40 agonists
Cytokines, e.g., type I interferons, thymic stromal lymphopoietin
Fcγ receptors and relatives including PIRs, TREMs
Necrotic cells, heat shock proteins, urate crystals, high mobility group box protein 1
Innate lymphocytes: NK, NKT, γδT

heightened CD86 expression to be synonymous with maturation. However, as discussed above, an increased level of CD86 is a useful way to monitor DC responses to inflammation or infection, but it is not equivalent to maturation (Sect. 4.2) [140, 143], which occurs in response to TLR ligands and many other stimuli (Table 3).

5.2 Distinct Maturation Stimuli Allow DCs to Initiate Distinct T Cell Responses

A good example of the distinct pathways induced by different maturation stimuli involves the myeloid DCs that are found in human blood. When these cells encounter two distinct stimuli—thymic stromal lymphopoietin (TSLP) vs CD40L—the cells differentiate in a manner that at first glance makes the mature DCs look very similar. These changes involve heightened MHC class II and CD86 expression, and acquisition of a highly dendritic appearance. However, the TSLP DCs cause naïve T cells to differentiate into inflammatory Th2 cells that produce TNF in addition to IL-4, 5, and 13, while CD40L DCs causes naïve T cells to differentiate into Th1 cells [161]. Deeper analysis reveals that the TSLP DCs make distinct chemokines from CD40L DCs (TARC and MDC vs Mig) and fail to make inflammatory cytokines like IL-1, IL-6, and IL-12. While much of the literature on maturation deals with the identification of stimuli that allow DCs to elicit strong Th1 type immunity, it is also necessary to understand how DCs induce a more classical "noninflammatory" Th2 pathway of T cell differentiation, where TNF is not produced. This likely involves a distinct maturation response to some microbial products, like the schistosome egg antigen [162, 163] or certain allergens [164, 165].

5.3 The Consequences of TLR Ligation on DCs

DCs respond rapidly to several ligands for TLRs, which are germline-encoded innate receptors for microbial products. Ligands for TLRs have now been

identified [166, 167], and these are discussed in detail in other chapters of this book. Importantly, there are subsets of DCs that express distinct TLRs. TLR7 and 9, the TLRs that respond to nucleic acids, are primarily expressed on plasmacytoid DCs and mediate the production of large amounts of type I interferons. TLR3 is expressed at highest levels on a subset of myeloid DCs, the $CD8\alpha^+$ subset in mouse lymphoid tissues, for example. DC subsets also need to be considered when one generates DCs in large numbers from bone marrow progenitors, as is often done to facilitate research. The use of flt-3L as the hematopoietin, rather than GM-CSF, leads to the development of plasmacytoid DCs as well as cells resembling $CD8\alpha^+$ DCs, and these subsets may express distinct TLRs [168, 169].

The prototype readout for a TLR response is the activation of nuclear factor (NF)-κB and the production of inflammatory cytokines, particularly TNF-α, IL-1, and IL-6. The signal transduction pathways for NF-κB activation in DCs and other cells originate from either MyD88 and/or TRIF (MyD88-independent) adaptor proteins. More research is needed to understand the value of TNF-α, IL-1, and IL-6 on adaptive immunity, but one possibility is that these account for the ability of mature DCs to overcome suppression [154] yet at the same time promote the expansion of existing suppressor T cells specific for self and environmental antigens [170, 171].

For adaptive immunity to develop, additional transcription factors and cytokines are vital. Two cytokines have significant immune-enhancing effects on T cells. IL-12, whose production is enhanced by the transcription factor IRF-5 [172], acts on $CD4^+$ T cells to enhance Th1 differentiation. Type I interferons (many α-interferons and a single β-interferon), whose production is enhanced by the transcription factors IRF-3 and IRF-7 [173], act on $CD8^+$ T cells [153, 174] and B cells [151], to enhance the development of cytotoxic T lymphocytes (CTL), memory, and antibody formation. For several RNA viruses, interferon production is likely to proceed via an intracellular receptor, RIG-I, rather than a TLR [175, 176]. The influence of the type of antigen-presenting cell on the pivotal production of IL-12 and interferons needs to be studied more in vivo.

Importantly, the differentiation of helper T cells can be influenced by the type of TLR ligand that acts on DCs. CpG DNA in mice, a TLR9 ligand, can be a strong adjuvant for Th1-type immune responses [177] while the TLR7 ligand imiquimod has similar properties [178]. The TLR2 ligand, Pam3Cys, and the TLR5 ligand, flagellin, in contrast, have been reported to induce Th2-type responses [179–181]. Since TLR ligands act on many different types of antigen-presenting cells, it will be important to dissect the immune responses that are induced when TLR ligands engage each cell type including DC subsets in vivo. This area will likely influence vaccine design in the future.

5.4
Negative Regulators of DC Maturation

In addition to positive external stimuli for maturation, DCs are subject to several different pathways of regulation. Negative molecules that can act on both TLR and cytokine receptor signaling are the suppressor of cytokine signaling (SOCS) proteins (reviewed in [182]). Many additional inhibitory pathways for TLR signaling have also been identified (reviewed in [183]). To date these pathways have primarily been studied as regulators of the innate cytokine-producing response rather than adaptive immunity. An important cell surface pathway that suppresses IL-12 production and enhances IL-10 involves CD47 and TSP [184, 185]. Reciprocally, IL-4 can act to dampen IL-10 and increase IL-12 production by DCs [186, 187]. Interestingly, the effects of microbial ligands on DCs may depend on their stage of development, e.g., lipopolysaccharide and bacteria can inhibit the formation and differentiation of DCs from monocytes in vivo [188], even though these stimuli are classical inducers of the differentiation of immature DCs.

5.5
Negative Regulation of the Immune Response by DCs

DCs at different stages of maturation are able to expand and/or differentiate different types of suppressive pathways. These include IL-10-producing foxp3$^-$ Tr1 cells [189] and CD25$^+$ CD4$^+$ foxp3$^+$ suppressors [170, 171]. A potential suppressive pathway in DCs entails the induction of active indoleamine dioxygenase, which can be toxic to lymphocytes [190–192].

5.6
Ways to Think About the Capacity of DCs to Mediate the Translation of Innate to Adaptive Immunity

DCs are able to produce a number of immune-enhancing cytokines, particularly IL-12 and type I interferons. These molecules likely play critical roles in the link between innate and adaptive T cell immunity [172, 173]. Interferons and IL-12 mediate many pathways for adaptive resistance including antibody responses [151], Th1 CD4$^+$ T cell development [193], macrophage activation [194, 195], and cytolytic T cell formation [153]. Another observation with respect to heightened cytokine production is that DCs can express higher levels of required signal transduction proteins, e.g., NF-κB [196]. In other words, because more NF-κB is available, TLRs and other transducers of maturation may lead to stronger and more rapid responses in DCs.

While it is possible that DCs, relative to other antigen-presenting cells, express unique membrane-associated costimulatory molecules for the adaptive immune response, it is more likely that DCs simply express higher levels of costimulators like CD86, as well as many different accessory molecules as summarized in Sect. 4. In vivo, the capacity of DCs to localize to the T cell areas and select T cell clones represents a valuable innate property that contributes to adaptive immunity. Another important attribute is the ability of DCs to capture, process, and present antigens, and for long periods. This may provide the time required for T cells to commit to differentiation. A corollary has recently appeared, which is that the stability of an MHC–peptide complex accounts for the immunogenicity of so-called immunodominant peptides [197]. This means that the amount and longevity of MHC–peptide complexes on cells, which is a special property of DCs, will also increase immunity.

5.7 Summary

The thymus produces a diverse repertoire of T cell clones in a resting or naïve state. T cells must then make several critical choices involving peripheral tolerance, development of effector functions, and memory. These decisions are influenced by the maturation of DCs that are presenting antigen. There are many stimuli that induce some features of DC maturation (Table 3). In the case of TSLP and CD40L stimulation mentioned in Sect. 5.2, the two maturation stimuli induce distinct chemokines and cytokines, which helps to explain the different T cell outcomes that the DCs bring about. For many maturation stimuli, particularly for TLR ligands, more research is required to assess their consequences in vivo for the establishment of a protective primary immune response, and for memory. A potentially critical feature of DC maturation is that the sustained presentation of MHC peptide allows time for T cell molecules to be induced and act back on the DCs such as CD40L, OX40, 4-BB, and GITR. At this time, much of the literature involves ex vivo studies, TCR transgenic T cells, model antigens, and simple readouts of T cell function. By emphasizing DC biology, one has an opportunity to work directly in vivo and with more demanding antigens. The in vivo control of autoimmune diseases and the establishment of protective immunity and memory represent challenges for future research.

6
Discussion of Some Emerging Links of Innate to Adaptive Immunity via DCs

6.1
Induction of Memory

Memory in the context of infection involves long-lived responsiveness to an antigen, microbe, or vaccine as a result of a primary exposure, but not requiring persistent infection or persistent antigen [198–200]. A new clue comes from experiments in which a protein antigen is targeted via DEC-205 to DCs in vivo; the mice exhibit $CD4^+$ T cell memory for 6 months [136]. This suggests that it should be valuable to consider the biology of the antigen-presenting cell to understand the establishment of memory, with the targeting of antigens to appropriate DCs being a potentially critical ingredient.

6.2
B Cell Responses

We have concentrated on T cells, but DCs influence B cell responses as well. The traditional pathway via $CD4^+$ helper T cells needs to be pursued further. For example, it has recently been demonstrated that the induction of strong $CD4^+$ helper T cells by DCs in vivo leads to more robust antibody responses to a boost with antigen [201]. This raises the possibility that antibody responses can be induced that will be stronger, longer lived, and of appropriate isotype through better control the DC helper–T cell interaction. Then there are pathways in which DCs interact directly with B cells and may require molecules like BAFF on the DCs [202]. Another example of B cell immunity, which is DC dependent but T cell independent, is stimulation of IgA antibody formation to commensal organisms [203]. In other studies, DCs are able to induce class switching on B cells in the presence [204] or absence [205] of CD40 ligation, the latter through BLys and April TNF family members. This new area of DC–B cell interactions raises some questions. Do DCs have special antigen-presenting capacities for B cells, such as retention and/or display of intact antigens? Do DCs or a subset of DCs have a means to access the B cell area? Do DCs use distinct costimulators to control B cells?

6.3
Mucosal Immunity

We summarized early in this review how DCs are positioned at mucosal surfaces and in mucosal-associated lymphoid tissues to capture antigens. This

is itself remarkable because epithelia are barriers, yet DCs may be readily sampling proteins and particulates. There are other enigmas. One relates to the function of the mucosal draining lymph nodes vs the mucosal-associated lymphoid organs themselves. How do DCs move to the T cell areas in the mucosal lymphoid organ, like the tonsil or Peyer's patch, and does this have different consequences than migration to mucosal draining lymph nodes in the gut, chest, or genital tract? Do DCs contribute to the mechanisms for inducing regulatory T cells at the gut surface, to dampen reactivity to harmless antigens in the intestine? Are there epithelial products that condition the DCs to induce different types of T regulatory cells to suppress environmental reactivity? Reciprocally, how can DC function be switched to induce stronger mucosal immunity, which is a requirement for vaccines against many prevalent infections?

6.4
Peripheral Tolerance

This compendium of review articles emphasizes resistance to infection, but resistance carries a risk that is inherent to the function of maturing DCs. When DCs capture microbial antigens, it is likely that the cells are also presenting many peptides derived from self and harmless environmental sources. As a result, it is important that tolerance mechanisms be in place prior to infection so that the DCs do not induce autoimmunity or chronic inflammation to environmental proteins [206]. It has become apparent that DCs are able to induce different types of peripheral tolerance, and this may appropriately condition the lymphocyte repertoire. Some of these tolerance mechanisms are intrinsic, where DCs induce deletion or anergy [69, 70, 207]. Others may be extrinsic or dominant and involve regulatory or suppressor T cells [170, 171, 189], and T-independent antibodies to IgA in the case of commensal organisms in the intestine [203]. These functions in peripheral tolerance may require the special innate features of DCs that have been reviewed here: the capture and processing of antigens, migration to lymphoid tissues, and we suspect, responses to environmental stimuli that allow DCs to produce IL-10 or transforming growth factor (TGF)-β.

6.5
DC Subsets

Although not emphasized in this review, there are specialized populations or subsets of DCs that exhibit different innate features with respect to location, expression of receptors for antigen uptake, intracellular antigen trafficking

pathways, and responses to environmental signals such as TLR ligands. In peripheral tissues, there are distinct markers on DCs that are associated with epithelial and interstitial sites, as illustrated by different endocytic receptors on epidermal LCs (Langerin/CD207 and DEC-205/CD205) and dermal DCs (DC-SIGN/CD209, mannose receptor/CD206) [208]. In the periphery and in lymphoid organs, there are distinct myeloid and plasmacytoid DCs in many species. Within so-called myeloid DCs there can be clear-cut subsets, the classical one being the $CD8\alpha^+$ and $CD8\alpha^-$ of mouse spleen. What is the raison d'être for these subsets? Are DC subsets specialized to respond to distinct classes of microbial insults, e.g., plasmacytoid DCs are designed to respond directly to viral infections with their heightened expression of the TLR7 and TLR9 receptors for microbial RNA and DNA, while some subsets of myeloid DCs are designed to interact with bacterial ligands for TLR 2 and TLR4? Is the $CD8\alpha^+$ subset of DCs designed to interact with dying cells of different types, where dying cells are a major potential source of antigens in self tissues, infectious foci, transplants, and tumors? Do DC subsets cooperate in some instances, e.g., myeloid DCs being better antigen capturing and processing cells and plasmacytoid DCs providing large amounts of adjuvant interferons [209]? Can any DC subset take part in the induction of tolerance, and the differentiation of T cells along Th1 or Th2 pathways, or are certain subsets more dedicated to some of these activities? These questions would all benefit from more in vivo approaches to discern what DCs are doing in intact tissues without having to isolate (and thereby perturb) them. This is becoming feasible using an approach in which antigens are delivered selectively to specific uptake receptors expressed by subsets of DCs in situ [69, 70, 136, 201, 210].

6.6 Vaccine Biology

Vaccine biology is another underlying theme of this compendium of reviews. The need to discover and develop vaccines against prevalent global infectious diseases emphasizes the need to better understand the link between innate and adaptive immunity. Vaccine biology demands not only that there be a measurable immune response, but also that the immunity be sufficient in quantity and quality to provide protection against a specific pathogen or tumor. Immunology needs to extend its scope from informative but simplified assays in mice to more demanding analysis of protection and memory, including in humans. In HIV/AIDS for example, there still are no reports of reliable immunization of T cells in humans exposed to safe forms of HIV vaccines, let alone trials designed to elicit protective immunity in humans.

The means are now available to exploit DC biology in vaccine discovery and development, something that has not been done in the past. A proposal would be that vaccines need to gain access to DCs that are matured appropriately for the pathogen in question, and that this is most likely to succeed if the vaccine-capturing, mature DCs are located in lymphoid organs, the sites for generating immunity. A new approach to achieve these ends has been put forward. It is to target vaccine antigens to antigen uptake receptors that are expressed by DCs in lymphoid organs along with a maturation stimulus. One method for selective delivery involves using antibodies to DC receptors, in which antibodies are engineered to include vaccine proteins. The first observations with this approach in naïve mice indicate that superior and protective immunity can be achieved by more directly considering DC biology during vaccine design [136, 201, 210].

7 Summary

The innate immune system provides many ways to quickly resist infection (Table 1). The two best-studied defenses in DCs are the production of protective cytokines—like IL-12 and type I interferons—and the activation and expansion of innate lymphocytes. IL-12 and type I interferons influence distinct steps in the adaptive immune response of lymphocytes, including the polarization of Th1-type $CD4^+$ T cells, the development of cytolytic T cells and memory, and the antibody response. DCs have many other innate features that do not by themselves provide innate resistance but are critical for the induction of adaptive immunity. We have emphasized three intricate and innate properties of DCs that account for their sentinel and sensor roles in the immune system: (1) special mechanisms for antigen capture and processing (Fig. 1; Table 2), (2) the capacity to migrate to defined sites in lymphoid organs, especially the T cell areas, to initiate immunity, and (3) their rapid differentiation or maturation in response to a variety of stimuli ranging from TLR ligands to many other nonmicrobial factors such as cytokines, innate lymphocytes, and immune complexes (Table 3). The combination of innate defenses and innate physiological properties allows DCs to serve as a major link between innate and adaptive immunity. DCs and their subsets contribute to many subjects that are ripe for study including memory, B cell responses, mucosal immunity, tolerance, and vaccine design. DC biology should continue to be helpful in understanding pathogenesis and protection in the setting of prevalent clinical problems.

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Helper T Cell-Regulated B Cell Immunity

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Abstract In this review, we will discuss the cascade of cellular and molecular events in the immune response to protein antigens that regulate the development of high-affinity B cell memory. The behavior of antigen-experienced pMHCII$^+$ dendritic cells DCs and the dynamics of their interaction with specific T-helper (Th) cells define the first developmental checkpoint for adaptive immunity in vivo. Recent studies provide insight into the basis of Th cell clonal selection and the requirements and consequences of antigen priming in this responsive Th cell compartment. Antigen-specific Th cells

expand to become the cognate regulators of effector B cell responses and initiators of the germinal center reaction and memory B cell development. We will discuss the development and role of these diverse mixtures of antigen-specific B cells in the control of B cell memory and long-term humoral immunity that underpin effective protein vaccination.

1 Development of pMHCII$^+$ DCs

1.1 Pre-existing Diversity in the DC Compartment

Dendritic cells (DCs) pre-exist antigen challenge as multiple cellular subsets with distinguishable cell surface phenotype [9]. In the murine system, three main DC subsets enter via the blood to reside at different levels in all secondary lymphoid organs [101, 40]. These blood-derived DCs all express CD11c and are distinguishable as CD8α^-CD11b^{hi}, CD8α^+, and 6B2$^+$ plasmacytoid DC (pDC) [41]. In lymph nodes (LNs) draining the skin, there are two further CD11c$^+$ subsets that include the emigrating progeny of Langerhans cells (LCs) and dermal DCs (dDCs) [41, 57, 50]. There are also reports of CD11c$^-$ monocyte-derived DCs in the mouse that have the capacity to activate naïve T-helper (Th) cells in vitro [92]. Hence, before antigen challenge, multiple subsets of DCs are available to process and present antigen to the adaptive immune compartment.

1.2 Antigen-Experienced pMHCII$^+$ DCs

DCs are acutely sensitive to inflammatory stimuli and uniquely efficient at antigen uptake, processing, and presentation [9, 39, 101, 89]. Upon local protein immunization, DC populations rapidly mature into cells capable of activating naïve antigen-specific Th cells. Processing and presenting antigenic peptides in the MHCII pathway is an elaborate, highly-regulated cellular activity that is not simply dependent on antigen uptake [111]. DC maturation also involves signals through pattern recognition receptors (PRR) such as the Toll-like receptors (TLR) that initiate substantial early changes in gene expression [1, 43]. Modulation of integrins, adhesion molecules, and chemokine receptors serve to direct the mature DCs to the T cell zones of draining LNs [9, 101, 40, 89]. In the draining LNs, chemo-attraction and inter-cellular adhesion helps to initiate first contact with naïve Th cells. DC production of cytokines, other growth factors, and the expression of a multitude of surface molecules can also impact and alter antigen-specific Th cell fate.

2
Antigen-Specific Th Cell Differentiation

2.1
Clonal Selection in the Th Cell Compartment

The recognition of pMHCII by the TCR is central to the development of adaptive immunity and B cell memory. Successful cognate interactions involve the dynamic rearrangement of cell surface molecules at the immune synapse [16]. As depicted in Fig. 1, the initial synapse between antigen-experienced DCs and naïve Th cells represents a major developmental checkpoint that can impact all facets of the adaptive response [55, 77, 97]. Valitutti and colleagues [25] recently demonstrated great versatility and discrimination in Th–antigen-presenting cells (APC) synapse formation. In these in vitro studies, T cells appear to form multiple contacts with different APC, but rapidly favor the stronger TCR-pMHCII interactions and remodel their contacts accordingly [25]. Glimcher and colleagues [61] recently demonstrated the cross-regulation of cytokine receptors with TCR polarization as a critical

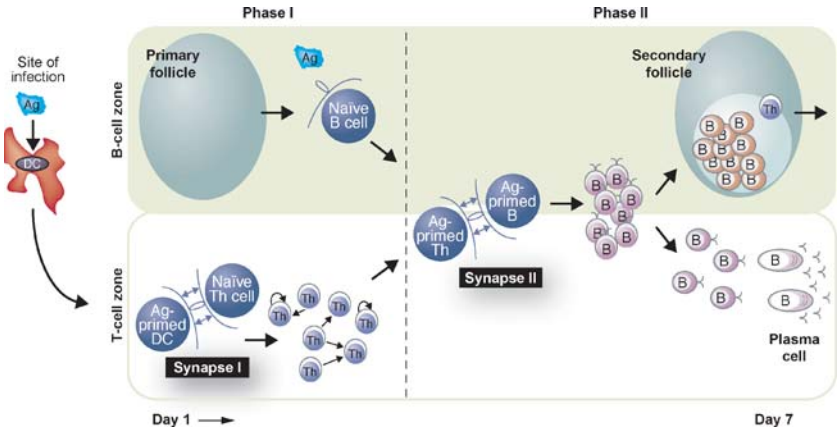

Fig. 1 Initiating and regulating adaptive immunity. Phase I begins with activating antigen-presenting cells at the site of vaccination and their migration into T cell zones of draining lymph nodes. The formation of immune synapse I between antigen-experienced APC and naive antigen-specific Th cells is the first major developmental checkpoint in this pathway. Th cell clonal expansion and migration toward the T–B borders initiates the search for antigen-experienced B cells. Phase II begins with Immune Synapse II between expanded effectors Th cells and antigen-primed B cells, the second developmental checkpoint in this pathway. A major division in B cell development ensues as isotype switch and plasma cell differentiation or secondary follicle formation

determinant in synapse formation. In this manner, physical co-polarization of either interferon (IFN)-γR or interleukin (IL)-4R directs antigen-specific Th cell functional commitment.

The nature of the T cell receptor (TCR)-pMHCII recognition events in vivo and the question of which ones are considered favorable are of fundamental importance but difficult to measure. Using an adoptive transfer model, we recently demonstrated clonal selection of antigen-specific Th cells that was controlled by TCR-pMHCII affinity thresholds [62]. Surprisingly, TCR-pMHCII off rates played no role in the initial selection of preferred clonotypes. Unlike B cell maturation, there was no preferential selection or propagation of higher affinity clonotypes above the limiting affinity threshold. This mechanism limits skewing of the responding Th cell repertoire during clonal expansion while maintaining some level of TCR diversity. Curiously, limiting antigen dose until expansion was truncated did not alter the affinity threshold. What determines the affinity threshold in vivo remains an intriguing open issue for the design of protein vaccination.

2.2 Priming pMHCII-Specific Naïve Th Cells

Benoist and colleagues [84] demonstrated that persisting antigen is required for continued Th cell clonal expansion in vivo. They used a powerful genetic model of inducible pMHCII complex expression on APC in vivo. These data contrast the transient need for antigen to induce CD8 T cell responses and highlight fundamental differences between these two T cell compartments in their response to antigen. Surprisingly, the effects on the specific Th clonal expansion that was reported occur in the absence of adjuvant and do not change with the addition of adjuvant. Interestingly, Swain and colleagues [44] addressed a related issue with a model of chronic pMHCII stimulation through repeated administration of antigen-pulsed APC every 24 h in vivo. In this situation, Th cell function and the capacity to protect in vivo are severely dampened. These studies emphasized the varied consequence of pMHCII recognition in vivo and hint at the many varied levels of control that are important to effective priming of adaptive responses.

The induction and regulation of Th cell function is controlled by a variety of cell surface molecules. Flavell and colleagues [4] have recently placed differential notch ligand expression on activated APC as a critical determinant in Th cell commitment. Different inflammatory stimuli, such as LPS and cholera toxin, induce delta or jagged on the APC, respectively. The differential expression of these notch ligands interacts with notch on Th cells to induce either Th1 or Th2 cell fate. The semaphorin Sem4a also appears involved in Th1 cell

priming in vivo. Using Sema4a-deficient mice, Kikutani and colleagues [54] demonstrated its role first on activated APC for Th cell priming, as well as its upregulation on primed Th cells that appears necessary for Th1 differentiation in vitro and in vivo. The list of molecules with impact on Th cell fate is extensive, and the coordinated influence of these molecules in vivo remains to be carefully elucidated.

2.3 Distinct Naïve Th Cells Control Plasma Cell Development

We have recently demonstrated a pre-existing functional division in the naïve Th cell compartment based on the differential expression of Ly6C [71]. While we provide no insight into the function of Ly6C itself, we demonstrated that 50% of CD4 SP thymocytes commit to the $Ly6C^{hi}$ compartment in the periphery. Interestingly, $Ly6C^{hi}$ and $Ly6C^{lo}$ Th cells with the same pMHCII specificity express different TCR repertoires before exposure to antigen. The LyC^{hi} Th cell compartment was uniquely specialized to support plasma cell development upon adoptive transfer and T cell-dependent (TD) immunization. We suspect the GPI-anchored Ly6C itself does play an early role in Th cell activation in vivo that has been demonstrated in vitro [94]. However, we also believe that this molecule is only one difference in a distinct program of naïve Th cell development that has broad implications for effector cell function and the regulation of B cell immunity.

3 Short-Lived Plasma Cell Development

As depicted in Fig. 1, Th cells expand, differentiate into effector cells, and migrate to the T–B borders of secondary lymphoid organs [72]. There they will interact with antigen-primed B cells that have internalized the protein antigen and processed and presented pMHCII molecules to form immune synapse II. This cognate interaction is quantitatively and qualitatively distinct from immune synapse I, representing the second major development checkpoint in Th cell-regulated B cell immunity.

3.1 Functional Heterogeneity Within Short-Lived PCs

One outcome of immune synapse II is the production of short-lived antigen-specific plasma cells. These B cells do not enter the germinal center (GC)

reaction, nor do they secrete germline-encoded antibodies of IgM and downstream non-IgM isotype under differential Th cell control [36, 42]. Induced CD40L on effector Th cells and the receipt of this signal through CD40 on B cells is required for class switch recombination (CSR) [6, 8]. Inducible costimulatory molecule (ICOS) expression on activated Th cells is thought to act upstream of CD40L in this temporally orchestrated set of events [99]. Some residual CSR in the absence of CD40/CD40L interactions may be explained by the action of Transmembrane activator and calcium modulating cyclophilin ligand interactor (TACI) and B cell-activating factor belonging to the tumor necrosis family (BAFF)-R [19]. OX40/OX40L interactions also quantitatively impact CSR while CD27-CD70 interactions promote plasma cell (PC) production [13]. Thus, the range of molecules expressed at the effector Th cell surface influences the quality of TCR-pMHCII contact on antigen-primed B cells in immune synapse II.

Th cell-derived cytokines have been considered the fine regulators of antibody isotype. IL-4 and IFN-γ are reciprocal regulators of IgG1 and IgG2a production [104]. Animals lacking IL-4 or Stat6 have decreased IgG1 levels and no IgE [53, 108]. IL-4 also acts together with IL-21 to control IgG subtypes and IgE levels [85]. Transforming growth factor (TGF)-β is implicated in the induction IgA, while IL-2 and IL-5 augment IgA production [20]. Similarly, IL-6 may selectively support IgG2a- and IgG2b-expressing B cells in vivo [52]. Each of these factors can exert its effects in vitro or in a bystander manner in vivo. However, it is thought that the directed delivery of these soluble molecules toward points of TCR-pMHCII contact allows immune synapse II to focus even soluble signals locally in an antigen-specific cognate manner.

Therefore, the spectrum of signals received at immune synapse II differentially directs PC development. More recently, elegant studies by Song and Cerny [105] demonstrated that both follicular (FO) B cells and marginal zone (MZ) B cells participate in the response to typical TD hapten-protein conjugates. Brink and colleagues [88] have had similar results using gene-targeted hen egg lysozyme (HEL)-specific B cells in adoptive transfer models. The differing rates and extent of responsiveness for MZ and FO B cells in these studies may reflect differences in B cell development, antigen, and immunization regimes across the different models. Together with the heightened capacity of MZ B cells to present antigen to Th cells [7], these studies introduce a new level of cellular heterogeneity that must be accounted for in the Th cell-regulated development of short-lived PC.

3.2 Molecular Regulation of PC Development

The transcriptional repressor prdm-1 (encoding Blimp-1) has been considered a master regulator of PC development [113]. Calame and colleagues [98] recently demonstrated that this molecule is required for antibody production in vivo. In the selective absence of Blimp-1 in B cells, total serum antibody levels were substantially reduced with little or no specific antibody in response to T cell-independent (TI) and TD immunization. In collaboration, we determined that the antigen-specific PC compartment was absent in these mice following initial priming and at antigen recall in the spleen and bone marrow (BM). Nutt and colleagues [47] have recently knocked-in green fluorescent protein (GFP) into the Blimp-1 locus to label all antibody secreting cells as GFP^+. They demonstrate heterogeneity for GFP levels across spleen, blood, and BM and use sorting and enzyme-linked immunoSPOT (ELISPOT) analysis to associate directly with secreting function. PC cell surface phenotype appears to vary substantially with evidence for $CD138^-$ PCs. This is a useful new model to access PCs directly ex vivo and has been useful to monitor antigen-specific PC numbers regardless of B cell receptor (BCR) expression [15]. Hence, powerful new animal models are now available to help dissect these complex developmental programs in vivo.

Structure/function analysis indicates the modular action of Blimp-1 as a central regulator that integrates a variety of environmental cues that lead to PC development [95]. The transcription factor Xbp-1 acts downstream of Blimp-1 and controls the unfolded protein response (UPR) and many secretory pathway components [96]. The transcriptional co-activator OBF-1 also appears important for B cells to complete the PC program [21]. In the absence of OBF-1, Bcl-6, Pax-5, and activation-induced cytidine deaminase (AID) are not repressed, blocking the induction of Blimp-1 and PC development. In contrast, ablation of the transcription factor MitF leads to the spontaneous development of PCs that appears independent of antigen stimuli [56]. Interestingly, re-expression of bcl-6 and its co-factor MTA3 re-activated the B cell program and increased CD19 and MHCII and decreased CD138 in plasma cell lines [27]. This remarkable study suggests that PC fate is not as terminal and passive as it has been thought to be and that this directional movement remains subject to gene expression dynamics. Further to this theme, using the GFP knockin model, some PC populations (without intentional immunization) clearly display short-term BrdU uptake indicative of cell turnover in vivo [47].

The appearance of short-lived PC is one developmental outcome of Th cell regulated B cell responses. Even within this developmental pathway there is a spectrum of functional options that reflect variations in the molecular

nature of cognate control at immune synapse II. It should be emphasized that antibody isotype is one clear functional distinction between PC subsets. Differential migration patterns as well as growth and survival requirements also contribute to variation and functional sub-specialization that represent separate immune clearance strategies.

4
The Germinal Center Reaction

4.1
Origins and Organization of GC B Cells

The second major outcome of immune synapse II is secondary follicle formation as the initiator of the germinal center pathway and memory B cell development (depicted in Fig. 1). There is evidence for pre-GC clonal assortment at this juncture [75, 100] that suggests some level of antigen-driven selection before somatic diversification of the BCR. Polarization of secondary follicles into

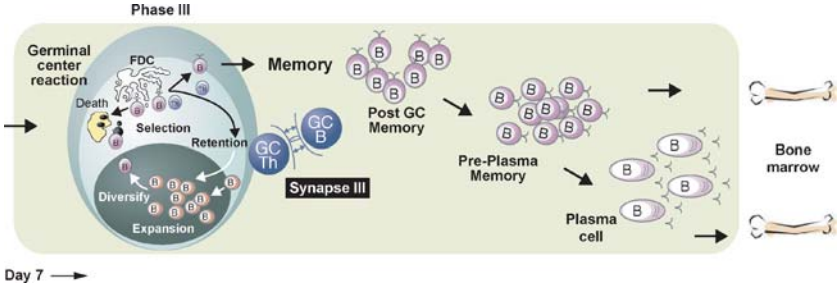

Fig. 2 The germinal center (GC) cycle of activity. Antigen-specific B cells enter the follicular area and rapidly expand into secondary follicles that polarize into the light and dark zones of the germinal center reaction. Clonal expansion is accompanied by somatic diversification of antibody variable region genes in the dark zone. Exit from cell cycle and expression of variant BCR allows for antigen-specific selection of centrocytes in the light zones. Negative selection leads to apoptosis while positive selection of high affinity variants results in GC cycle re-entry or exit from the GC into the memory B cell compartment. Multiple subsets of memory B cells emerge from the GC reaction of the primary immune response. We propose that $6B2^{+}$ post-GC memory B cells are the first cellular product that exits the GC. These cells can give rise to $6B2^{-}$ pre-plasma memory B cells that are phenotypically and functionally distinct non-secreting memory response precursors. The $6B2^{-}$ pre-plasma memory B cells are the immediate cellular precursors of long-lived plasma cells that are a terminally differentiated, antibody-secreting memory B cell compartment. Both pre-plasma memory B cells and the long-lived plasma cells preferentially home to the bone marrow

a T cell zone proximal region of expanding centroblasts (dark zone) and an opposing region of non-cycling centrocytes (light zone) defines the beginnings of the GC reaction (see Fig. 2). This polar organization of GC B cells appears to be at least partly controlled by chemokine expression and chemokine receptor distribution [2]. Higher expression of CXCL12 in the dark zone assorts CXCR4-expressing centroblasts, while higher CXCL13 in the light zone attracts CXCR5-expressing centrocytes. These studies used flow cytometry and cell cycle status to identify GC B cell subsets and demonstrate the aberrant behavior of GC B cells from various genetically modified host animals.

GCs are characteristic of Th cell-regulated B cell responses; however, TI antigens can also promote these structures [23]. The truncated dynamics of the TI GC and lack of somatic diversification imply differing requirements for GC Th cells in the TD GC reaction. Interestingly, MZ B cells that are recruited into TD responses are also able to form GCs [105]. Unlike the TI GCs, these MZ B cells somatically diversify their BCR within the GC and display evidence for affinity-based selection and subsequent memory B cell development. Thus, there appears to be flexibility to the origins of B cells that form the GC reaction, and a spectrum of cellular activities within that are subject to the differential control of antigen-specific Th cells.

4.2 Antigen-Driven Clonal Evolution in the GC Reaction

The GC reaction is a cycle of cellular activity and molecular change that regulates antigen-specific clonal evolution during the development of B cell memory. Extensive clonal expansion of antigen-specific B cells in secondary follicles precedes induction of somatic hypermutation (SHM) [59, 90, 73, 72]. SHM is a diversification mechanism that introduces single base pair substitutions with rare insertions and deletions into the VDJ segments of rearranged antibody genes [37]. Approximately one mutation is introduced into the BCR with each cell cycle. The variant BCR is then expressed by centrocytes that migrate toward the light zone. Centrocytes with high-affinity variant BCR appear positively selected, while those that express deleterious changes are lost to apoptosis. Together, these processes are referred to as affinity maturation (AFM) and give rise to the high affinity memory B cell compartment.

Follicular dendritic cells (FDC) reside at high density in GC light zones. FDC express trophic factors for GC B cells, chemokines such as CXCL13 to attract centrocytes and overexpress a range of adhesion molecules such as ICAM1 and VCAM1 to promote intercellular contacts [38]. Their activated cell processes can be densely coated with immune complexes (IC) trapped by FcγR and complement receptors [109]. It is reasonable to propose that these

IC play a role in antigen-affinity based selection. However, Shlomchik and colleagues [33] demonstrated that affinity maturation could proceed in animals that express only membrane-bound antibody, suggesting that secreted antibody is not required for AFM. In support of this position, we have evidence for SHM and AFM in the B cell selective Blimp-1 knockout (KO) animals. It is still possible in both these models that even very small amounts of sIg could complex antigen and deposit on FDC. We await sensitive functional assays for the detection of IC on FDC to resolve this important issue.

4.3 Molecular Regulation of the GC Reaction

Typically, GC formation is Th cell regulated and can be blocked by interfering with early Th cell-derived signals such as CD40L and ICOS [8, 99]. Absence of CD28 co-stimulation to Th cells also results in impaired GC responses. Under normal physiological conditions the GC reaction emerges as the most efficient means to control AFM and memory B cell development. However, in the disorganized or absent pre-immune lymphoid sub-compartments of mice lacking lymphotoxin (LT)α , LTβ, TNFRI, and LTβR, the activities of the GC reaction remain disorganized but largely intact [65, 66, 51, 28]. Vestiges of the GC or small cell aggregates manage the expansion, diversification, and selection steps required for AFM and memory B cell development. Selective re-expansion of high-affinity variants upon antigen recall appears to continue the selection of the best memory B cell compartment in these less-than-ideal priming conditions.

The transcriptional repressor Bcl6 is highly expressed in the GC and is necessary for GC formation [24]. Interestingly, Tokuhisa and colleagues [110] carefully demonstrated the presence of IgM and IgG1 antigen-specific memory B cells in the absence of Bcl6. These studies were based on direct labeling with antigen and their capacity to respond to soluble low dose antigen recall. However, there were no GC formed, the memory B cells expressed no SHM or evidence for AFM. Bcl6 has been recently shown to repress *p53*, the tumor-suppressor gene that controls DNA damage-induced apoptosis [87]. Regulation of p53 in this manner may protect GC B cells to allow for the DNA breaks that are necessary intermediates in SHM and CSR. Bcl6 also directly represses Blimp-1 [112] and hence must be lost at some point during the GC reaction to allow subsequent development of pre-plasma memory B cells and long-lived PC in vivo [98].

AID most likely initiates SHM upon deamination of cytidine to uracil [37, 80]. Error-prone DNA polymerases used for excision repair introduce mutations at all base positions and can result in C-T and G-A exchanges. Fur-

thermore, evolved biases in codon usage are compounded by targeting bias for AID and DNA polymerases to maximize the introduction of SHM into the complementarity-determining region (CDR) of antibody genes [116]. Double-stranded DNA breaks also appear as intermediates to this process. Interestingly, AID is also necessary for CSR [37]. In CSR, the mismatch produced by AID is processed to produce double-strand DNA breaks as necessary CSR intermediates that are then repaired by non-homologous end joining. In a recent study, Nussenzweig and colleagues [91] demonstrated that AID is also required for the c-myc/IgH translocations associated with plasmacytoma development.

The majority of centrocytes expressing variant BCR die in situ. Overexpression of anti-apoptotic molecules, such as Bcl-2, results in the accumulation of low-affinity B cells and argues for apoptosis as the clearance mechanism in the GC [106, 103]. Takemori and colleagues [107] demonstrated a role for CD95 (Fas) in controlling selection in the GC reaction. In the absence of CD95, SHM was intact, but there was also a decreased presence of high-affinity changes in the memory B cell compartment. Curiously, these studies also provided evidence for a persistent GC B cell compartment that continues to mutate in the wildtype (WT) animal but does not contribute to the memory compartment [107]. In contrast, $CD95^{def}$ GCs also persisted and continued SHM, but these GC contributed cells into the memory B cell compartment that diluted the high-affinity memory B cells. These more complicated and careful analyses highlight how little is still understood about the dynamics and regulation of antigen-driven affinity maturation.

4.4
GC Th Cells and Immune Synapse III

In the GC reaction, FDC provide stromal support and act as an antigen depot, while GC Th cells may regulate B cell-positive selection in a cognate manner. Th cells found predominantly in the light zone of the GC reaction (XXX) are a clonally restricted population of antigen-specific Th cells [31, 114, 115, 68]. MacLennan and colleagues [31] suggested the sequential appearance of Th cells first in the T zones and then the GC. Kelsoe and colleagues [115] demonstrated a similar pattern and also suggested free movement between different GC [114]. The latter group also reported that GC Th cells were highly sensitive to apoptosis and may undergo their own selection process in the GC [115]. Our work using TCR repertoires and immunofluorescence [68] indicated that antigen-specific Th cells in the LN shifted location from the T cell zones at day 7 to more than 75% in the GC by day 9 after initial priming. Interestingly, in a separate study [79], we demonstrated that not all antigen-

specific Th cells enter the GC reaction. These data suggested a level of pre-GC selection for Th cells based on the quality of TCR-pMHCII binding, but also excluded the GCs as a requisite phase in memory Th cell development.

The phenotype and function of GC Th cells has been more elusive. Down-regulation of CD90 (Thy1) was evident in tissue section [114], but we find this is not so clear by flow cytometry. In collaborative studies with Cyster and colleagues [5], we demonstrated that CXCR5 is upregulated upon antigen-experience, but too early and on too many specific Th cells to be considered a marker for GC Th cells. Butcher and colleagues [49] combined CXCR5 up-regulation and expression of CD57 to localize effector Th cells in the GC reaction. These cells appear specialized for B cell help and produce elevated levels of IL-10. Interfering with CD40L-CD40 interactions in vivo disrupts established GCs, while blocking B7-2 interactions impairs SHM and entry in the memory compartment [32]. These latter studies suggest that GC Th cells may play a central role in the positive selection of memory B cells. However, clarification of cognate GC cellular interactions and the existence of immune synapse III (Fig. 2) remains a fascinating open question for the field.

5
B Cell Memory

5.1
Cellular Products of the GC Reaction

Over the course of the primary immune response, GC B cells expressing high-affinity BCR variants are selected into the memory B cell compartment. In mathematical models of AFM, the most reasonable means to create a high-affinity memory compartment is through reiterative cycles of diversification and selection [48]. In this model, a centrocyte that has been positively selected early during the GC reaction can return to the dark zone to re-diversify and re-expand. Introduction of point mutations can then produce a second pool of progeny with variants of the high-affinity receptor in a step-wise cell cycle-dependent manner. New models suggest a one-way process of continued diversification and selection and no requirement for continued dark zone presence [78]. These divergent predictions highlight the inadequate resolution of the current experimental data.

Under conditions of normal physiology, TD responses give rise to affinity matured memory B cells as a cellular product of the GC reaction [72]. As discussed, there is now clear evidence that both FO B cells and MZ B cells can give rise to GC and produce affinity-matured memory B cells [105]. It is also

possible that memory B cells can develop in the absence of SHM and visible GC [110] as well as in responses to TI immunogens [3]. Memory B cell compartments vary at the level of expressed BCR repertoires and antibody isotype in ways that depend on the type of immunization regime used. The nature of the antigen, the pre-existing balance of precursors, the type of adjuvant used, and the route of immunization will all impact the quality of antigen-specific T cell help and consequently influence the development of B cell memory.

At a more fundamental level, the GC produces two broad categories of memory B cells. The affinity-matured precursors for the response to antigen recall are most typically considered memory B cells [72]. However, the long-lived PCs that contribute to persisting serum levels of specific high-affinity antibody can also be regarded as a cellular component of B cell memory. These specialized PCs are affinity-matured products of the GC reaction, but will not be recruited into the memory response to antigen recall.

5.2 Memory Response Precursors

Memory response precursors are defined as affinity-matured post-GC B cells that are often isotype switched and capable of responding to low-dose soluble antigen recall. Antigen binding has been a useful marker for these cells directly ex vivo, where they have often been described as $IgM^{-}IgD^{-}$ $6B2^{+}$ B cells [46, 35, 76]. Early studies indicated long-lived populations of cells that persist without cell turnover [30], and more recent genetic models [64] indicate no requirement for persisting immunogen in vivo. Our early studies focused on the $6B2^{+}IgG1^{+}$ antigen-binding cells [76, 75] and estimated the purity of target cell populations by single-cell RT-PCR-based BCR analysis for SHM and evidence of AFM. Most recently, Tarlinton and colleagues [15] isolated rare mutated antigen-binding B cells that entered the blood as a post-GC memory B cell compartment. These studies present the phenotypic resolution of a post-GC memory B cell compartment using antigen binding levels, IgG1 expression, CD19 and CD38 expression, high peanut agglutinin (PNA) binding, and low Blimp-1 expression. All cellular assessments are verified using BCR repertoire analysis with evidence for AFM in vivo as the best molecular indicator of memory B cell development in vivo.

More recently, animal models using other antigens have emerged to dissect the progression of cellular development in humoral immunity. Peptides in tetramer arrays have been useful [81] as well as fluorophore-labeled whole proteins following immunization with protein–sheep red blood cell (SRBC) preparations [88]. Bachmann and colleagues [29] recently labeled viral particles from the bacteriophage Qβ to assess the role of Cr2 in memory develop-

ment. Their antigen-labeling, function, and gene-expression assays identify early short-lived PC activity and GC and post-GC compartments of B cells based on PNA expression levels. Cr2 played no role in the early PC response or the extent of the GC reaction but was required for the development of Qβ-specific post-GC B cells that upregulated Blimp-1 and XBP-1 expression. Concomitantly, there was almost complete loss of long-lived PC without Cr2 expression on the B cells within the GC reaction. The authors concluded that the development of functional sub-classes of memory B cells depends on the strength of signal received by GC B cells prior to exit for the GC reaction.

5.3 Pre-plasma Memory Cells

We have identified phenotypically separable compartments of antigen-binding post-GC B cells in a hapten-specific B cell response model [67, 26, 98]. These $IgM^{-}IgD^{-}$ $CD138^{-}$ Ag^{+} (also $PI^{-}CD4^{-}CD8^{-}$) non-secreting cells could be distinguished by differential labeling with 6B2 (detects a glycovariant of the CD45R isoform B220). Upon adoptive transfer of these different putative memory cell subsets into $RAG2^{def}$ recipients, antigen-specific clonal expansion and differentiation into PCs indicated that both compartments contained memory response precursors. The cellular and humoral immune response pattern on transfer and challenge and the broad parent-to-progeny relationship allowed us to propose a model of linear development for B cell memory. In this model, the $6B2^{+}$ memory compartment lies developmentally upstream from the $6B2^{-}$ pre-plasma memory compartment that itself appears to be the cellular precursors of long-lived PCs. The dynamics and details of the primary response to this antigen were similar [26], suggesting that this heterogeneous memory cell development was established as a consequence of the primary response GC reaction.

There is evidence for this atypical $6B2^{-/low}$ pre-plasma memory compartment in the periphery of the BCR quasimonoclonal mouse model [17, 18]. Noelle and colleagues [82] suggested these cells were more correctly considered $CD138^{-}$ pre-plasma cells because they could give rise to PCs upon adoptive transfer in the absence of antigen. However, these workers demonstrated the need for clonal expansion before differentiation into PCs for these cells, an attribute more akin to memory response precursors than pre-committed PCs. Bachmann and colleagues [29] also described a $PNA^{lo}6B2^{lo}$ compartment of post-GC B cells that appear without Cr2, but fail to develop in the absence of GCs after immunization of $TNFR1^{-/-}$ animals. They concluded that these Qβ-specific pre-plasma memory B cells are products of the GC reaction and the precursors of long-lived PCs that home to the BM.

Diamond and colleagues [93] demonstrated the presence of $6B2^-$ peptide-specific B cells that emerge in response to DNA mimotope immunization in BALB/c mice. Unfortunately, the presence of already differentiated $6B2^-CD138^+$ PCs was not excluded from the analyses of subset function or gene expression. Cambier and colleagues [45] also reported the accumulation of $6B2^{lo/-}$ cells that have lost the high molecular weight isoform of B220. These cells accumulate to approximately 50% of the Id^- B cells in aged 3-83μδ transgenic mice. The 6B2 antibody also labels more than 95% of naïve human B cells, but was absent on the majority of $CD27^+$ human memory B cells [14]. Hence, there is evidence for a pre-plasma memory B cell compartment that persists as antigen-experienced B cells in both mouse and human.

A recent study by Bell and Gray [10] questioned the existence of a $6B2^-$ memory cell compartment. These studies focused on antigen-binding cells lacking 6B2 expression and demonstrated that the $6B2^-$ compartment more closely resembled myeloid cells capturing serum antibody and masquerading as memory B cells. These antigen-capturing $6B2^-$ cells could be found in recombination activating gene 1 $(RAG1)^{-/-}$ animals after transfer of immune serum and were lost in animals that lacked FcγRIII and the common FcγR subunit. A second study by Schlondorff and colleagues [58] followed up on the initial analysis to identify the antigen-capturing compartment as basophilic granulocytes. In contrast to the initial study, this second analysis focused on cells that captured antigen-specific plasma IgE via FcεR and were revealed through antigen-binding and anti-IgE labeling. These cells expressed IL-3R and CD45, but not B220, CD16/32, and CCR2. Crosslinking the surface IgE resulted in the production of IL-4 and IL-6. These data allowed the authors to conclude that the $B220^-$ antigen-capturing cells studied by Bell and Gray were basophils and potentially important regulators of humoral immunity.

We agree that non-B cells can capture antibody in vivo and can contaminate flow cytometric analysis using antigen binding. However, we also believe that direct antigen binding is the most logical physical and functional attribute of memory B cells that should be used to purify these rare cells directly ex vivo. Using high-resolution cell sorting strategies together with estimations of cellular function or gene expression (or both) will serve to purify B cell compartments away from non-B cell contaminants.

5.4 Long-Lived Plasma Cells

Efficient immune memory protects the recipient from pathogen re-infection before the appearance of overt symptoms of disease. After the resolution of the primary response, most antigen-specific PCs are found in the BM. These

BM-resident PCs express high-affinity BCR with evidence for SHM and AFM. The post-GC memory compartment appears long-lived and does not self-replenish through cell turnover [11, 36, 63, 102]. It is possible that memory response precursors may contribute to this compartment through a mechanism of asymmetric division during homeostatic proliferation, contributing one daughter cell into terminal development. However, it has been proposed that because memory B cells are sensitive to inflammatory stimuli at times of infection to other antigens, some memory cells differentiate into long-lived PCs in an antigen-nonspecific manner [12].

In the absence of Cr2, long-lived PCs do not develop [29]. This is also true in the absence of the transcription factor Aiolos [22]. This B cell intrinsic defect may have its origins in the efficacy of the GC reaction and AFM rather than as a direct effect of long-lived PC development. Based on gene ablation studies, long-lived PC use a variety of redistribution mechanisms such as upregulation of CXCR4 and α4β1 integrin binding to VCAM-1 to get to the BM [34]. In the BM, these cells need signals through B cell maturation antigen (BCMA) for survival [83]. In our studies with B cell selective Blimp-1-deficient animals, no long-lived PCs appeared in the BM. Curiously, there was also a substantial diminution of the pre-plasma memory B cells in these animals that may be the indirect cause of the long-lived PC defect [98]. These trends further support the linear progression model for memory B cell development.

5.5
Memory Response to Antigen Recall

The memory B cell response to TD antigen recall requires helper T cell regulation [60, 59]. We monitored the dynamics of this response from the memory Th cell standpoint [74, 68, 86] and the memory B cell response [70]. When using priming doses of antigen and adjuvant, both compartments emerged more rapidly than their primary counterparts. Memory Th cells reached peak levels more rapidly but to similar levels as the primary response. The memory B cell response also displayed accelerated kinetics but reached substantially higher maximal levels compared to the primary response. Under re-challenge conditions of soluble protein antigen in the absence of adjuvant, the antigen-specific memory B cells most likely act as the mainAPCs (depicted in Fig. 3). Even without adjuvant, circulating high-affinity antibody can bind the protein immunogens and form IC that may be used to recruit the innate system in presentation for recall responses. These issues have not been adequately addressed experimentally.

As depicted, immune synapse IV occurs between antigen-primed memory B cells and pMHCII-specific memory Th cells (Fig. 3). Even in the presence of

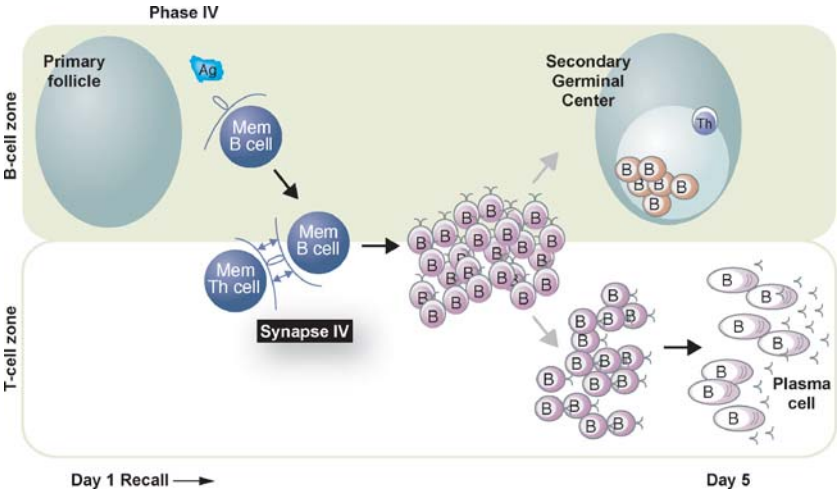

Fig. 3 The response to antigen recall. Low-dose soluble antigen in the absence of adjuvant can induce a rapid and exaggerated humoral immune response. Hence, antigen-specific B cells are the most likely APC for pMHCII complexes at immune synapse IV. Interactions between memory Th cells and memory B cells are the critical developmental checkpoint for the memory response. Memory cell expansion is vigorous, producing a large number of high-affinity memory response plasma cells as the dominant cellular outcome. Secondary GC reactions are also part of the memory response, although more a minor outcome than seen in the primary response

priming doses of antigen and adjuvant, the memory responders dominate the recall response [68, 67], out-competing naïve lymphocytes that may express specific Ag-R. In the memory B cell compartment, there is rapid expansion of memory response precursors with emerging populations of both $6B2^{+}$ post-GC memory B cells and $6B2^{-}$ pre-plasma memory B cells [67]. There is also an exaggerated production of $CD138^{+}$ antigen-specific PCs that largely express high-affinity BCR. The memory response PCs found in the spleen appear to have short half-lives based on accumulation and decline in situ [67]. There is also the rapid appearance of pre-plasma memory B cells and BM-homing PCs seen in the first few days after re-challenge [67]. Hence, the cellular response to antigen re-challenge is rapid and extensive, producing large numbers of high-affinity memory response PCs. Antigen recall also increases the steady state levels of all memory B cell compartments.

6 Concluding Remarks

Understanding the cellular and molecular details of memory B cell development is a high priority in the quest for efficient and rational protein sub-unit vaccine design. Antigen-specific B cell memory develops as a consequence of multiple cellular programs of development that serially progress and intersect at critical junctures in vivo. These junctures act as the critical developmental checkpoints for immune efficacy and provide points of divergent evolution for the emerging adaptive response. In this review, we have emphasized the innate-to-adaptive interface and the profound impact of these earliest events on the quality and quantity of B cell memory and humoral immunity. We have highlighted the extensive cellular heterogeneity that underpins immune memory, and we believe separable antigen-experienced cellular compartments sub-specialize in function to provide multi-layered, long-term protection in vivo.

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T Cell Memory

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Abstract T cell memory induced by prior infection or vaccination provides enhanced protection against subsequent microbial infections. The processes involved in generating and maintaining T cell memory are becoming better understood due to recent technological advances in identifying memory T cells and monitoring their behavior and function in vivo. Memory T cells develop in response to a progressive set of cues—starting with signals from antigen-loaded, activated antigen-presenting cells (APCs) and inflammatory mediators induced by the innate immune response, to the poorly defined subsequent signals triggered as the immune response wanes toward homeostasis. The persistence of the resting memory T cells that eventually develop is regulated by cytokines. This chapter discusses recent findings on how memory T cells develop to confer long-term protective immunity.

Abbreviations

APC	Antigen-presenting cells
IFN	Interferon
LM	*Listeria monocytogenes*
LCMV	Lymphocytic choriomeningitis virus
TLR	Toll like receptors
NOD	Nucleotide oligomerization domain
DC	Dendritic cells
MP	Memory phenotype
AS	Antigen-specific
Treg	Regulatory T cells
TNF	Tumor necrosis factor
LP	Lamina propria
T_{EM}	Effector memory cells
T_{CM}	Central memory cells
TRAIL	TNF-related apoptosis-inducing ligand

1 Introduction

The T cell response to an acute infection can be divided into three successive phases: expansion, contraction, and maintenance. The expansion phase starts when antigen-specific naïve cells undergo activation and prodigious expansion in response to antigen stimulation in the context of costimulatory molecules and inflammatory mediators. T cell activation is manifested by acquisition of effector function including the ability to secrete cytokines, mediate cytotoxic activity, and upregulate tissue homing receptors that allow migration into non-lymphoid tissues. The expansion of effector T cells plateaus after the infection is eliminated, typically between 1 and 2 weeks after infection. The contraction phase commences from this point and proceeds over the next 2–4 weeks when most effector T cells undergo cell death, leaving only 10%–20% of the responding cells as differentiated memory cells. How such wide-scale death is regulated is poorly understood, but it involves participation of effector molecules, such as interferon (IFN)-ɣ, and receptors associated with apoptosis. The third maintenance phase refers to the subsequent period when memory cells persist almost indefinitely, poised to respond to the same infectious agent in a more vigorous manner. Unlike naïve T cells, which are sustained in interphase, memory T cells are maintained by periodic cell turnover, termed homeostatic proliferation, presumably reflecting a higher state of activation (Tough and Sprent 1994). Regulation of memory T cell homeostasis is also slightly different from naïve T cells in that it is independent of antigen and mostly dependent on a set of common gamma chain (ɣc) family cytokines.

Upon re-exposure to the pathogen, i.e., in the "secondary recall response," memory T cells respond faster and stronger than naïve T cells to eliminate the pathogen. The enhanced immune response is due to an increased precursor frequency of antigen-specific memory cells and the increased responsiveness of memory T cells compared to naïve T cells. In addition, memory T cells, unlike naïve T cells, are dispersed throughout the body even in non-lymphoid tissues, and can mediate pathogen clearance at the site of infection.

2
CD8 T Cell Memory

The bulk of recent advances in the understanding of T cell memory arose from studying CD8 T cell responses in the mouse. Facilitating these studies is the availability of infectious pathogens, such as lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* (LM), where the characteristics of the CD8 response to various dominant and subdominant T cell epitopes have been well characterized. The availability of CD8 T cell receptor (TCR) transgenic mice and MHC class I/peptide tetramers allowed the identification of antigen-specific effector and memory cells directly ex vivo without the need for further cell expansion. Furthermore, the recent advent of the intravital microscopic technique permits the visualization of T cells under in vivo conditions. It is only a matter of time before many of the events in memory T cell generation and maintenance are followed in real time.

2.1
CD8 Memory Generation: T Cell Expansion

It is becoming increasingly clear that transition through the effector stage is a prerequisite for memory cell development. Strong evidence that memory cells are selected from effectors arises from the findings that the TCR repertoire of memory cells closely mirrors that of effector cells (Sourdive et al. 1998) and that adoptive transfer of effectors that had undergone strong antigen stimulation and extensive division gave rise to memory cells (Opferman et al. 1999). A more direct demonstration for their transiting through the effector stage before becoming memory cells was provided by Jacob and Baltimore who used an elegant genetic system to show that memory cells permanently carry a reporter gene specifically induced only during the effector cell stage (Jacob and Baltimore 1999). In light of these findings, a discussion of how effector cells are generated is important for understanding memory T cell development.

Naïve cell differentiation into effector cells is controlled by a confluence of signals, including the density and duration of antigen stimulation, the availability of costimulatory molecules, and the activation state of the innate immune response. Recent studies indicate that only a brief period of antigen stimulation is required to trigger a program in naïve cells to differentiate into effector cells. Even exposure to the antigen for as little as 24 h is sufficient to drive naïve cells to proliferate and acquire effector function (Kaech and Ahmed 2001; van Stipdonk et al. 2001). Naïve cells stimulated for a shorter duration (4 h) or with suboptimal intensity, as in the absence of costimulation, undergo fewer cell divisions and appear to perish through apoptosis (Gett et al. 2003). These findings indicate that once T cells receive a sufficient duration and intensity of activation signals, the bulk of the differentiation process from naïve to memory cells is programmed to occur without further contact with antigen. Indeed, prolonged exposure to antigen can be detrimental to the generation of functional memory cells. For instance, memory cells formed after a chronic infection do not exhibit all the hallmarks of mature memory cells including self-renewal and efficient recall responses (Wherry et al. 2004). Recent intravital microscopic imaging in live mice largely confirms the notion that a relatively short duration of stimulation is required for T cell activation under in vivo conditions (Mempel et al. 2004). Thus, after briefly sampling the antigen-loaded dendritic cells (DC), naïve T cells formed a stable conjugation with DC for several hours before dissociating from the DC. The activated T cells then began proliferating and differentiating into functional effector cells.

The TCR signals triggered by the antigen are modified by other extrinsic factors that shape the overall quality and magnitude of the T cell response. Expansion and differentiation of antigen-specific naïve T cells are augmented by interactions with costimulatory molecules on DC, such as those arising from B7-CD28, CD40-CD40L, and IL-12–IL-12R interactions (Curtsinger et al. 2003; Whitmire and Ahmed 2000). Upregulation of costimulatory molecules and production of inflammatory cytokines, including type I IFN, are induced on antigen-presenting cells (APC) through the interaction of pattern recognition receptors, such as the Toll-like receptors (TLR), with microbial components, such as LPS and double-stranded RNA (Badovinac et al. 2004; Hoebe et al. 2003). Collectively, the primary signals from the TCR and the secondary signals from costimulatory molecules and cytokine receptors integrate to induce the differentiation and expansion of effector cells.

In contrast to naïve cells that recirculate within the confines of the blood and secondary lymphoid organs, effector cells are capable of migrating into non-lymphoid tissues. Thus, naïve and effector cells express different sets of chemokine and homing receptors. For instance, whereas naïve T cells express

CCR7 and CD62L that direct their migration into lymph nodes (LN), effector cells downregulate these receptors and upregulate CCR5 and CCR2, which allow entry into inflamed tissues (Campbell et al. 2003). Effector T cells are able to migrate into nearly all non-lymphoid sites, but their migratory capacity is influenced by the resident DC in the LN draining the site of inflammation. These DC appear to induce the expression of specific tissue-homing receptors on activated T cells that direct the cells to enter different non-lymphoid tissues (Campbell and Butcher 2002; Haddad et al. 2003; Johansson-Lindbom et al. 2003; Mora et al. 2003). Thus, intestinal draining DC promote expression of gut-homing receptors, such as $\alpha 4\beta 7$, and skin-draining DC favor the expression of skin-homing receptors, like P-selectin ligand and cutaneous lymphocyte antigen (CLA). Moreover, it appears that the tissue microenvironment at the site of antigen entry is the determining factor in imprinting homing capability on T cells (Calzascia et al. 2005; Dudda et al. 2004; Mullins et al. 2003). Thus, bone marrow (BM)-derived DC can imprint intestinal or skin tropism based on the site of antigen delivery, and multiple homing capacities can be generated in the same LN if the antigen originates from multiple non-lymphoid tissues.

Upon arrival in the inflamed tissues, effector cells kill infected cells by releasing perforin and granzyme (Heusel et al. 1994; Kagi et al. 1994). Upon antigen encounter, effector cells can also rapidly secrete the anti-viral cytokines IFN-γ and tumor necrosis factor (TNF) that directly inhibit viral replication and promote expression of MHC molecules and antigen presentation in infected cells (Slifka and Whitton 2000b). Mature effector cells can quickly turn on or turn off cytokine production in the presence or absence of antigen, respectively (Slifka et al. 1999). This allows them to specifically target infected cells while minimizing damage to uninfected areas. The molecular mechanisms that promote rapid cytokine secretion in response to antigen are not completely understood, but some aspects are conferred by epigenetic changes—alterations in gene expression patterns that do not modify DNA base sequences, but are nonetheless inherited from one generation to the next. Hence, alterations in covalent modifications on DNA and changes in histone content are evident on cytokine genes and are reflective of DNA accessibility and increased cytokine responsiveness of effector cells (Ansel et al. 2003).

2.2 CD8 Memory Generation: Selection and Contraction

The majority of the effector cells are no longer needed upon pathogen elimination and hence, most of the effector cells die off, leaving only a small fraction of these cells to differentiate into memory cells (Fig. 1). Recent work

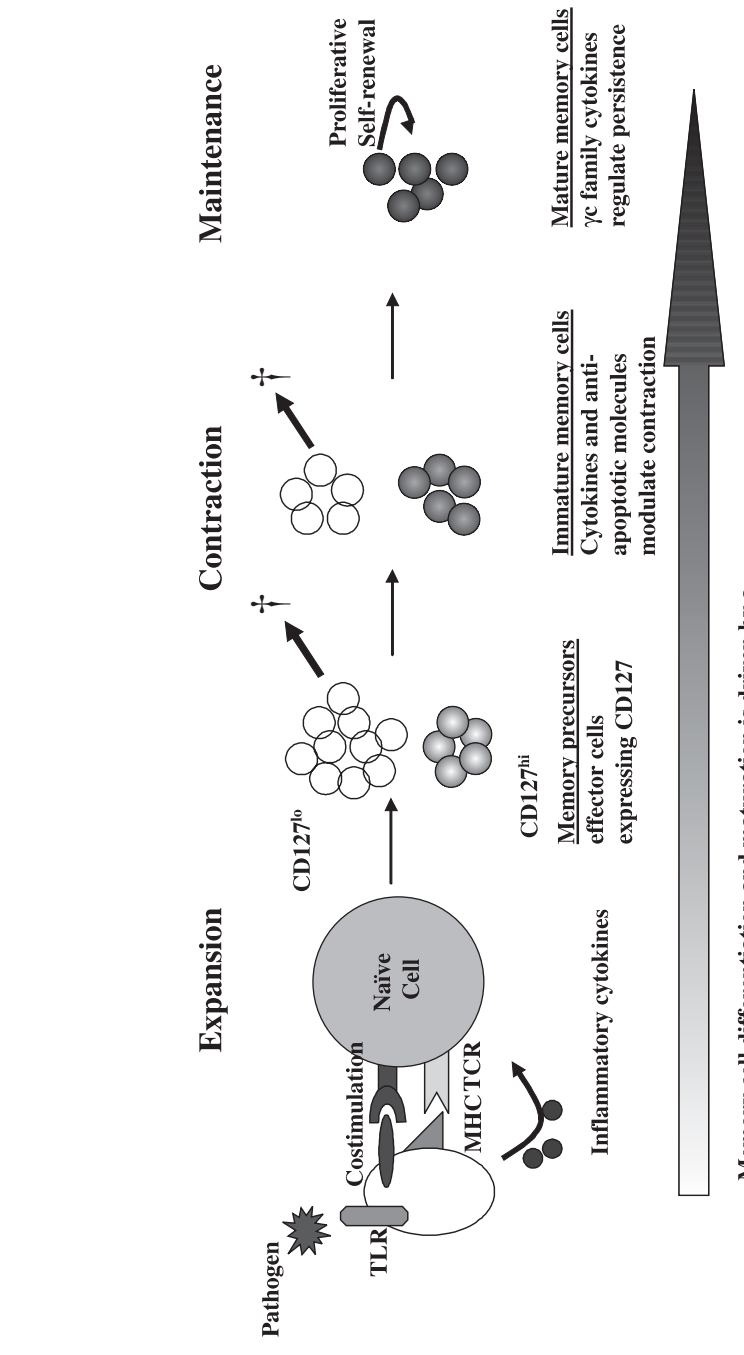
Expansion
Contraction
Maintenance
Pathogen
TLR
Costimulation
MHC TCR
Naïve Cell
Inflammatory cytokines
CD127lo
CD127hi
Memory precursors
effector cells
expressing CD127
Immature memory cells
Cytokines and anti-
apoptotic molecules
modulate contraction
Proliferative
Self-renewal
Mature memory cells
γc family cytokines
regulate persistence
Memory cell differentiation and maturation is driven by a
progressive set of cues over time

Fig. 1 Memory T cell generation and maintenance. Memory T cells develop from the integration of a progressive set of cues. Naïve cells receive cues including the duration and intensity of antigen stimulation, costimulatory interactions, and inflammatory signals. These signals drive the expansion of T cells and may also program changes in T cells that influence the ensuing contraction phase. The developing effector cells can be segregated into $CD127^{hi}$ expressing memory precursors or the $CD127^{lo}$ effector cells that are destined to die. The signals that are provided during the contraction phase not only control the quantity, but also the quality of the memory cells that develop. Mature memory cells are characterized by their ability to undergo proliferative self-renewal and efficient recall responses. The maintenance of high numbers of memory cells is controlled independently of antigen contact and regulated by the γc family cytokines. White cells indicate effector cells that die during contraction. The differentiation of naïve into mature memory cells is indicated by the progression of *light to dark gray* cells

has shown that only the effector cells that express high levels of the receptor for the cytokine interleukin (IL)-7, CD127, representing about 10% of effector cells at the peak of the response, give rise to functional memory cells (Kaech et al. 2003). Expression of CD127 is required on naïve and memory cells for their long-term survival, but CD127 is rapidly downregulated upon activation. Since effector cells require at least 3–4 weeks to differentiate into fully functional memory cells (Kaech et al. 2002), the expression of CD127 at the effector stage appears to ensure the survival of memory-precursors through the prolonged transition period. In addition to CD127, the expression of the CD8αα homodimer was also reported to mark the memory precursors among effector CD8 cells (Madakamutil et al. 2004). Interaction of CD8αα with its ligand, the non-classical MHC thymic leukemia (TL) molecules, is known to deliver survival signals for $CD8\alpha\alpha^{+}$ cells in the intestine, and such a mechanism has been proposed to promote survival of memory-precursor effector cells. However, recent evidence demonstrating that CD8 memory cells can be efficiently generated in the absence of non-classical class I molecules, such as TL, is calling into question the validity of such a model (Williams and Bevan 2005).

Even with the identification of memory precursors among effector cells, the crucial question as to how only a fraction of effector cells survives to become memory cells remains unknown. A few models have been put forward to explain this selection process. One model proposes that the fate of the effector cells is largely controlled by the overall strength of stimulation, which is determined by many factors including the density of the antigen and the duration of TCR signaling. According to this model, naïve T cells receiving strong stimulation produce terminally differentiated effector cells that are destined to die, whereas naïve cells activated with a lower intensity of stimulation survive to become memory cells. This notion is consistent with the finding that effector cells either perish with time or persist with defective

functional capability under conditions that induce prolonged T cell activation, as in chronic LCMV, human immunodeficiency virus (HIV), and hepatitis C infections (Appay et al. 2000; Lechner et al. 2000; Matloubian et al. 1999; Zajac et al. 1998). In a typical immune response, relatively weak stimulation is likely to be delivered to naïve cells that arrive at the site of antigenic stimulation late in the immune response when antigen levels are reduced. However, compelling evidence for or against the notion that straggler cells generate the bulk of the memory precursors is sparse, despite a recent study showing that generation of memory CD8 cells declined significantly when the interval of bacterial infection is shortened with antibiotics (Williams and Bevan 2004).

A more recent variation of the above model proposes that the overall intensity of the stimulation, with the duration and strength of stimulation being the key variables, determines the ultimate fate of effector cells (Lanzavecchia and Sallusto 2002). Accordingly, improperly activated T cells die after an abortive response, while T cells exposed to the full throttle of stimulation become terminal effector cells. Naïve cells primed at intermediate stages of activation give rise to memory cells, with relatively low intensity of signaling inducing production of central memory cells, whereas slightly stronger stimulation mediates the generation of effector memory cells (memory cell subsets are discussed in Sect. 4.2).

Instead of being selected on the basis of subtle differences in the strength of stimulation, it is also possible that memory precursors are chosen largely through a stochastic process. One deciding factor could be the random exposure to survival factors, such as IL-7 and IL-15 that generally exist at limiting amounts. Hence, an opportunistic encounter with a survival factor may be sufficient for an effector cell to survive longer than other effector cells or to be better at competing for survival factors by upregulating the expression of the IL-7 receptor. The idea that contact with survival cytokines influences the memory selection process is supported by the finding that infusion of IL-2 for several days from the peak of the immune response prolonged the survival of effector cells and led to generation of increased numbers of memory cells (Blattman et al. 2003). Considering their role in supporting survival of memory T cells (discussed in Sect. 2.3), it is likely that infusion of IL-7 or IL-15 may be even more efficacious than IL-2 in promoting generation of memory cells. In support of this idea, in vitro activated CD8 cells cultured in the presence of IL-15 developed into memory cells upon adoptive transfer into naïve hosts, whereas culturing with high doses of IL-2 mostly induced differentiation into short-lived effector cells (Manjunath et al. 2001; Weninger et al. 2001). It should be mentioned, however, that random competition for homeostatic cytokines is unlikely to be the sole deciding factor in selection of memory precursor cells. This is because the number of newly generated mem-

ory cells that incorporate into the pre-existing memory pool after an immune response is not constant, but varies according to the magnitude of the primary response (Murali-Krishna et al. 1998). What is consistent is the proportion (~10%) of effector cells that survives to differentiate into memory cells. One way to achieve such a constant proportional level of incorporation could be through a mechanism whereby reciprocal numbers of the pre-existing memory T cells are eliminated to accommodate the new memory cells. Since the magnitude of the primary response dictates the extent of the attrition of pre-existing memory T cells, it is possible that such pruning could be mediated by inflammatory and cytotoxic cytokines generated during the immune response, which are likely to be produced at levels proportional to the overall magnitude of the T cell response.

The memory precursor cells are presumably retained during T cell contraction as the rest of the effector cells (~90%) are eliminated. Although the initiation of the contraction phase was initially thought to be dependent on clearance of antigen, recent work suggests that the contraction phase, similar to the expansion phase, is programmed to occur shortly after T cell activation. Thus, the onset and the pace of T cell contraction are largely independent of the magnitude of T cell expansion and antigen availability (Badovinac et al. 2002). Interestingly, much of the effector cell death appears to be mediated by inflammatory cytokines produced during the activation of the innate immune response and by the effector cells themselves. Thus, the contraction of antigen-specific CD8 cells in response to *Listeria* infection was significantly delayed in IFN-γ-deficient mice (Badovinac et al. 2000) and preventing inflammation altogether by pre-treating mice with ampicillin prior to *Listeria* infection completely abolished the contraction phase, even though the expansion of the antigen-specific CD8 cells was severely reduced (Badovinac et al. 2004).

Though key features of T cell contraction are regulated early in the immune response, events that occur during the contraction phase have significant effects on the quality and quantity of memory cells generated. During this period, a balance of multiple survival and death-promoting pathways presumably regulates the elimination of unwanted effector cells and the retention of memory cells. The bulk of the effector cells are probably pruned through apoptosis by growth factor removal and the modulated expression of apoptotic molecules, including the members of the tumor necrosis factor receptor (TNFR) superfamily containing death receptor domains. Although initially thought to be important, it is now clear that in infectious models, the FAS-FASL interaction does not affect T cell contraction (Lohman et al. 1996; Zimmermann et al. 1996), but recent evidence shows that TNFR promotes T cell contraction and apoptosis after LCMV infection (Suresh et al. 2005). Involvement of multiple pathways for inducing effector cell death is also

suggested by the recent finding that upregulation of a nuclear factor (NF)-κB-dependent serine protease inhibitor, spi2A, protects effector cells from cell death and promotes the differentiation of memory cells (Liu et al. 2004). In terms of molecules that promote cell survival, the retention of memory cells is associated with the increased expression of the pro-survival molecules Bcl-2 and Bcl-xL on memory precursors (Grayson et al. 2000). However, enforced Bcl-2 or Bcl-xL expression did not change the magnitude of T cell contraction consistent with the notion that multiple pathways influence effector cell death (Razvi et al. 1995).

In addition to eliminating unwanted cells, events that occur during the contraction phase are also important for maturation of the surviving effector cells into memory cells. This appears to be due to the fact that recently generated effector cells require about 3–4 weeks to gradually differentiate into memory cells. Thus, effector cells isolated shortly after the peak of the expansion phase do not display all the characteristics of resting memory cells, including the capacity to undergo homeostatic proliferation, to undergo efficient expansion, and to mediate effector function upon re-stimulation with antigen (Kaech et al. 2002). The progressive transition of effector to memory cells over several weeks was also evident from the comparison of gene expression profile between effector cells and memory cells (Kaech et al. 2002). Hence, alterations in gene expression continue to occur over several weeks, in close correlation with the gradual acquisition of functional characteristics of memory cells.

The fully functional memory cells that arise after an immune response endow the host with increased immunity against infection with the same pathogens. The enhanced immunity is due to an increased number of antigen-specific memory cells, often enriched for high-affinity clones, and the capability of memory cells to respond to antigen in a much more efficient manner than naïve T cells (Ahmed and Gray 1996). Thus, memory cells can become activated and undergo proliferation more readily in response to a lower density of antigen, and their requirement for activation, such as costimulation, is less stringent than that for naïve T cells (Slifka and Whitton 2001; Suresh et al. 2001). Moreover, memory cells display effector function, such as the release of IFN-γ and perforin, much faster than activated naïve T cells (Slifka and Whitton 2000a; Veiga-Fernandes et al. 2000). The increased responsiveness of memory cells compared to that of naïve cells appears to be mediated by multiple mechanisms. For instance, rapid proliferation is attributed to the presence of pre-activated kinases required for cell division (Veiga-Fernandes and Rocha 2004), and some of the inflammatory mediators are pre-synthesized and sequestered intracellularly for immediate release upon TCR engagement (Catalfamo et al. 2004; Slifka and Whitton 2000a; Swanson et al. 2002; Veiga-

Fernandes et al. 2000). Furthermore, the epigenetic changes initiated at the effector cell stage also facilitate accelerated expression of cytokine genes (Fitzpatrick et al. 1998, 1999).

2.3 Maintenance of CD8 Memory: Role of Cytokines

Once established, memory CD8 cells are maintained at relatively constant numbers over a long period of time, providing the host with prolonged protective immunity. Although there is no net change in the overall size of the memory pool, memory cells under normal steady state conditions undergo periodic homeostatic proliferation balanced by compensatory death of excess cells. Recent work has defined the key factors that regulate homeostasis of memory CD8 cells and found them to be different from those that regulate naïve T cells (Table 1). Unlike naïve T cells, which require contact with self-MHC/peptide ligands and the cytokine IL-7 for homeostasis (Ernst et al. 1999; Goldrath and Bevan 1999; Schluns et al. 2000; Tan et al. 2001), memory CD8 cells do not require contact with self-MHC/peptide molecules (Murali-Krishna et al. 1999; Polic et al. 2001), but only with cytokines for survival or homeostatic proliferation. These cytokines are members of the γc family cytokines, namely, IL-7, IL-15, and IL-2.

The role for soluble factors in controlling homeostasis of memory CD8 cells was first obtained from the finding that activation of innate immunity by injecting poly I:C or LPS in mice caused expansion of host polyclonal memory phenotype (MP) CD8 cells, as defined by $CD44^{hi}$ expression (Tough et al. 1996, 1997). These studies were extended to show that poly I:C and LPS acted by

Table 1 Summary of phenotypic markers and factors that regulate memory cells

Cell type	Phenotypic markers		Cytokine receptor expression levels			Factors required for persistence			
	CD44	CD62L	CD127	CD122	IL-15Rα	MHC	IL-7	IL-15	IL-2
Naïve CD8	Low	Hi	Int	Low/int	Low	+	+	+/o	o
Naïve CD4	Low	Hi	Int	Low	Low	+	+	o	o
Memory CD8	Hi	Hi/low	Hi	Hi	Hi	o	+	+	−
Memory CD4	Hi	Hi/low	Hi	Int	Hi	o/+	+	+	+

Naïve and memory T cells express different levels of activation and cytokine receptors. Accordingly, different factors regulate the survival and proliferation of these cells
Abbreviations: +, positive effect; −, negative effect; o, no dependence for the listed factor; Hi, high; Int, intermediate

eliciting the production of type I and II IFN, which in turn induced production of IL-15 by APC that acted directly on MP CD8 cells (Tough et al. 1997; Zhang et al. 1998). Consistent with the notion that IL-15 is the terminal regulatory factor of CD8 cell homeostasis, mice deficient in either IL-15 or IL-15Rα possessed severely depressed numbers of MP CD8 T cells. In particular, these mice were devoid of cells that express high levels of CD122, the receptor for IL-15 (Judge et al. 2002; Kennedy et al. 2000; Lodolce et al. 1998). Moreover, $CD122^{hi}$ MP CD8 cells, which constitute about two-thirds of $CD44^{hi}$ cells, transferred into IL-15^{-} mice failed to undergo a basal level of homeostatic proliferation and disappeared within 1–2 weeks (Judge et al. 2002).

Despite the finding that the bulk of MP CD8 cells have a very short lifespan in the absence of IL-15, subsequent experiments showed that normal numbers of antigen-specific (AS) memory CD8 cells can be generated in IL-15^{-} and IL-15Rα^{-} mice upon immunization with infectious agents, such as LCMV and vesicular stomatitis virus (Becker et al. 2002; Goldrath et al. 2002; Ku et al. 2000; Schluns et al. 2002). Nonetheless, AS memory CD8 cells generated in the absence of IL-15 signaling failed to undergo periodic homeostatic proliferation and gradually disappeared over several months, presumably from the inability to undergo occasional cell division. Thus, IL-15 is crucial for both MP and AS memory cells to undergo homeostatic proliferation, even though these two types of cells display variable dependency for IL-15 in terms of cell survival. Why this exists is currently unknown, but this trait appears to be dependent on how memory CD8 cells were generated, with weak activation of innate immunity favoring the generation of cells heavily dependent on IL-15. Hence, AS memory CD8 cells generated in the absence of infectious agents, i.e., upon immunization with soluble antigen mixed with purified adjuvant, were found to be as dependent on IL-15 for survival as MP CD8 cells (Burkett et al. 2003).

In addition to IL-15, IL-7 also plays a major role in supporting homeostasis of memory CD8 cells. IL-7 was originally identified as a non-redundant cytokine crucial for early T cell development (Peschon et al. 1994; von Freeden-Jeffry et al. 1995) and recently for its essential role in regulating survival and maintaining normal numbers of peripheral naïve T cells (Schluns et al. 2000; Tan et al. 2001). Like naïve T cells, memory CD8 (and CD4) cells also express high levels of IL-7R, implicating a role for this cytokine in homeostasis of memory T cells. Indeed, at elevated levels, IL-7 can replace IL-15. Thus, whereas $CD122^{hi}$ MP CD8 cells are absent in IL-15^{-} mice, these cells were present in large numbers when IL-15^{-} mice were backcrossed to an IL-7 transgenic background (Kieper et al. 2002). Moreover, both MP and AS memory CD8 cells were able to undergo efficient homeostatic proliferation in irradiated IL-15^{-} mice, presumably from increased availability of IL-7, but not in the absence of both IL-7 and IL-15 (Goldrath et al. 2002; Tan et al. 2002). Col-

lectively, these findings indicate that basal levels of IL-7 in normal T-sufficient hosts can keep memory CD8 cells, especially AS memory cells, alive at a resting state, but these cells also require IL-15 to undergo homeostatic proliferation. The presence of IL-15 thus appears to ensure the survival of memory CD8 cells without risking competition for IL-7 with naïve T cells, which are $CD122^{lo}$, and to maintain memory CD8 cells at a higher state of activation.

In terms of its physical properties, IL-15 appears to be unique in how it is expressed by APC and recognized by T cells. As with IL-2, IL-15 was initially thought to be recognized by a trimeric αβɣ receptor complex on T cells; note that the same βɣ chains is complexed with another α chain (CD25) to bind to IL-2. Recent work, however, demonstrated that IL-15Rα chain is dispensable for recognition of IL-15 by T cells, but is crucial for expression of the IL-15 cytokine by APC (Dubois et al. 2002; Lodolce et al. 2001; Schluns et al. 2003). For this reason, $IL\text{-}15R\alpha^{-}$ mice are phenotypically identical to $IL\text{-}15^{-}$ mice (Kennedy et al. 2000; Lodolce et al. 1998), even though $IL\text{-}15R\alpha^{-}$ CD8 cells are just as capable of responding to IL-15 as wild-type CD8 cells (Burkett et al. 2003). Indeed, more recent work has shown that both IL-15 and IL-15α chain have to be synthesized by the same cells for functional expression of IL-15, indicating that the IL-15/IL-15Rα complex is pre-associated prior to expression of the cell surface (Burkett et al. 2004; Sandau et al. 2004). Such expression modality also explains why soluble IL-15 is virtually undetectable in culture supernatants (Bamford et al. 1996).

In contrast to the positive effects mediated by IL-7 and IL-15, IL-2 appears to inhibit survival and expansion of memory CD8 cells. Thus, injection of anti-IL-2 antibodies increased the production of proliferating memory CD8 in normal and $IL\text{-}15^{-}$ mice (Dai et al. 2000; Ku et al. 2000). How IL-2 mediates its negative role is not clear, but it is unlikely to be through direct interaction with memory CD8 cells, as most of these cells do not express CD25. A more tantalizing possibility is that depletion of IL-2 removes $CD4^{+}$ $CD25^{+}$ regulatory T cells (Treg) (Murakami et al. 2002). Considering that Treg are able to suppress the response of other T cells, it is possible that Treg inhibit memory CD8 cell expansion either by consuming IL-15 or another unknown IL-15-like cytokine.

3 CD4 T Cell Memory

In contrast to CD8 cells, whose major effector function is to kill infected target cells and secrete inflammatory cytokines like IFN-ɣ and TNF, the primary effector function of CD4 cells is to direct the activity of other cells, including B, DC, and CD8 (Bevan 2004; Coulie et al. 1985). CD4 cells are

capable of differentiating into multiple types of effector cells, with the two best characterized subsets defined as T-helper type 1 (Th1) cells that secrete inflammatory cytokines, such as IFN-ɣ and TNF, and Th2 cells that produce IL-4, IL-5, and IL-13 (Glimcher and Murphy 2000). Th1 cells tend to provide protection against intracellular bacterial or viral infections, whereas Th2 cells confer immunity against extracellular pathogens such as parasites and worms. CD4 effector cells can also drive B cell activation and maturation into plasma cells and promote generation of effector CD8 responses. For cell-associated antigens and certain viral infections, CD4 cells, via CD40L–CD40 interactions, induce activation of APC and secretion of cytokines like IFN-ɣ and IL-2 that regulate CD4, CD8, and B cell expansion and differentiation (Bevan 2004; Heath and Carbone 2001).

3.1 CD4 Memory Generation

Like CD8 responses, the CD4 response can be delineated into three phases: expansion, contraction, and maintenance. However, the magnitude of the CD4 response is generally lower than that seen for CD8 cells (Homann et al. 2001; Kamperschroer and Quinn 1999; Whitmire and Ahmed 2000). The early CD4 response is influenced by different cues including the duration of antigen stimulation, costimulation, and inflammation. For example, a brief TCR signaling can drive a few rounds of CD4 cell proliferation, but sustained TCR signaling is required for their full differentiation into cytokine-producing effector cells (Bajenoff et al. 2002; Jelley-Gibbs et al. 2000; Lee et al. 2002). This contrasts with what is seen for CD8 cells, where brief antigen exposure is sufficient to trigger a full T cell differentiation program all the way to the memory stage (Kaech and Ahmed 2001; van Stipdonk et al. 2001). Another distinction between CD4 and CD8 cells lies in their dependence for costimulation. CD4 T cell activation is heavily dependent on upon CD40L–CD40, B7-CD28, and OX40L–OX-40 costimulatory interactions. In contrast, CD8 expansion in Th-independent systems, such as several types of viral and bacterial infections, does not depend on these interactions (Whitmire and Ahmed 2000). However, CD8 expansion is slightly reduced in the absence of the 4-1BB–4-1BBL costimulatory interaction (Tan et al. 1999). The innate immune system also influences the differentiation and polarization of CD4 cells. Activation through TLR and other innate immunity mediators, such as the nucleotide-binding oligomerization domain (NOD) proteins, is essential for efficient CD4 activation and generation of effector and memory cells (Kobayashi et al. 2002; Pasare and Medzhitov 2004; Schnare et al. 2001; Sporri and Reis e Sousa 2005; Watanabe et al. 2004).

There are at least two pathways of differentiation leading to fully polarized effector cells, defined as IFN-γ-secreting Th1 vs IL-4-secreting Th2 cells. However, the population of effector CD4 cells in a typical immune response is heterogeneous, as they comprise cells suspended at varying degrees of polarization. This heterogeneity includes cells that have committed to the Th1 or Th2 subset or the multi-potential IL-2 producers that have yet to make a commitment toward any lineage (Roman et al. 2002). Multiple factors affect the polarization to Th1 vs Th2 effectors, including the strength of TCR signaling, co-stimulation, and probably most importantly, the cytokine milieu, with IL-12 promoting Th1 cells and IL-4 favoring Th2 lineage. Exactly how these conditions initiate and sustain the differentiation pathway has yet to be fully defined, but it involves the induction of the transcription factors T-bet for Th1 cells and GATA-3 for Th2 cells (Glimcher and Murphy 2000).

As with CD8 cells, memory CD4 cells are believed to originate from a fraction of effector CD4 cells generated during the peak of the immune response, presumably soon after the cessation of any stimulatory signals (Harbertson et al. 2002; Hu et al. 2001). Considering that these effector CD4 cells constitute a heterogeneous population of cells in terms of the extent of differentiation and polarization, the question arises as to whether all effector cells have equal chances of becoming memory cells. This could be the case if the effector-to-memory conversion occurs in a stochastic manner. In support of this idea, fully polarized effectors appear to be capable of converting into memory cells and maintain their fixed trait upon conversion into memory cells (Harbertson et al. 2002; Hu et al. 2001; Swain 1994); hence, it is quite possible that the composition of memory cells reflects the heterogeneity at the effector stage. However, it is also possible that the capacity to become memory cells is determined by the strength of initial stimulation. Thus, fully differentiated IFN-γ-producing effector cells are less likely to differentiate into memory cells than their partially polarized counterparts that do not secrete IFN-γ (Wu et al. 2002). Nonetheless, it is clear that memory CD4 cells are heterogeneous and comprise both partially and fully polarized populations of cells. Many of the partially differentiated cells are found in the secondary lymphoid tissues and are capable of becoming fully polarized Th1 or Th2 effector cells, depending on the stimulatory conditions (Ahmadzadeh and Farber 2002; Marzo et al. 2002). In contrast, the majority of fully polarized memory cells are found in non-lymphoid tissues and appear to be the descendents of effector cells that had migrated into these tissues during the peak of the immune response (Marzo et al. 2002).

As with CD8 cells, effector CD4 cells undergo apoptosis during the contraction phase, and this process is probably mediated by multiple mechanisms, including the cytotoxic activity of the effector cytokine IFN-γ. A role for IFN-γ

is indicated by the finding that activated CD4 cells, like CD8 cells, accumulate in IFN-γ-deficient mice after an immune response to a pathogen or an autoantigen (Chu et al. 2000; Dalton et al. 2000; Refaeli et al. 2002; Xu et al. 2002). In addition, activity of pro-apoptotic Bcl-2 family molecules, such as Bim, Bak, and Bax, appear to be particularly relevant for timely contraction of activated CD4 cells. Prolonged survival of activated CD4 cells is thus apparent in mice deficient in Bim and in mice deficient in both Bak and Bax (Bouillet et al. 1999; Hildeman et al. 2002; Rathmell et al. 2002). Nonetheless, it is unclear whether the death pathways induced by the pro-apoptotic molecules are part of a cell autonomous program triggered with T cell activation (Marrack and Kappler 2004) or from a lack of extrinsic survival factors that keeps cells alive.

3.2 Maintenance of CD4 Memory

In contrast to the consensus that memory CD8 cells can persist almost indefinitely, there is controversy about whether such a similar longevity applies to memory CD4 cells. Early studies of responses to viral infections, including LCMV and Sendai virus, have concluded that CD4 cell memory in the mouse is stable for as long as 1–2 years (Sierra et al. 2002; Topham and Doherty 1998; Varga and Welsh 1998; Whitmire et al. 1998). Similarly, studies in humans also led to the estimation that the half-life of CD4 memory cells against vaccinia virus is about 10–15 years, similar to that of CD8 cells (Hammarlund et al. 2003). Contrary to these results, more recent analysis of memory cell persistence over a longer duration found that CD4 memory cells specific to LCMV gradually disappear over time, whereas CD8 memory cells persist in stable numbers (De Boer et al. 2003; Homann et al. 2001). The attrition rate of memory CD4 cells was found to be slow, at a half-life of about 500 days, indicating that CD4 memory cells can nonetheless persist for a significant amount of time.

As with other T cell subsets, γc cytokines and self-MHC/peptide ligands probably provide survival signals for memory CD4 cells, but a clear consensus has yet to be firmly established. MHC class II molecules were initially believed to be irrelevant based on the finding that in vitro generated TCR transgenic CD4 effectors survive upon adoptive transfer into MHC II-deficient hosts (Swain et al. 1999). Consistent with this finding, abolishing TCR expression on mature T cells did not affect survival of MP CD4 cells (Polic et al. 2001). Despite these studies, three lines of investigation implicated a role for MHC molecules in memory CD4 cell homeostasis. First, although MHC class II molecules were not essential for survival of TCR transgenic memory CD4 cells, these cells could not display a full range of effector function (Kassiotis

et al. 2002). Second, when the expression of TCR was extinguished on mature T cells, the rate of spontaneous homeostatic proliferation of MP CD4 cells in normal T-sufficient conditions declined significantly, implying that most of the turnover of MP CD4 cells is driven by signaling through the TCR (Polic et al. 2001). Third, acute homeostatic proliferation of MP CD4 cells in lymphopenic hosts was dependent upon TCR signaling, but only when IL-7 was concurrently removed (Seddon et al. 2003).

As with MHC, the role of the cytokines in regulating the maintenance of memory CD4 cells has yet to be fully defined. Early studies indicated that γc cytokines were not involved in regulating homeostasis of memory CD4 cells. Thus, γc-deficient TCR transgenic CD4 cells, which die rapidly as naïve cells, survived efficiently upon activation by antigen (Lantz et al. 2000); one caveat with this study, however, is that γc-deficient CD4 cells may have developed a physiologically irrelevant mechanism to survive without γc cytokines. Nonetheless, another study showed that wild-type MP CD4 cells did not require either IL-7 or IL-15 to undergo efficient homeostatic proliferation in irradiated hosts (Tan et al. 2002). Other analysis, however, strongly indicated that IL-7 is essential for homeostasis of memory CD4 cells. First, in the absence of TCR signaling, MP CD4 cells were found to be acutely dependent on IL-7 for survival and homeostatic proliferation (Seddon et al. 2003). Second, antigen-specific TCR transgenic memory CD4 cells were found to be dependent on IL-7 for survival under in vitro and in vivo conditions, even in hosts with normal expression of MHC class II molecules (Kondrack et al. 2003; Lenz et al. 2004; Li et al. 2003). Third, injection of anti-IL-7R mAb caused significant decline in the survival and turnover of de novo generated polyclonal LCMV-specific memory CD4 cells (Lenz et al. 2004).

The reason for a lack of consensus in the above studies could be due to the fact that the rate of proliferation and the homeostatic requirements for antigen-specific memory CD4 cells are different from those for spontaneously generated MP CD4 cells. A direct comparison of these two types of memory CD4 cells revealed that MP CD4 cells as a population turn over at a much faster rate than antigen-specific memory CD4 cells (Lenz et al. 2004). For antigen-specific memory CD4 cells, we interpret the above studies to indicate that these cells turn over at a slower rate than MP cells and are mainly dependent on IL-7, and possibly on MHC, for survival and homeostatic proliferation. This may also apply to antigen-specific memory CD4 cells within the MP CD4 cell population. In contrast, MP CD4 cells also appear to contain a population of recently activated cells that has a fast rate of turnover. These cells appear to be MHC dependent, as ablation of TCR signaling significantly decreases the proliferation rate of these cells (Polic et al. 2001; Seddon et al. 2003; Tan et al. 2002). The factors that drive the fraction of fast-dividing MP CD4 cells is

currently under investigation, but it is possible that proliferation of these cells is driven by antigens that cannot be cleared, such as those from the gut flora.

4
Heterogeneity of Memory T Cells

4.1
Memory T Cell Homing

Effector T cells generated during an immune response give rise to memory cells that are found in various non-lymphoid tissues. Memory T cells can therefore be arbitrarily categorized according to their differential capacity to home to different tissues. For instance, expression of α4β7 is required for migration of effector and memory cells to the intestine, and expression of CLA allows migration to the skin, and these tissue-specific homing capacities appear to be imprinted by the microenvironment of the local tissue (Calzascia et al. 2005; Dudda et al. 2004; Mullins et al. 2003). Migration into non-lymphoid tissues is also regulated by the expression of appropriate chemokine receptors (Campbell and Butcher 2000). Despite their homing tendencies, it appears that memory cells in some, but not all, tissues have the capacity to recirculate through the lymph and the blood. MP CD4 cells were thus found to be the predominant cells in the afferent lymph draining the skin (Mackay et al. 1990). More recently, parabiosis studies also found that memory CD8 cells rapidly migrated into the lung, liver, and lymphoid tissues of the naïve parabiont, but not into lamina propria (LP) and the brain (Klonowski et al. 2004). This finding suggests that memory CD8 cells in the blood, but not in LP and brain, recirculate readily into the lung and lymphoid tissues. According to this idea, the memory cells in LP and brain could be generated from activated CD8 cells that infiltrated these tissues at the peak of the immune response.

4.2
Phenotypic and Functional Memory T Cell Subsets

Memory T cells are found in both lymphoid and non-lymphoid tissues. The cells in the lymphoid tissues, especially in the lymph nodes, express the chemokine receptor CCR7 and the adhesion molecule CD62L. In contrast, memory cells in non-lymphoid tissues do not express these molecules. This finding, coupled with the finding that effector memory cells (T_{EM}) cells display effector function faster than central memory cells (T_{CM}) cells, led to the paradigm that memory T cells can be divided into $CCR7^{+}CD62L^{hi}$ central memory (T_{CM}) and $CCR7^{-}CD62L^{lo}$ effector memory subsets (T_{EM}) (Masopust

et al. 2001; Reinhardt et al. 2001; Sallusto et al. 1999). Recent studies, however, indicate that such a categorization may be overly simplistic. Thus, both subsets were found to be equally efficient in producing cytokines and mediating cytotoxic activity (Bouneaud et al. 2005; Ravkov et al. 2003; Unsoeld et al. 2002; Wherry et al. 2003). Moreover, under in vivo conditions, T_{CM} cells were found to confer better protective immunity from re-infection than T_{EM} cells (Bouneaud et al. 2005; Ravkov et al. 2003; Unsoeld et al. 2002; Wherry et al. 2003; Zaph et al. 2004). The division of memory cells into T_{CM} and T_{EM} subsets thus appears to be more useful for distinguishing anatomical location rather than functional capability.

In terms of their lineage relationship, it was initially proposed that T_{CM} cells are precursors of T_{EM} cells, serving as a pool of long-lived memory cells that give rise to T_{EM} cells upon re-exposure to antigen (Sallusto et al. 1999). Recent studies, however, indicate that at least two other possible lineage relationships exist. First, it is possible that T_{CM} and T_{EM} cells arise from independent lineages. This notion is suggested by the finding that Vβ repertoires of human memory T_{CM} and T_{EM} cells are largely unrelated, even when individuals were analyzed over a considerable span of time (Baron et al. 2003). Nonetheless, human T_{CM} cells can be converted to T_{EM} cells in vitro after stimulation through the TCR or cytokines, indicating a relationship between the two subsets, at least under in vitro conditions (Champagne et al. 2001; Geginat et al. 2001; Rivino et al. 2004), Second, instead of the T_{CM} to T_{EM} order of transition, adoptive experiments in the mouse indicate that T_{EM} cells convert to T_{CM} cells rather than vice versa (Bouneaud et al. 2005; Wherry et al. 2003; Zaph et al. 2004).

The underlying reasons for the contradictory results remain unknown, but could reflect differences between mouse and human cells, differences between memory generated after acute and chronic infections, or differences between CD4 and CD8 cells. Nonetheless, an intriguing recent finding suggests that the proliferative capacity of T cells during priming is the key factor determining the fate of T_{CM} vs T_{EM} cells. Specifically, the activation of a low precursor frequency of antigen-specific naïve cells (with a high proliferative potential) results in the generation of a stable population of T_{EM} cells that do not convert into T_{CM} cells, whereas a high precursor frequency of naïve cells (with a low proliferative potential) induced the generation of memory cells with T_{EM} to T_{CM} transitional capability (L. Lefrancois, personal communication).

5
Influence of CD4 T Cells on the Quality of CD8 Memory

For many infectious systems, activation of CD8 cells does not require CD4 cell help because infectious agents can directly activate APCs and induce CD8 cell responses (Bevan 2004). Viruses or intracellular bacteria could activate APCs by directly stimulating TLRs, inducing the secretion of inflammatory mediators and upregulation of costimulatory molecules required for CD8 cell activation. In contrast, CD8 cell responses to non-inflammatory antigens are described as CD4-dependent because CD4 cell help is required to drive efficient CD8 responses. In these models, APCs need to be "licensed" by CD4 cells, through the CD40–CD40L interaction, in order to acquire the capacity to induce activation-naïve CD8 cells (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998). In addition to the licensing role of CD4 cells, recent work indicates that CD4 cells are also crucial for the generation of functional CD8 memory, even in immune responses typically considered CD4-independent. Notably, in the absence of CD4 cells, primary CD8 cell responses were normal, but the ensuing memory CD8 cells failed to persist on a long-term basis and were impaired in their ability to undergo expansion and to display effector function, including cytokine production and cytotoxic activity upon antigenic re-stimulation (Bourgeois et al. 2002; Janssen et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003; Sun et al. 2004; von Herrath et al. 1996). The memory CD8 cells generated in the absence of CD4 help also exhibited significantly lower expression of key surface markers, CD62L, CD44, CD127, and CD122 (Khanolkar et al. 2004; Sun et al. 2004).

How CD4 cells provide "help" for the development of fully functional CD8 memory cells is unclear. Initial work suggested that CD4 cells provide a signal during the primary response to program activated CD8 cells to differentiate into fully functional memory cells. Thus, depletion of CD4 cells during the primary response, but not during the secondary response, resulted in the generation of memory CD8 cells with an impaired ability to display recall responses (Janssen et al. 2003; Shedlock and Shen 2003). However, a more recent study indicated that the "help" signal from CD4 cells is not provided during the priming stage, but at the maintenance phase after the primary response has waned and resting memory cells have developed (Sun et al. 2004). Hence, "helped" memory CD8 cells generated in wild-type mice became functionally impaired and gradually disappeared upon adoptive transfer into mice deficient in CD4 cells, and conversely, "unhelped" memory CD8 cells regained effector function upon adoptive transfer into wild-type hosts (Sun et al. 2004). Moreover, the help provided by CD4 cells was not antigen-specific, as adoptive transfer involved only the memory CD8 cells. The nature of the CD4 cell help

is unclear, but it may somehow ensure that memory CD8 cells continue to respond to the homeostatic cytokines IL-7 and IL-15. This notion is supported by the finding that "unhelped" memory CD8 cells, unlike "helped" memory CD8 cells, gradually lose the expression of the receptors for IL-7 and IL-15 over time (Sun et al. 2004).

Irrespective of the nature of the proximal signal provided by the CD4 cells for memory CD8 cells, much of the distal negative effect on "unhelped" memory CD8 cells appears to be mediated through the TNF-related apoptosis-inducing ligand (TRAIL). Thus, in the absence of TRAIL, "unhelped" memory CD8 cells displayed normal secondary response, and the presence of exogenous TRAIL effectively inhibited secondary response of "helped" memory CD8 cells (Janssen et al. 2005). This study also found that both "unhelped" and "helped" memory CD8 cells upregulate DR5, the receptor for TRAIL, after secondary stimulation, but only "unhelped" cells also produced TRAIL. The implication therefore is that the demise of "unhelped" memory CD8 cells is mediated through an autocrine negative effect mediated by TRAIL. How the proposed role of TRAIL can be reconciled with the fact that CD4 cells are required during the maintenance phase is unclear, but there is no doubt that this will be an issue of further investigation.

6
Concluding Remarks and Perspectives

The collective work of many investigators in recent years has provided further insight into some of the intriguing and novel mechanisms that regulate memory cell generation and differentiation. While the innate immune response precedes the memory response, it is apparent that memory cell differentiation is influenced by the inflammatory mediators and cells traditionally associated with the innate system. Memory differentiation appears to progress over a continuum of time, so that the magnitude and quality of memory is tailored by different co-stimulatory molecules and cytokines. Once established, memory is actively maintained by cytokines, and competition for these factors influences the survival of these memory cells. Future challenges will be to explore which signaling pathways determine the selection of memory cells and when these events occur. The interaction of the innate immune system with adaptive immunity will surely be an important aspect of this selection. Additionally, improved abilities to track CD4 memory will certainly lead to greater understanding of how these cells are generated and maintained and how they influence CD8 memory.

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The Privacy of T Cell Memory to Viruses

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Abstract T cell responses to viral infections can mediate either protective immunity or damaging immunopathology. Viral infections induce the proliferation of T cells spe-

cific for viral antigens and cause a loss in the number of T cells with other specificities. In immunologically naïve hosts, viruses will induce T cell responses that, dependent on the MHC, recognize a distinct hierarchy of virus-encoded T cell epitopes. This hierarchy can change if the host has previously encountered another pathogen that elicited a memory pool of T cells specific to a cross-reactive epitope. This heterologous immunity can deviate the normal immune response and result in either beneficial or harmful effects on the host. Each host has a unique T cell repertoire caused by the random DNA rearrangement that created it, so the specific T cells that create the epitope hierarchy differ between individuals. This "private specificity" seems of little significance in the T cell response of a naïve host to infection, but it is of profound importance under conditions of heterologous immunity, where a small subset of a cross-reactive memory pool may expand and dominate a response. Examples are given of how the private specificities of immune responses under conditions of heterologous immunity influence the pathogenesis of murine and human viral infections.

Abbreviations

APC	Antigen-presenting cell
CDR	Complementary determining region
CMV	Cytomegalovirus
DC	Dendritic cell
EBV	Epstein–Barr virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IFN	Interferon
J	Joining
LCMV	Lymphocytic choriomeningitis virus
MHC	Major histocompatibility antigen
NK	Natural killer
PKR	Protein kinase R
PV	Pichinde virus
TCR	T cell receptor
V	Variable
VV	Vaccinia virus

1 Introduction

Immunological memory is a function of expanded clones of antigen-specific T and B cells. Its purpose is to protect a host from a second encounter with a pathogen, to keep low-grade persistent infections under control, and, by passive transfer of antibody, to protect a fetus or neonate from infection. The repertoire of T and B cells that constitutes a memory population is influenced by genetic and epigenetic factors, prior infection history, and innate response regulatory mechanisms (Welsh et al. 2004).

It has long been noted that an acute host response to viral infection comes in two waves, an early innate response associated with the induction of cytokines and activation of natural killer (NK) cells, dendritic cells (DCs), and macrophages, and a later response associated with the expanded clones of antigen-specific T and B cells (Welsh 1978; Biron 1995). A very important innate system cytokine characteristic of viral infections is type 1 interferon (IFN), which exerts many activities, including the direct inhibition of viral replication in infected cells (Welsh 1984; Biron and Sen 2001). Other important innate system cytokines are interleukin (IL)-12, IL-18, and inflammatory cytokines IL-1, IL-6, and tumor necrosis factor (TNF)-α (Biron 1995). Studies in the past 10 years have implicated toll receptors in the induction of many of these cytokines (Compton et al. 2003; Haynes et al. 2001). Viral proteins, RNA, and DNA can engage many of these receptors, as reviewed elsewhere in this volume. This receptor engagement triggers signal transduction events that release transcription factors such as interferon regulatory factor (IRF)-3 and nuclear factor (NF)-κB to activate cytokine genes (Jiang et al. 2004). In addition, viral double-stranded RNA can activate protein kinase R (PKR), whose phosphorylated products release cytokine transcription factors into the nucleus (Biron and Sen 2001). The cytokines produced in response to these events can skew T cell responses into the type 1 (IL-2, IFN-γ) vs type 2 (IL-4, IL-5, IL-13) cytokine direction, and, as a consequence, influence the antibody isotype ultimately produced by the B cells. Viral infections tend to be strong inducers of type 1 cytokine responses, perhaps because of the early induction of IL-12 and IL-18, which themselves induce IFN-γ from NK cells and T cells.

The most important cellular interaction at the advent of a new immune response is the engagement of a naïve T cell with an antigen-presenting DC. DCs exposed to antigen in the periphery can become activated due to toll receptor engagement or to other "danger" signals, which induce the expression of co-stimulatory proteins such as CD80 (B7.1) and CD86 (B7.2) and of the CCR7 chemokine receptor, which directs their migration into the lymph nodes, where they encounter naïve antigen-specific T cells (Sallusto et al. 2000). CD80 and CD86 on the DCs engage CD28 on the T cells and induce CD154 (CD40L) expression on the T cells and the release of growth factors such as IL-2 (Harris and Ronchese 1999). This sets up a programmed proliferation of the T cells (Kaech and Ahmed 2001; Mercado et al. 2000; van Stipdonk et al. 2001), which may divide as many as 15 times before their peak in acute infection (Selin et al. 1994; Blattman et al. 2002; Welsh and Selin 2002). The $CD4^+$ T cells through CD40L/CD40 interactions can provide help to B cells, which also proliferate (Liu et al. 1997). After clearance of viral antigens, the T cell response contracts by apoptosis (Razvi et al. 1995a) and

by dissemination into peripheral tissue (Marshall et al. 2001; Masopust et al. 2001; Reinhardt et al. 2001; Wiley et al. 2001), leaving the host with a stable population of memory T cells, which slowly divide but do not increase in number (Razvi et al. 1995b; Zimmermann et al. 1996). The B cell response is more complex, as some develop into long-lasting antibody-secreting plasma cells, and some become memory B cells, which do not secrete antibody but which can rapidly proliferate and produce antibody on antigen re-challenge (Han et al. 1997; Lin et al. 2003). Developing B cells triggered by viral antigens enter germinal centers and undergo somatic mutations and selection for higher affinity antibody responses (Han et al. 1997; Muramatsu et al. 2000). The high-affinity antibodies are more effective at the neutralization of viruses, though some viruses seem to induce high-affinity responses without affinity maturation (Clarke et al. 1990; Roost et al. 1995). This is likely because of germ line immunoglobulin sequences that encode antibody that is already high affinity.

This review will focus on the repertoire of cells constituting a T cell memory pool, how this repertoire is generated, how it is modulated by innate immune system cytokines, and how it is modulated in response to other infections. In particular, it will describe how properties of an immune system unique to an individual can alter the pathogenesis of infections. A more comprehensive though less focused review on immunological memory to viral infections can be found elsewhere (Welsh et al. 2004).

2
Diversity of Memory T Cell Repertoires

2.1
Immunodominance and TCR Repertoire Diversity

Generation of T cell repertoires in the immunologically naïve host is initially a stochastic process dependent on the randomness of DNA recombination events. There are about 10^{15} possibilities for the generation of T cell receptors (TCRs) with paired α- and β-TCR chains (Casrouge et al. 2000; Nikolich-Zugich et al. 2004). Since it is impossible to accommodate all possibilities within one body, only a subset of this repertoire is generated and present in any given host. The initial randomly generated repertoire is trimmed down as it passes through the positive and negative selection phases in the thymus. This has been elegantly shown in a transgenic mouse where a single β-TCR transgene is expressed along with an α-TCR artificial rearrangement substrate transgene that must undergo a recombination event to express an α-TCR that

would pair with the β-TCR and be expressed on a mature T cell. This approach, which provided a manageable number of α-TCR gene complementary determining regions 3 (CDR3) to sequence, showed a very high clonal diversity in $CD4^+CD8^+$ thymocytes (with few duplicates), which became less diverse in single positive thymocytes, with some sequences overrepresented 5–40 times, and even less diverse in peripheral T cells, perhaps as a consequence of homeostatic proliferation of some T cells but not others (Correia-Neves et al. 2001). In addition to the role of CDR3 in repertoire selection, the other TCR variable (V) regions, CDR1 and CDR2, may be involved in narrowing the repertoire due to their differences of affinity in binding to specific class I and class II MHC molecules. The consequence of this is an uneven Vα and Vβ family distribution in the pre-immune TCR repertoires in the peripheral organs of mice or humans expressing different class I or II MHC antigens (Battaglia and Gorski 2002; Gulwani-Akolkar et al. 1991). Despite all of these trimming events, however, the naïve TCR repertoire in the immunologically naïve host remains quite diverse.

CD8 T cells recognize processed 8–9 amino acid viral peptides presented by class I MHC molecules (Townsend et al. 1986). For the most part these peptides are loaded into newly synthesized class I molecules in virus-infected cells, though there are examples of the "cross-presentation" of exogenous viral peptides on uninfected antigen-presenting cells (APC) (Sigal et al. 1999). Although hundreds of peptides in any viral infection have appropriate sequences to bind class I MHC, usually only a small number stimulate "immunodominant" T cell responses. What causes immunodominance is a function of (1) the number of T cells with receptors that recognize the peptide-MHC combination, (2) the expression level of the peptide and its affinity for binding the MHC, (3) how early during virus infection the epitope is expressed (earlier is better), and (4) whether there is "immunodomination" by T cells of other specificities (Yewdell and Bennink 1999). CD4 T cells recognize longer, usually 12–15 amino acid, viral peptides expressed by either endogenously or exogenously loaded class II MHC molecules. Similar issues are involved in CD4 T cell immunodominance as with CD8 T cells (Wang et al. 1992).

2.2 Public Vs Private Specificities

Despite the events that narrow the TCR repertoire, genetically identical individuals are still bequeathed with very different TCR repertoires because of the stochastic processes during T cell development. In the mouse, where it has been calculated that there are fewer than 10^7 T cell clones per host, there is substantial variability of the TCR repertoire from host to host (Cas-

rouge et al. 2000). Humans have about 3,000 times the body size of a mouse and will have many more clones of T cells, but this number will still be orders of magnitude below the theoretical possibility (Arstila et al. 1999). The repertoire selected in genetically identical environments, while being distinct between individuals, has similar potential specificities. When genetically identical, immunologically naïve mice are infected with a virus such as lymphocytic choriomeningitis virus (LCMV), they generate quantitatively similar responses to immunodominant epitopes, and their responses to these epitopes usually have similar hierarchies among individual mice. For example, most C57BL/6 mice will generate a range in T cell frequencies directed at a reproducible hierarchy of peptide epitopes, in the order of $NP_{396–404} > GP_{34–41} > GP_{33–41} > GP_{276–286} > NP_{205–212} > GP_{92–101}$ (Kim et al. 2005; Fuller et al. 2004). This is a common "public specificity" (Cibotti et al. 1994) that can be predicted ahead of time in these genetically identical mice. Examination of TCR usage between mice will also show preference patterns for TCR V domains per epitope (Blattman et al. 2000). This is another manifestation of public specificity influenced in part by the importance of CDR1 and CDR2 regions in generating the pre-immune repertoire (Battaglia and Gorski 2002; Gulwani-Akolkar et al. 1991).

Sequencing of the CDR3 shows, however, that different TCRs are used by different hosts for similar types of antigen recognition. This is referred to as the "private specificity" of TCR repertoires (Cibotti et al. 1994; Maryanski et al. 2001). The CDR3 of antigen-specific TCR pools from different mice will have some similar CDR3 amino acids which establish a recognition "motif," but many amino acids will be unshared. These private T cell responses are probably mostly accounted for by the diversity of T cells that emigrate from the thymus. In addition, there may be stochastic events involved with chance encounters between T cells and APC during early stages of infection. One might think that the T cell that is the first to encounter its antigen may become a dominant clone (Butz and Bevan 1998), whereas a T cell that first encounters antigen 2 days later may develop into a subdominant clone or else may never have a chance due to "immunodomination" by other clones competing for domains on APC.

2.3 Repertoire Selection During Infection

Events that shape the antigen-specific TCR repertoire occur very early in infection. T cell responses to *Listeria monocytogenes* epitopes occur rather normally if mice are treated with antibiotics 1 day after infection to prevent further antigen synthesis (Mercado et al. 2000). T cells that engage APC bind

to them for hours, but after disengagement the APC tend to die off, as has been elegantly visualized by in vivo videomicroscopy (Huang et al. 2004) and with APC infected with green fluorescent protein (GFP)-expressing recombinant viruses (Norbury et al. 2002). A question is how efficient this process is for stimulating potentially reactive T cells. This has been studied in mice with implanted 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled transgenic T cells, where LCMV or *L. monocytogenes* infections were shown to easily stimulate programmed expansions of nearly all of the cells (Mercado et al. 2000; Kaech and Ahmed 2001; van Stipdonk et al. 2001). These experiments would suggest that the stimulation of T cells, at least those of high affinity, can be quite efficient, and this probably means that the private specificity of the TCR repertoire is more a function of the pool of thymic emigrants than it is a function of inefficient random encounters with the APC. Nevertheless, limited titration of immunogen resulted in situations where only a subpopulation of the transgenic T cells responded (Kaech and Ahmed 2001). One should keep in mind that we are talking about systemic infections in mice, and the dynamics might be different with more limited localized infections or in humans. Due to their much larger thymic output, two MHC-matched humans may be 3,000 times more likely to have similar T cell clones than two mice and may be less likely to stimulate all of their antigen-specific clones during an infection. Perhaps in that case, stochasticity due to random encounters of T cells with APC may play a somewhat greater role in private specificities of T cell responses between humans.

The private specificity phenomenon complicates TCR studies between individuals but allows for longitudinal studies within an individual. Studies using CDR3 "spectratype" or "immunoscope" techniques, PCR-based techniques which analyze receptor diversity based on the CDR3 lengths of the expressed α and β TCRs derived from particular V families (Pannetier et al. 1993; Gorski et al. 1994), along with studies based on sequencing of the CDR3 region, all showed that genetically identical C57BL/6 mice infected with LCMV generated distinct TCR repertoires, as analyzed either by total leukocytes, total CD8 T cells, total CD4 T cells, or MHC tetramer or MHC-dimer defined and sorted antigen-specific T cells (Lin and Welsh 1998; Blattman et al. 2000; Wang et al. 2003). Sequential sampling of peripheral blood showed that the repertoire during an acute infection evolved until antigen was mostly cleared at about day 7 post-infection, after which it became relatively fixed as the T cell response contracted and entered the memory pool (Lin and Welsh 1998; Xiong et al. 2001). Similar spectratypes, with some minor variations, were noted when the memory pool was re-challenged with antigen.

2.4 The Complexity of Virus-Specific TCR Repertoires

Analyses of the TCR repertoires specific to viral peptides have been done in a number of systems, with perhaps the most extensive analyses being with human T cells recognizing an HLA-A2-restricted influenza $M1_{58–66}$ peptide (Lehner et al. 1995; Moss et al. 1991; Naumov et al. 1998). This is an invariant peptide present in all 35 influenza A virus strains that have infected humans since 1918 (Park et al. 1997). Over their lifetimes, people encounter many different influenza infections, which repeatedly should boost the response to the $M1_{58–66}$ peptide. During these repeated exposures the TCR repertoire changes. In young children only a small component of the repertoire is associated with Vβ17 T cells, but by age 15 Vβ17 T cells dominate the response (Lawson et al. 2001b). A detailed analysis of limiting dilution clones from 5 HLA-A2.1$^+$ adult donors showed that 85% belonged to the Vβ17 family, and a CDR3 sequencing analysis of 38 Vβ17$^+$ long-term clones revealed that 74% had a highly conserved XRSX motif (Lehner et al. 1995). Other $M1_{58–66}$-specific Vβ families (23, 13.6, 8.1) were found at lower frequencies and seemed of lower affinity, as they required high concentrations of epitope for cytotoxicity (Lawson et al. 2001a). This reflects a narrowing of a TCR repertoire that can occur on repeated exposure to antigen, and one might expect that this repeated exposure would select for a small number of dominant clones. However, a more extensive analysis of the full range of $M1_{58–66}$-restricted clones within individuals revealed a repertoire that is still quite diverse (Naumov et al. 1998, 2003). This showed that literally hundreds of clones constitute the $M1_{58–66}$-specific repertoire, but with no overwhelmingly dominant clones.

Molecular analyses of the Vβ 17 receptor repertoire from tetramer-identified $M1_{58–66}$-specific T cells or from $M1_{58–66}$-stimulated T cell lines revealed a power-law-like distribution in clonal composition, where a small number of clones were present at high frequency and larger numbers of clones were present at ever decreasing frequencies. Why this distribution occurs is unclear, though a power law distribution of the clonal ranks and rank frequencies can be mathematically generated simply by taking virtual clones of comparable affinity and allowing for proliferation after chance encounter with antigen (E. N. Naumova, in preparation). This might suggest that there are many clones of comparable affinities in the highly evolved $M1_{58–66}$-specific T cell response, but this is not definitive, as power law distributions can be generated on the basis of other paradigms. Similar power law distributions have been noted for the mouse repertoire specific to a mouse hepatitis virus epitope (Pewe et al. 2004). Mathematical analyses have also shown that the distribution of $M1_{58–66}$-specific TCR β regions in humans can be described in terms of fractals (Naumov et al. 2003, 2006). The reason for this is not clear, though influenza virus

hemagglutinin-specific B cell repertoires based on immunoglobulin structural diversity can similarly be mathematically described in terms of fractals (Burgos 1996). All of these analyses imply a repertoire that is quite complex.

Despite the high numbers of antigen-specific clones, the influenza $M1_{58–66}$-specific response conforms to distinct structural features, even when examined between different healthy individuals (Fig. 1; Clute et al. 2005). Within the very dominant antigen-specific Vβ17 population lies a hierarchy of joining region (J)β usage, with Jβ2.7 > Jβ2.3 > Jβ2.1 (Fig. 1). This percentage distribution of Jβ usage is remarkably similar between individuals and within samples from the same individual over a number of years. The amino acid sequences of the CDR3 within most of the Jβ-defined subpopulations share the IRSS amino acid sequence, with amino acid substitutions frequently occurring in the first and fourth positions. Thus, the specific clones of T cells are different between individuals, but the structure of the T cell repertoire is very similar, and apparently obeying the same rules. The similarity between individuals is likely due to the structural properties of HLA-A2.1-positively selected T cells that emigrate from the human thymus.

A major question is whether all T cell repertoires distribute themselves in patterns similar to or different from the influenza $M1_{58–66}$-specific repertoire, but insufficient analyses have been made to clearly answer this question. What is known is that the T cell repertoire can have different levels of complexity. During viral infections, T cell responses are sometimes directed against a small number of immunodominant epitopes, but in other cases directed against a large number of epitopes. The CD8 T cell response to the LCMV infection in the C57BL/6 mouse is directed against at least seven epitopes, whereas the vesicular stomatitis virus infection and Sendai virus infections in mice are each directed mostly at one epitope (Oldstone 1991). An epitope-specific response may involve several Vβ families, such as Vβ7, Vβ8, Vβ13, and others for the HLA-A2-restricted HTLV-1 $Tax_{11–19}$ epitope (Lim et al. 2000b), and Vβ2, Vβ4, Vβ16, and Vβ22 for the HLA-A2-restricted Epstein–Barr virus (EBV) immunodominant epitope $BMLF1_{280–288}$ epitope (Annels et al. 2000; Lim et al. 2000a), or there may be a predilection for the use of one Vβ family, such as the dominance seen for Vβ17 directed against the $M1_{58–66}$ epitope (Lehner et al. 1995). Within a Vβ family the repertoire may be composed of literally hundreds of clones, such as that seen with influenza $M1_{58–66}$ (Naumov et al. 2003, 2006), or it may be "oligoclonal," indicating a predominance of a smaller number of clones. Examples of oligoclonal responses have been reported, particularly in persistent viral infections, such as with human immunodeficiency virus (HIV), hepatitis C virus (HCV), cytomegalovirus (CMV), and EBV (Lim et al. 2000a; Annels et al. 2000; Meyer-Olson et al. 2004; Wilson et al. 1998; Manfras et al. 2004; Pantaleo et al. 1994; Khan et al. 2002).

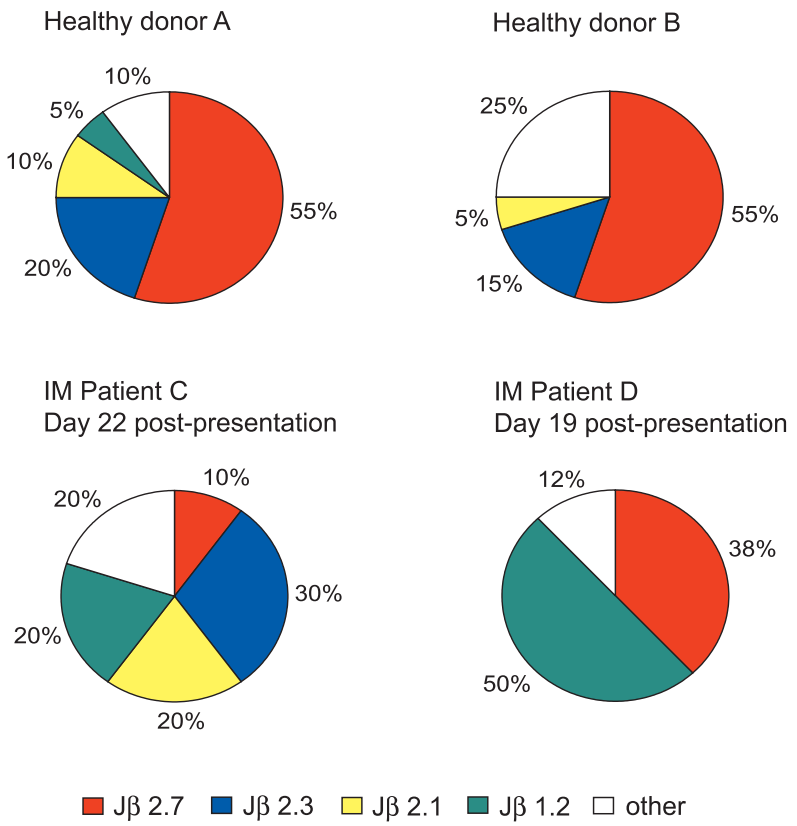


Fig. 1 CD8 T cells isolated from two healthy donors, with previous exposure to Epstein–Barr virus (EBV) and influenza A virus, and from two patients, presenting with infectious mononucleosis (*IM*) during an acute EBV infection, were cultured for 3–4 weeks in the presence of 1 μM $M1_{58–66}$ peptide-pulsed T2 cells transfected with and expressing HLA-A2.1. Following RNA isolation and cDNA synthesis of those M1-specific T cell lines, the CDR3 regions of Vβ17 clones were sequenced. Each Vβ clone was defined by its unique nucleotide sequence, and the pie charts illustrate the percentage of unique clones using each Jβ family (*A* 88 clones out of 152 sequences; *B* 20 clones out of 104 sequences; *C* 20 clones out of 31 sequences; *D* 8 clones out of 17 sequences). Based on Clute et al. (2005)

Narrowing of the diversity of TCR repertoires for a viral epitope may occur by evolution for the most perfect fit during persistent virus infections, such as those with HIV, HCV, CMV, and EBV, or with repeated antigenic challenges, such as with influenza virus. A repertoire could theoretically also be narrowed because of clonal exhaustion and activation-induced cell death that may occur under conditions of antigen excess (Zhou et al. 2002). The repertoire may be restricted if the host is partially tolerant to the epitope. Transgenic expression of LCMV proteins can cause complete or partial tolerance to the epitope when the host is later challenged with virus (von Herrath et al. 1994). The repertoire may also be restricted for structural reasons. The TCR repertoire generated in response to the mouse influenza virus epitope $PA_{224–233}$ tends to be far more diverse than to the influenza epitope $NP_{366–374}$. Crystal structures showed that the $PA_{224–233}$ epitope had an arginine in position four sticking out of the MHC groove, whereas the $NP_{366–374}$ epitope was more buried into the MHC (Turner et al. 2005). "Flattening" the $PA_{224–233}$ epitope by way of alanine substitutions resulted in a more restricted repertoire, leading to the suggestion that epitopes that structurally blend into the MHC may induce a narrower repertoire. Further examples are needed to confirm this hypothesis.

There are many examples of T cell cross-reactive peptides encoded by different viruses (Welsh et al. 2004), and another major cause of TCR repertoire restriction could be due to cross-reactive T cell responses. Here, a rather narrow subset of an epitope-specific T cell memory pool is selectively stimulated to proliferate on exposure to a cross-reactive pathogen (Haanan et al. 1999). On infection with a heterologous virus, these high-frequency, but not very diverse set of clones, may immunodominate an emerging T cell response from naïve precursors and cause a further restriction of the repertoire. This will be discussed in Sect. 6.

3 Distribution of Repertoire in Different Tissues

T cells can freely circulate throughout the body and can migrate into peripheral tissues. Probably because of different levels of viral antigen expression in different tissues, T cells of some specificities may be at relatively higher frequencies than T cells of other specificities (Wang et al. 2003). Tissue-dependent differences in the TCR repertoire of antigen-specific T cells, at least to the degree in which they have been studied, are minor at best (Wang et al. 2003; Turner et al. 2003). Perhaps of greater importance are the biological properties of these antigen-specific T cells. T cells in lymphoid organs tend to proliferate faster, express higher levels of Fas (CD95) and Fas L, and are more prone to

apoptosis than T cells in, for example, lungs, adipose tissue, or peritoneal cavity (Wang et al. 2003). Lung T cells appear to be protected from apoptosis by signals derived from the engagement of very late antigen (VLA)-1 on T cells with collagen in the lung parenchyma (Ray et al. 2004). T cells from peripheral organs also express more IL-7 receptor α, or CD127 (Wang et al. 2004). These tissue differences can occur independently of the TCRs, as transgenic T cells distributed into these areas have comparable phenotypic differences (Wang et al. 2003, 2004). Some gene array data have suggested that T cells responding to antigen in peripheral tissues may have enhanced expression of genes regulating cytotoxic and cytokine effector functions (Marshall et al. 2005).

Nevertheless, T cells of different specificities have different "personalities," in that they may have quantitative differences in their expression of apoptotic properties, such as mitochondrial electron transport potential, annexin V-reactive phosphatidyl serine, an early indication of apoptosis, Fas and Fas L, and the expression of IL-7R (Grayson et al. 2003; Wang et al. 2004). The reason for this remains unclear, as these personality differences are found during acute infection, the resting memory state, and the recall response (Wang et al. 2004). They occur under conditions of various forms of immunization—leading to the suggestion that they may relate to the inherent properties of the epitope—that are perhaps influenced by the genetic background of the host, either in selecting the repertoire in the first place or else in interacting with T cells in the periphery.

After the contraction phase of the immune response, memory T cells can be found in lymphoid organs, including bone marrow, and in peripheral tissues. There are some tissue-dependent phenotypic differences, in that "central memory" cells, which are $CCR7^{high}$ $CD62L^{high}$, are preferentially found in lymphoid organs and bone marrow and gradually undergo homeostatic division (Wherry et al. 2003; Sallusto et al. 2000; Razvi et al. 1995b; Tough and Sprent 1994). CCR7 is the chemokine receptor that directs lymphocytes into lymphoid tissue (Sallusto et al. 2000). "Effector memory" cells, which are $CCR7^{low}$, $CD62L^{low}$, tend to be more in peripheral tissues and are thought to be less proliferative and have a higher level of effector function (Sallusto et al. 2000). Adoptive transfer studies have indicated that these populations can be somewhat interchangeable (Wherry et al. 2003).

4 Homeostasis of Memory T Cells

At any given moment, a small subpopulation of memory CD8 T cells is undergoing division and is cytolytically active (Razvi et al. 1995b; Selin and Welsh

1997; Zimmermann et al. 1996). In vivo studies using bromodeoxyuridine to label dividing cells indicate that after a few weeks most of the memory T cells have divided at least once, but they do not increase in number (Zimmermann et al. 1996; Selin et al. 1996). The limits in their number could be imposed by the available space in the lymphoid organs. Alternatively, with each division there could be one surviving and one dying daughter cell. This steady state homeostatic division appears to be mediated by IL-15 and IL-7 (Prlic et al. 2002; Tan et al. 2002; Kieper et al. 2002). CD8 T cell memory tends to wane in IL-15 knockout (KO) mice (Becker et al. 2002) and is poorly generated in the first place in mice lacking IL-7 (Bradley et al. 2005). Of note is that cells from the acute infection that survive into the memory state are those with the highest expression of IL-7 receptors (Kaech et al. 2003). Recent work has indicated that there may be a higher turnover of memory CD8 T cells in the bone marrow than in other organs (Becker et al. 2005).

So far as anyone can tell, the steady state turnover of CD8 T cells in a replete and unchallenged immune system is generally across-the-board, affecting all $CD44^{high}$CD8 T cells somewhat equally, but in a non-synchronous manner. Though not extensively studied, there is little evidence for TCR repertoire changes. There appears to be quite a different dynamic if a host is rendered lymphopenic and if the immune system needs to replenish itself, as some of the T cells undergo several cycles of division and appear to compete with each other. In fact, bona fide virus-specific memory cells do relatively poorly in this competition, and it is thought that T cells that are either self-reactive or else reactive with foreign environmental antigens may proliferate the best (Peacock et al. 2003). This is clearly different from a typical foreign antigen-stimulated response, which is driven by IL-2, and where there is a transient enlargement of the lymph nodes and a considerable expansion and then apoptosis of the T cells. Instead, the proliferation is more IL-7 and IL-15-dependent, and it occurs without a lymph node expansion phase or a discrete apoptotic deletion phase. One is left with many $CD44^{high}$ CD8 cells that are not true memory cells, but instead are something else that is derived from a $CD44^{low}$ naïve cell (Kieper and Jameson 1999) but is neither a naïve cell nor a bona fide memory cell.

The most significant point about the above findings in the context of this review is the depletion of bona fide virus-specific memory cells under conditions of homeostatic proliferation in the lymphopenic host. CSFE-labeled LCMV-immune spleen leukocytes were transferred into lymphopenic environments caused by genetics (severe combined immunodeficient or T cell-deficient mice), irradiation, or toll receptor stimulation by the type 1 IFN-inducer poly I:C, and the proliferation of the LCMV antigen-specific memory cell population was monitored in comparison with the rest of the donor cells. In each

case there was substantially less proliferation of the bona fide memory cells; in some cases their frequency within the donor population dropped from about 20% to as little as 3% after 2 weeks (Peacock et al. 2003). Irradiation studies were particularly intriguing. Because of their enhanced expression of Bcl-2 (Grayson et al. 2000), bona fide memory cells were initially more resistant to irradiation than the rest of the T cell population and were enriched in number (Grayson et al. 2002), but with the passage of time even they were significantly diluted out (Peacock et al. 2003). A second interesting point is that lymphopenia and a subsequent loss of bona fide memory T cells occurs with toll-like receptor (TLR) agonists, and many viral infections can stimulate TLR and simulate the effects of their agonists (McNally et al. 2001; Jiang et al. 2003b; Kim and Welsh 2004; Peacock et al. 2003). Under these conditions, the TCR repertoire of the host most certainly changes, due to the failure of bona fide memory cells to recover from the deletion (Peacock et al. 2003).

5
Virus-Induced Lymphopenia and Loss of Memory T Cells

Although new thymic emigrants continually cause repertoire shifts in the naïve T cell compartment, the memory cell repertoire remains relatively constant, providing that there are no antigenic challenges or events that cause lymphopenia. When these antigenic challenges occur there are substantial reductions and alterations in memory T cell populations (Selin et al. 1996, 1999; Brehm et al. 2002). In general, an infection with an unrelated virus will induce the formation of new memory cells specific to the second virus and will delete the frequencies of memory cells specific to the previously encountered virus (Brehm et al. 2002). This is a permanent change that remains for the lifetime of the mouse, though it has never been demonstrated or sufficiently studied in the human. We have proposed two models to explain this loss in memory T cell frequency: the passive attrition model, whereby old memory cells are lost simply by their competition with newly formed memory cells for survival niches in the immune system after immune response silencing, and the active attrition model, whereby there is a directed apoptosis of the pre-existing memory cells (Selin and Welsh 2004). Most of our data support the active attrition model (Kim and Welsh 2004).

The early phases of many acute human and experimental animal viral infections is characterized by a profound lymphopenia, occurring throughout the body's organs, and particularly affecting the memory-phenotype ($CD44^{high}$) CD8 T cell population (McNally et al. 2001). These T cells appear to be undergoing apoptosis, as reflected by their reactivity with annexin V, their activation

of caspases, and their staining with TdT-mediated dUTP-X nick end labeling (TUNEL) assay (McNally et al. 2001; Jiang et al. 2003b). This apoptosis seems at least in part due to type 1 IFN, as it correlates with the IFN response and does not occur in type 1 IFN receptor-deficient mice (McNally et al. 2001). There also can be concomitant severe effects of type 1 IFN on DC, preventing their expansion and development (Hahm et al. 2005). The significance of this lymphopenia remains unresolved. Clearly, extremely severe lymphopenia may be an indicator of an overwhelming infection that may lead to death of the host. A more moderate lymphopenia does not seem to impair the development of the T cell response. Given that pathogens which are some of the strongest inducers of lymphopenia stimulate some of the strongest CD8 T cell responses, it is possible that, by making room in the immune system, the lymphopenia serves to stimulate the new T cell response. Creation of lymphopenic environments by irradiation or cytotoxic drug treatment can enhance immune responses to antigens (Oehen and Brduscha-Riem 1999; Pfizenmaier et al. 1977), possibly either by making space or reducing the number of regulatory T cells. It is noteworthy that infections cause less lymphopenia in older mice (Jiang et al. 2003a), and influenza virus and LCMV inducer weaker CD8 T cell responses in older mice (Po et al. 2002; Kapasi et al. 2002).

A second function of the lymphopenia may be to kill off some memory cells to allow more naïve T cells to participate in a new immune response. When a host immune to one pathogen is infected with a second pathogen, any memory T cells cross-reactive with the second virus will dominate the new immune response, by virtue of their higher starting frequency (Klenerman and Zinkernagel 1998; Haanan et al. 1999; Brehm et al. 2002). Reducing the numbers of these memory cells would result in less immunodomination and allow for more naïve T cells to participate in the response (Bahl et al. 2006). Thus, lymphopenia may create conditions allowing for a more diverse immune response to a pathogen, and studies have linked better prognosis with more diverse responses (Meyer-Olson et al. 2004; Borrow et al. 1997).

The third important effect of lymphopenia is the ultimate loss of preexisting memory. Mouse kinetic studies have shown that the memory T cells depleted during the early lymphopenia stage of infection never recover to their original frequencies as the infection progresses (Kim and Welsh 2004). This argues on behalf of the active deletion model of memory cell loss during infections.

6
Heterologous Immunity and Memory TCR Repertoire Shift

Experimental viral immunologists go to great lengths to assure themselves that their animal colonies are free of endogenous pathogens in order to design reproducible experiments. Results from those experiments are then thought to provide the basis for human immune responses to viruses. Indeed sometimes they are, but humans are not immunologically naïve, and they often have memory T cells than can cross-react with and respond to a new infectious agent. Cross-reactivity is a common property of the TCRs. Crystal structural studies reveal the general principles of TCR engagement with peptide-presenting MHC molecules (Ding et al. 1999; Kjer-Nielsen et al. 2003; Rudolph and Wilson 2002; Reiser et al. 2002, 2003), but thermodynamic studies of TCR peptide–MHC interactions have provided new insights into the kinetics of T cell recognition (Boniface et al. 1999; Borg et al. 2005; Willcox et al. 1999; Wu et al. 2002). Using surface plasmon resonance and calorimetry assays to define energy consumption during TCR binding to peptide-MHC, several groups have reported that the TCR undergoes significant conformational changes for proper accommodation to cognate antigen. These conformational modifications involve the TCR CDR3, as shown for human T cells binding flu-$M1_{58–66}$/HLA-A2.1 and EBV-EBNA3 $_{339–347}$/HLA-B8 epitopes (Willcox et al. 1999; Borg et al. 2005) and mouse T cells binding a cytochrome C epitope $MCC_{88–103}$/H2-E^k (Boniface et al. 1999). An "induced fit" model has been proposed, where αβ-TCRs with low conformational complementarity to peptide-MHC initially contact the MHC molecule using the more rigid CDR1 and CDR2 loops and then readjust the flexible CDR3 loops for particular shapes and charges created by the peptide-MHC complex (Wu et al. 2002). This wobbling effect of the CDR3 may enable it to accommodate structurally diverse peptides.

T cell cross-reactivity can be seen between closely related viruses, such as different strains of influenza virus (Haanan et al. 1999; Effros et al. 1977; Boon et al. 2004) or dengue virus (Mongkolsapaya et al. 2003; Zivny et al. 1999), and between different members of the same virus group, such as hantaviruses (Maeda et al. 2004), arenaviruses (Brehm et al. 2002), and flaviviruses (Spaulding et al. 1999). Cross-reactivity between evolutionarily conserved sites within virus groups may not be surprising, but examples of cross-reactivity between completely unrelated viruses such as LCMV and vaccinia virus (VV) (Kim et al. 2005), influenza virus and HCV (Wedemeyer et al. 2001), influenza virus and EBV (Welsh et al. 2004), influenza virus and HIV (Acierno et al. 2003), and human papillomavirus and coronavirus (Nilges et al. 2003), have now been shown. When cross-reactive immune responses are present, they can

Table 1 Potential pathological aspects of heterologous immunity

1. Alterations in immunodominance and amplification of an ineffective response
a. Deviation of a response toward non-protective epitopes
– Weakly expressed epitopes
– Epitopes expressed late in infection
– Epitopes cross-presented on uninfected cells
b. Deviation toward a low-affinity response
Less effective at cytotoxicity and viral clearance
Less likely to have a full complement of cytokine production
2. Cytokine deviation—replacement of a type 1 with a type 2 cytokine response
a. Reduced clearance of virus
b. Altered immunopathology (e.g., eosinophilia)
3. TCR repertoire narrowing
a. Increased probability of T cell-escape variants

alter the pathogenesis of infection and either inhibit or enhance the replication of a newly encountered heterologous virus (Selin et al. 1998; Chen et al. 2001, 2003; Ostler et al. 2003). This alteration in T cell dynamics can have considerable pathogenic consequences (Table 1).

6.1 Heterologous Immunity Between LCMV and VV

The most explored experimental model of heterologous immunity has been between LCMV and VV in the mouse (Selin et al. 1998; Yang et al. 1985; Chen et al. 2001). Immunity to LCMV can provide resistance to an otherwise lethal VV infection, and cause a substantial 10- to 100-fold lowering of viral titers early during infection. However, there often are marked changes in immunopathology. On intraperitoneal challenge, VV-infected mice develop panniculitis, presenting as severe inflammation and fatty necrosis of visceral fat pads. In humans, panniculitis can occur in Weber–Christian disease but more commonly presents as erythema nodosum, a disease of unknown etiology sometimes occurring after viral infections or vaccinations and involving painful lesions on the shins (Di Giusto and Bernhard 1986; Bolognia and Braverman 1992). On intranasal challenge, VV-infected mice may develop a blockage of the airways with cells and fibroid tissue in a pathology known as bronchiolitis obliterans, another human condition of unknown etiology occurring in association with viral infections or during lung transplant rejection (Schlesinger et al. 1998). In these models both the protective immunity, i.e., en-

hanced clearance of virus, and the immunopathology are mediated by T cells producing IFN-γ (Selin et al. 1998; Chen et al. 2001). Altered pathogenesis of VV infection also occurs in mice previously exposed to influenza virus, murine cytomegalovirus, and Pichinde virus (PV) (Selin et al. 1998; Chen et al. 2003).

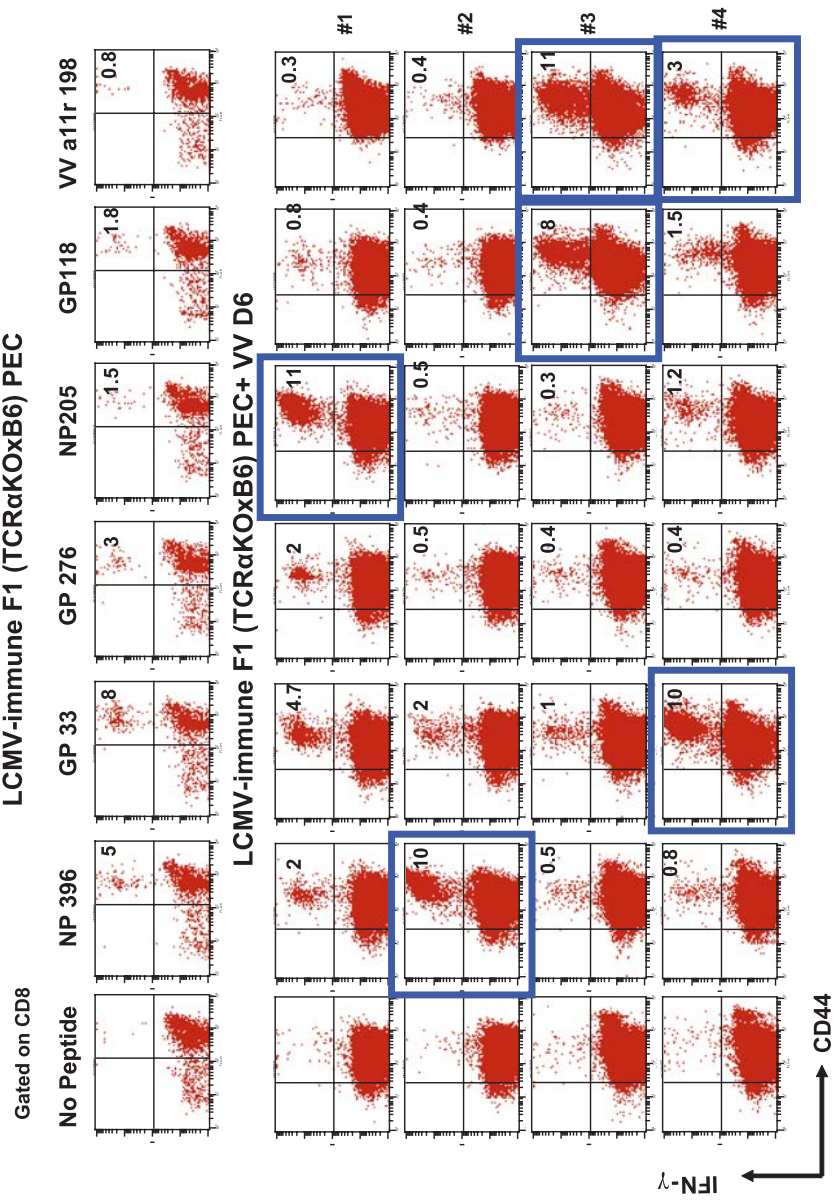
6.1.1 Lack of Reciprocity

The LCMV+VV model shows that heterologous immunity is not necessarily reciprocal. LCMV protects against VV but VV does not protect against LCMV (Selin et al. 1998). Also, VV elicits the proliferation of subpopulations of a CFSE-labeled adoptively transferred LCMV-specific memory cell population, but LCMV stimulates very little proliferation of a VV-immune population (Kim et al. 2002). A possible explanation for this lack of reciprocity is that VV, encoding over 200 proteins and perhaps thousands of potential epitopes, is probably much more likely to encode an epitope that would activate some cells from an LCMV-immune T cell population, whereas LCMV, which encodes only four proteins and a far more limited number of epitopes, may be less likely to encounter a VV-immune T cell to stimulate. This may in fact be why so many large DNA viruses have evolved to encode gene products that interfere with class I antigen presentation (Ploegh 1998). Other factors may also be involved. For instance, VV might be a better inducer of IL-12 than LCMV, and this might augment the ability of any cross-reactive T cells to produce IFN-γ (Chen et al. 2001).

6.1.2 Private Specificity of Heterologous Immunity Between LCMV and VV

Studies on the heterologous immunity between LCMV and VV can be flawed by high variability among the VV-challenged mice in regards to

Fig. 2 Private specificities of the VV-induced T cell response in LCMV-immune F1 (TCRα KO×B6) mice. *Top row*: Hierarchy of CD8 T cell responses to LCMV-encoded and a VV-encoded ($a11r_{198–205}$) epitope in the peritoneal cavity (*PEC*) of LCMV-immune mice, as shown by a peptide-induced intracellular IFN-γ assay. The hierarchy is very similar among individual LCMV-immune mice (Kim et al. 2005). *Bottom four rows*: CD8 T cell responses in four LCMV-immune mice challenged with VV for 6 days intraperitoneally. This shows the differences in the specificities of the LCMV-immune T cells amplified in the individual mice. It also shows that the response to a cross-reactive VV-encoded epitope ($a11r_{198–205}$) is amplified in two of the four mice, indicating that private specificities can dictate immunodominance of T cells specific to the challenge virus. The use of the F1 (TCRα KO×B6) mice ensures that cross-reactivity is not mediated by T cells bearing two α-TCRs


LCMV-immune F1 (TCRαKOxB6) PEC
Gated on CD8
No Peptide
NP 396
GP 33
GP 276
NP205
GP118
VV a11r 198
LCMV-immune F1 (TCRαKOxB6) PEC+ VV D6
#1
#2
#3
#4
IFN-γ
CD44

immunopathology and immune response (Chen et al. 2001). In a study to predict which LCMV-encoded epitopes might be driving cross-reactive responses to VV, substantial variability was noted when individual mice were tested. For instance, in 50% of mice the VV infection stimulated strong expansion of T cells specific to the LCMV $NP_{205–212}$ epitope, in 23% of mice they were specific to either the GP33 or 34 overlapping epitopes, and in 15% of mice specific to the $GP_{118–125}$ epitope. Often there was expansion of T cells specific to only one LCMV epitope, but sometimes T cells specific to more than one epitope were expanded (Kim et al. 2005). In other cases there was no expansion at all.

Figure 2 shows the strong but very different specificities of expansions of LCMV-specific memory T cells in individual mice challenged with VV. This experiment was also performed with F1 progeny of wildtype C57BL/6 mice crossed with α-TCR-deficient mice, to rule out the presence of two α-TCRs as accounting for cross-reactivity.

The question was whether these variations in expansion represent random stochastic events in an LCMV-immune mouse challenged with VV, where only a limited number of the cross-reactive T cells actually engage antigen, or whether each mouse had a unique T cell repertoire in regards to its potential cross-reactivity with VV. To address this point, CFSE-labeled splenocytes from different donor LCMV-immune mice were adoptively transferred into three recipients, which were each then challenged with VV. The pattern of epitope-specific T cell expansion was virtually identical among the recipients of a single donor, but different in recipients from different donors. This showed that these variations in T cell responses were reflections of the private specificities of the individual immune host (Kim et al. 2005).

6.1.3 Matrix of Cross-Reactivity Between LCMV and VV

How then could this cross-reactivity pattern between LCMV and VV be explained? In a quest for cross-reactive epitopes based on searching for VV sequence homology with the LCMV $NP_{205–212}$ epitope, an epitope (VV $a11r_{198–205}$) was found that cross-reacted with three LCMV epitopes: $NP_{205–212}$, $GP_{34–41}$, and $GP_{118–125}$ (which, incidentally, showed no cross-reactivity with each other) (Kim et al. 2005; Cornberg et al. 2006). VV $a11r_{198–205}$ also cross-reacted with a PV epitope (PV $NP_{205–212}$) and an immunodominant VV epitope ($e7r_{130–138}$). Hence, a whole matrix of cross-reactivity was revealed, and this was directed at only one of possibly many cross-reactive VV epitopes. Of significance, however, was when $a11r_{198–205}$-stimulated cell lines were derived from individual LCMV-immune donors, each line had different patterns of

cross-reactivity, with some high for one epitope and others high for a different epitope, again reflecting the private specificity of cross-reactivity (Cornberg et al. 2006).

6.2 Heterologous Immunity Between LCMV and PV

LCMV and PV are arenaviruses which encode nucleoprotein $NP_{205–212}$ epitopes that share 7 of 9 amino acids in an evolutionarily conserved site (Brehm et al. 2002). T cell responses to these epitopes are normally subdominant in either infection, but when mice immune to one virus are infected with the second, the T cell responses to the $NP_{205–212}$ epitope become dominant and T cell responses to the normally dominant epitopes are much subdued (Brehm et al. 2002). Protective heterologous immunity occurs between these viruses, with LCMV protecting against PV more than PV protects against LCMV, perhaps in part due to higher frequencies of $NP_{205–212}$-specific memory cells induced by LCMV (Selin et al. 1998; Brehm et al. 2002). There is a high level of T cell cross-reactivity between the two epitopes in regards to peptide induced IFN-γ production, but double tetramer staining and peptide dilution experiments suggest many affinity differences. Of note is that a heterologous virus challenge selects for a very small subset of the cross-reactive T cells, leading to a substantial narrowing of the TCR repertoire (Fig. 3). This narrowing of the repertoire has different patterns between individuals, and adoptive transfer studies have indicated that this variation is again a reflection of the private specificities of the immune system that developed after the primary infection (Cornberg et al. 2006). It is noteworthy that, with a recent exception in the HIV system (Dong et al. 2004), most studies have linked narrow TCR repertoires to poor clearance of virus and to the enhanced probability of selecting for epitope escape variants (Wilson et al. 1998; Pantaleo et al. 1994; Meyer-Olson et al. 2004; Borrow et al. 1997).

6.3 Immune Deviation

A byproduct of heterologous immunity may be immune deviation caused by shifts in cytokine production. Three days after acute infection of naïve mice with VV, there are high levels of IL-6 and low levels of IFN-γ produced. This contrasts to VV infection of LCMV-immune mice, where there are much higher levels of IFN-γ and lower levels of IL-6 (Chen et al. 2001). In general, studies in murine models with several virus infections have shown alterations in cytokine responses to a virus caused by previous virus infections (Chen et al. 2003).

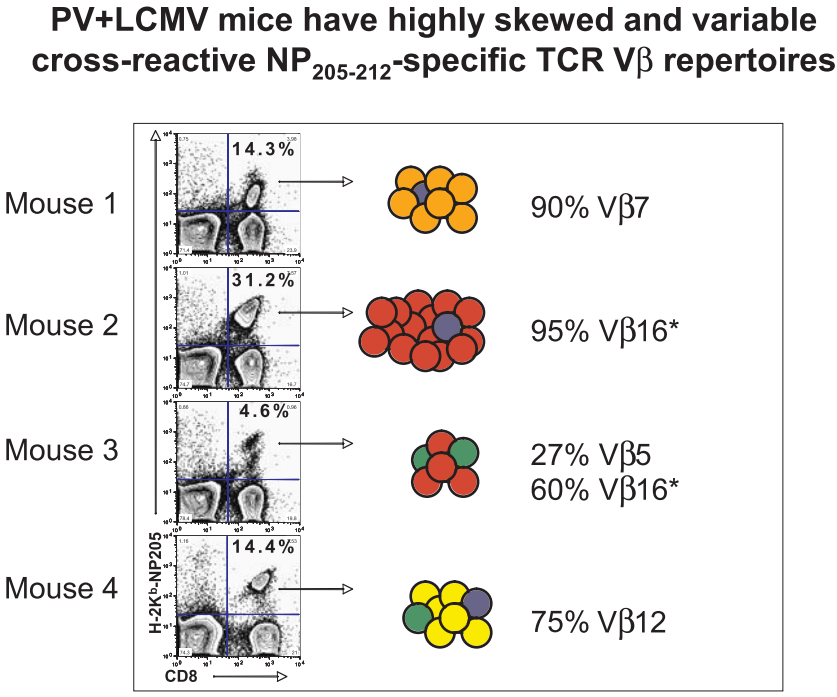


Fig. 3 TCR repertoire narrowing during heterologous immunity between PV and LCMV. T cells specific to the subdominant LCMV epitope $NP_{205–212}$ are mostly in the Vβ16 family and seldom represent more than 5% of the acute CD8 T cell response to LCMV in naïve C57BL/6 mice. This figure shows that acute LCMV infections of PV-immune mice elicit responses that can be immunodominant yet highly variable between mice, reflecting the private specificities, and that they can result in a narrowing of the repertoire by stimulating expansions of T cell Vβ families that would never be prevalent in an acute response in naïve mice. *The percentage of $Vβ^+$ cells was calculated by staining tetramer-defined T cells with Vβ-specific antibodies. An exception was Vβ16, for which no antibodies are available, and which was detected less quantitatively by PCR amplification. Based on a manuscript submitted by Cornberg et al. (2006)

Immune deviation away from type 1 responses is a problem in respiratory syncytial virus (RSV) infection and may have been what occurred when children in the 1960s contracted RSV infections after being immunized by an ineffective vaccine (Kapikian et al. 1969). Mice immunized with a VV recombinant expressing the RSV G protein and later challenged with live RSV developed a type 2 response and severe eosinophilia in the lung. However, if mice had been immune to influenza virus prior to the recombinant VV im-

munization, no such deviation occurred on RSV challenge, and the pathology was much less severe (Walzl et al. 2000). It is also interesting to note that prior immunization with RSV G led to an extreme repertoire narrowing of Vβ14 CD4 T cells specific to the dominant epitope $G_{185–193}$ on live RSV challenge (Varga et al. 2001).

6.4 Heterologous Immunity in Human Infections

Evidence is accumulating for heterologous immunity in humans between commonly occurring viruses.

6.4.1 Influenza Virus Strains and Variants

It has been noted for some time that a prior history of an influenza virus infection can lead to an altered immune response to a different but related strain.

The original observation involved assessment of antibody responses, where antibodies cross-reactive between the strains dominated the immune response and squelched new immune responses to non-cross-reactive antigens. These alterations of B cell repertoires were referred to as "original antigenic sin," (Fazekas de St. Groth and Webster 1966) and a similar phenomenon can happen with T cell responses (Klenerman and Zinkernagel 1998; Mongkolsapaya et al. 2003). Different strains and variants of influenza are commonly cross-reactive at the human and mouse T cell level, leading to speculations that these cross-reactive cells may be involved in the pathogenesis of influenza virus infections (Effros et al. 1977; Haanan et al. 1999; Boon et al. 2004). Perhaps of even greater conceptual interest is the observation of T cell cross-reactivity between influenza and other viruses, as discussed in the following sections.

6.4.2 Hepatitis C Virus

The pathogenesis of HCV in humans is remarkably variable, ranging from asymptomatic to fulminant, with most patients undergoing long-term persistent infections and others clearing the virus (Farci et al. 1996). The reasons for this extreme variability are unknown, and it has been questioned whether heterologous immunity may play a role (Urbani et al. 2005; Rehermann and Shin 2005). HCV encodes an HLA-A2-restricted epitope ($NS3_{1073–1081}$) that shares 7 of 9 amino acids with the influenza epitope ($NA_{231–239}$), and T cells from influenza-immune individuals with no evidence of a past HCV infection

can often respond to the HCV epitope in vitro (Wedemeyer et al. 2001). Hence, the human population may be partially immune to HCV as a consequence of this cross-reactivity. We know that LCMV and PV similarly share 6 of 8 amino acids in their cross-reactive peptides and that sequential infections can lead to immunodomination of the normally protective epitope-specific T cell responses, as well as to a substantial narrowing of the repertoire, and that the degree of this is influenced by the private specificity phenomenon (Brehm et al. 2002; Fig. 3). In 2 of 8 patients examined, a very pronounced cross-reactive T cell response between influenza and HCV was noted; and in these same individuals there was a narrowing of the repertoire, in that T cell responses to other epitopes were minimal (Urbani et al. 2005). These same patients experienced a hepatitis far more severe than patients who mounted a more diverse T cell response against a variety of epitopes. The fact that these patients were all immune to the ubiquitous influenza virus suggests that private specificities may have dictated the altered immune responses.

6.4.3 Epstein–Barr Virus

EBV-associated mononucleosis is one of several viral diseases that are more severe in teenagers and young adults than in children. Others that come to mind are mumps, chicken pox, polio, and measles. What is unique about mononucleosis is that the characteristic diagnostic feature of the disease is an overzealous CD8 T cell response (Silins et al. 2001). Could this relate to the reactivation of memory T cells? It may, as a subset of T cells directed against a major HLA-A2.1 restricted immunodominant EBV peptide, BMLF-$1_{280–288}$, cross-react with the invariant HLA-A2.1-restricted influenza A virus epitope $M1_{58–66}$, even though they share only 3 of 9 amino acids (Welsh et al. 2004). Our recent studies have shown activation of these cross-reactive T cells in some but not all acute mononucleosis patients, perhaps again reflecting private specificities in the host response (Clute et al. 2005). Of note, as mentioned above in Sect. 2.4, is that the TCR repertoire to influenza $M1_{58–66}$ normally has a consistent structure in regards to the dominance of Vβ17 and its hierarchy of utilization of Jβ genes (Fig. 1). Analyses of the $M1_{58–66}$ repertoire from two individuals experiencing EBV-associated acute infectious mononucleosis, however, revealed a substantially different hierarchy of Jβ usage, suggesting that a skewed subset of the $M1_{58–66}$-specific TCR repertoire, probably those cross-reactive with EBV, were being stimulated to proliferate (Fig. 1).

6.4.4 Dengue Virus

Perhaps the most recognized human examples of heterologous immunity come from dengue virus infections. Dengue viruses occur as four distinct but cross-reactive serotypes which fail to elicit neutralizing antibody effective against each other (Morens 1994). A host immune to one serotype when challenged with a second serotype may, instead of having protective immunity, develop a much more severe disease known as dengue hemorrhagic fever and shock syndrome. One theory for its occurrence is that non-neutralizing cross-reactive antibodies can cause "immune enhancement" and increase the uptake of virus into target cells via Fc receptors, resulting in a higher frequency of cells infected (Morens 1994). These could also inhibit the formation of effective neutralizing antibodies to the second virus. A second theory is that original antigenic sin of T cells may be the cause of disease (Mongkolsapaya et al. 2003). A recent study with cases of dengue hemorrhagic fever has shown that some T cell responses were of a higher avidity to a strain of dengue virus other than the one causing the disease. The interpretation is that weakly effective T cells dominated the repertoire to a second dengue virus infection because of their high frequency in the memory pool after an infection with a different serotype.

7 Why Private Specificities Are Important in Pathogenesis

Studies have shown that immunologically naïve mice, while using different TCRs to mount an immune response, generate responses with similar epitope hierarchies, similar effector functions, and similar outcomes. This is because primary responses are likely to select for T cells with suitable CDR1 and CDR2 and which have CDR3 amino acid motifs at positions important for engagement of the peptide MHC complex. The other CDR3 amino acids reflected in the private specificities of the response may be less relevant for those epitopes, which will select the best T cell fits from the highly diverse naïve TCR repertoire. The relevance of the private specificities may increase when a cross-reactive epitope generated by a second virus engages the TCRs of expanded pools of memory cells (Fig. 4). Expanded clones harboring these non-motif amino acids would vary from host to host, a result of the private specificity of the repertoire, but these other amino acids could have great impact on a cross-reactive read-out. Cross-reactive stimulations may result in repertoires not ideal to fight infection, but they are generated nevertheless because of the high frequency of those cells in the memory pool.

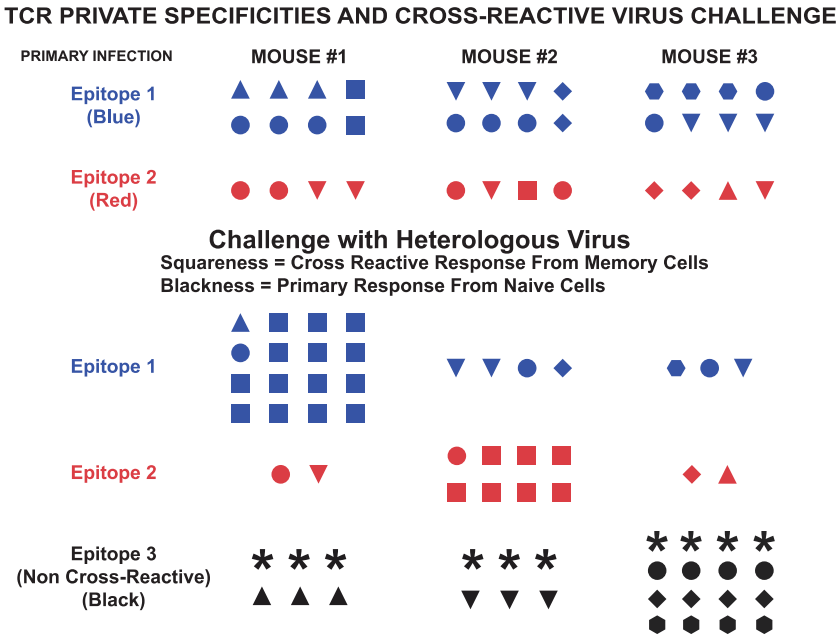


Fig. 4 Model demonstrating how private specificities can alter and narrow the repertoire in a cross-reactive memory response but not in a primary naïve response. Here the color and shape of the epitope determine its specificity. Three naïve mice generate quantitatively similar but qualitatively different repertoires against an immunodominant (*blue*) and subdominant (*red*) epitope. When challenged with a cross-reactive heterologous virus, which responds to either "square" or "black," the private specificities of the memory response dictate immunodominance and epitope specificity and repertoire diversity. T cells specific to non-cross-reactive epitopes are deleted, while cross-reactive T cells can become dominating and oligoclonal

The potential consequences of this stimulation, depicted in Fig. 4, are summarized in Table 1. Exposure of a memory T cell population to a cross-reactive epitope from a heterologous virus may cause a suppression or immunodomination of T cell responses to normally protective epitopes (an effect subdued somewhat by the early lymphopenia). Thus, an immune response to a poorly presented epitope might dominate and be ineffective at eliminating the virus. The T cell response may have a very narrow repertoire, possibly leading to the selection of escape variants. That subset of T cells that is selected may be skewed in terms of its functional capacities and may be ineffective at controlling infection. An immune deviation of type 1 or type 2 cytokines could also ensue. Depending on the private specificities of the host's memory pool, it is

possible that either a strong protective response or a response more likely to cause immunopathology would develop (Table 1).

8
Revisiting the Interactions Between Innate and Adaptive Immunity

Innate immunity is thought to provide the host with time for the differentiation and proliferation of low-frequency antigen-specific clones of naïve T and B cells to reach sufficient numbers to attack and clear the pathogen. Cytokines produced by innate effector mechanisms may retard the replication of the pathogen and influence the deviation of an immune response into a type 1 or type 2 direction. These distinctions between the timing and the roles of innate vs adaptive immunity are not so clearly delineated in the context of heterologous immunity, a phenomenon that we would argue is quite common. A cross-reactive memory cell population may be constitutively effective and not need the time for clonal expansion that a naïve population does. If the cross-reactive memory population is deviated in a type 1 or type 2 direction, it, rather than innate mechanisms, may dictate the deviation of the immune response in the newly developing T and B cells. The laws of innate immune system effects on antigen-presenting cells for the generation of immunodominance hierarchies become perverted in the presence of high frequencies of pre-existing cross-reactive T cell clones. One might also suspect that the rapid production of memory T cell cytokines, such as IFN-γ, might curb the replication of the pathogen, thereby reducing the induction of type 1 IFN and its subsequent effects on lymphopenia, memory cell loss, DC suppression, and NK cell activation. Superimposed on the uncertainty of these events is the variation due to the memory pool's private specificities that are unique to an individual host.

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Enhancement of Infectious Disease Vaccines Through TLR9-Dependent Recognition of CpG DNA

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Abstract The adaptive immune system—with its remarkable ability to generate antigen-specific antibodies and T lymphocytes against pathogens never before "seen" by an organism—is one of the marvels of evolution. However, to generate these responses, the adaptive immune system requires activation by the innate immune system. Toll-like receptors (TLRs) are perhaps the best-understood family of innate immune receptors for detecting infections and stimulating adaptive immune responses. TLR9 appears to have evolved to recognize infections by a subtle structural difference between eukaryotic and prokaryotic/viral DNA; only the former frequently methylates CpG dinucleotides. Used as vaccine adjuvants, synthetic oligodeoxynucleotide (ODN) ligands for TLR9—CpG ODN—greatly enhance the speed and strength of the immune responses to vaccination.

1 Introduction

The innate immune system has developed various strategies to detect pathogens based on the recognition of specific patterns. As noted elsewhere in this volume, the family of Toll-like receptors (TLRs) plays a pivotal role in the detection and initial classification of infectious agents by detecting conserved pathogen-specific molecules. One of the most important questions faced by the innate immune system when it detects the presence of an infection may be to determine whether any pathogen that is present is extracellular or intracellular. In the former case, the "correct" type of immune response is a T-helper type 2 (Th2)-like response, in the latter the immune system should induce a Th1-like immune response capable of killing infected cells. Recent studies indicate that the innate immune system accomplishes this determination at least in part by making use of the ligand specificity and the cellular site of expression of the TLRs. Specifically, TLR2, 4, 5, and 6 are thought to be expressed on the cell surface of certain innate immune cells, where they detect components of extracellular pathogens. TLR3, 7, 8, and 9 are expressed within the endosomal compartments of some innate immune cells, where they appear to be "looking inward" to detect nucleic acid components of intracellular pathogens [1].

TLR9 is used by the innate immune system for detecting unmethylated CpG dinucleotides, which are relatively common in bacterial and viral genomes but are highly methylated and uncommon in vertebrate genomes [2]. Although TLR9 appears in general to be highly conserved through evolution, the gene seems to be missing in chickens [3]. Nevertheless, chicken immune cells are activated specifically by CpG oligodeoxynucleotides (ODN) [4], so further studies will need to be performed to determine whether in some species there could be TLR9-independent pathways for the detection of CpG motifs. In mice, the great majority of evidence supports the conclusion that TLR9 is required for all known CpG-specific responses to synthetic phosphorothioate ODN [2, 5, 6]. However, native phosphodiester DNA can activate immune cells not only through TLR9, but also through TLR9-independent pathways, if the DNA is delivered into the cells via transfection [7]. It also is noteworthy that DNA vaccines can induce strong early antigen-specific immune responses in the absence of TLR9, and even in the absence of MyD88, indicating that DNA can exert immune effects through other pathways [8–10], as also has been described for plasmid DNA transfected into cells [11].

In certain experimental systems such as B cell activation, non-CpG ODN also can be shown to have immune stimulatory effects, especially when the ODN are synthesized with a nuclease-resistant phosphorothioate backbone and used at relatively high concentrations of 1 μM or above [12]. However,

even these "non-specific" effects of "control" phosphorothioate ODN recently have been shown to be almost completely TLR9-dependent [6].

Each of the TLRs has a unique pattern of cellular expression, and these patterns vary between different species. Among resting human immune cells, TLR9 is expressed exclusively in B cells and in plasmacytoid dendritic cells (pDC), which produce most of the type I interferon (IFN) that is made in response to viral infection (reviewed in [13]). Some studies have also reported functional TLR9 expression in activated but not in resting human neutrophils [14] and pulmonary epithelial cells [15, 16], but the in vivo level and functional significance of this TLR9 expression are uncertain. In any case, the pattern of TLR9 expression is clearly broader in mice than in humans, since mice also express TLR9 in monocytes and myeloid DC (reviewed in [13]). This makes it difficult at best to predict the effects of TLR9 activation in humans from extrapolating results in mice.

In addition to species-specific variations in the cell types expressing the TLRs, there are species-specific differences in the optimal CpG motif for activating TLR9. Empiric studies defined the CpG motif as a hexamer comprising the two bases flanking the CpG dinucleotide to the 5′ and 3′ sides [17]. The optimal immune stimulatory ODN hexamer CpG motif is GACGTT for mice [4, 17, 18], but it is GTCGTT for humans [19] and apparently for many other vertebrate species including cow, sheep, cat, dog, goat, horse, pig, and chicken [4, 20]. Besides the hexamer CpG motif, the immune stimulatory activity of an ODN is determined by:

1. The number of CpG motifs in an ODN. Two to four are usually optimal.
2. The spacing of the CpG motifs. Usually at least two intervening bases are optimal, preferably Ts.
3. The presence of poly G sequences or other flanking sequences in the ODN. The effect depends on ODN structure and backbone.
4. The ODN backbone. Nuclease-resistant phosphorothioate backbone is the most stable and best for activating B cells, but gives relatively weaker induction of IFN-α secretion from pDC compared to native phosphodiester linkages in the CpG dinucleotide [17–19, 21–24].

In addition, the immune stimulatory effects of the ODN are enhanced if there is a TpC dinucleotide on the 5′-end and if the ODN is pyrimidine-rich on the 3′-side [6, 18, 19, 22].

There are at least three main families of immune stimulatory CpG ODN with distinct structural and biological characteristics. The A-class CpG ODN are potent activators of natural killer (NK) cells and IFN-α secretion from

pDC, but only weakly stimulate B cells [25] (reviewed in [12]). A-class ODN have polyG motifs at the 5′- and/or 3′-ends that are capable of forming complex higher-ordered structures known as G-tetrads, which are required for the strong IFN-α induction characteristic of this ODN class. A-class ODN are inactive if synthesized with a completely modified phosphorothioate backbone: They must have at least a central phosphodiester region containing one or more CpG motifs, and these motifs are preferably in a self-complementary palindrome for optimal induction of IFN-α secretion (reviewed in [12]). B-class ODN have a completely phosphorothioate backbone, typically form no higher-ordered structures, and are strong B cell stimulators, but are weaker for induction of NK activity or IFN-α secretion [12]. The C-class CpG ODN have immune properties intermediate between the A and B classes, inducing both B cell activation and IFN-α secretion [26–28]. These properties appear to result from the unique structure of these ODN, with one or more 5′ CpG motifs, and a 3′ palindrome, which allows formation of duplex or hairpin structures. All three ODN classes require the presence of TLR9, as they have no immune stimulatory effects in mice deficient in TLR9 [26].

An important question is how the different classes of CpG ODN can induce such divergent immune effects, since they all appear to act through TLR9. The fact that maximal induction of pDC IFN-α secretion requires the ODN to adopt a secondary structure, apparently dimeric in the case of the C-class and multimeric in the case of the A-class, suggests the hypothesis that these higher ordered structures might either induce TLR9 crosslinking, or perhaps promote the recruitment of one or more additional cofactors or adaptor proteins into the TLR9 signaling complex. The possibility that additional TLR9 cofactors may take part in the recognition of one or more CpG ODN classes has not been excluded. Recent studies have also pointed to the possibility that the presence of the different ODN classes in distinct intracellular compartments may contribute to their differential immune effects [29]. Transfection of a B-class ODN can induce levels of IFN-α secretion similar to those induced by an A-class ODN, providing new insights into the biology of these pathways, but also raising new questions about the immune effects of the transfection step [29].

Most published studies using CpG ODN as vaccine adjuvants have been carried out with B-class CpG ODN, so although the following sections refer to “CpG ODN” generically, this is actually referring to the B-class unless otherwise stated.

2 Mechanism of Action of CpG ODN

Even before the discovery that TLR9 was an intracellular protein, it was apparent that immune stimulation by CpG ODN requires internalization by the target immune cells, which occurs spontaneously in culture without need for uptake enhancers or transfection [17]. The cellular uptake of ODN is temperature- and energy-dependent, and appears to be relatively sequence-independent (reviewed in [30]). In resting cells, CpG ODN are taken up into an acidified endosomal compartment. TLR9 initially is present within the endoplasmic reticulum, but upon CpG ODN uptake the TLR9 translocates into the same endosomal compartment as the CpG ODN, thus allowing binding and initiation of signal transduction [31, 32]. Inhibitors of endosomal acidification/maturation completely block the immune effects of CpG ODN, demonstrating an essential role for this compartment in the TLR9-induced signal transduction pathways [33–36]. Immune stimulation by CpG ODN also is blocked by inhibitors of phosphatidylinositol 3 kinase (PI3-kinase), which also appears to have a role in the internalization of the DNA [37].

In the endosome, TLR9 binding and recognition of a CpG motif leads to the rapid recruitment of the adaptor molecules MyD88, IL-1 receptor-associated kinase (IRAK)-1, and TNF-α receptor activated factor 6 (TRAF6) [32, 34, 38–41]. These events lead to activation of several mitogen-activated protein kinases including extracellular receptor kinase (ERK), p38, and Jun N-terminal kinase as well as the IκB complex, the pathways of which converge on the nucleus to alter gene transcription [19, 42–47]. The balance between these pathways controls the activity of the DC, with activation of the ERK pathway generally favoring higher levels of interleukin (IL)-10 secretion that limits the IL-12 secretion and Th1 response [1, 47]. IFN-α secretion by pDC is interferon-regulatory factor 7 (IRF7)-dependent and requires direct interactions between IRF7 and MyD88, TRAF6, and IRAK-1 [48–51].

The immune effects of activating TLR9-expressing cells in humans (B cells and pDC) with CpG ODN are Th1-like and may be considered in two stages; an early innate immune activation and a later enhancement of adaptive immune responses. Through the signal transduction pathways described above, TLR9-stimulated B cells and pDC show increased expression of costimulatory molecules, resistance to apoptosis, upregulation of the chemokine receptor CCR7 that causes cell trafficking to the T cell zone of the lymph nodes, and secretion of Th1-promoting chemokines and cytokines such as MIP-1, IP-10, and other IFN-inducible genes [12]. PDC activated by A-class ODN secrete especially high levels of type I IFN [25], but B-class ODN also induce substantial IFN-α secretion. In vivo in mice, TLR9 activation induces the migration

and clustering of pDC in the marginal zone and outer T cell areas, which is dependent on IFN-α [52]. Although in general the CpG ODN effect is Th1-like, it is important to note that the CpG-activated B cells produce IL-10, which limits the DC Th1 priming [53–55].

The secondary innate immune effects of TLR9 activation can have therapeutic anti-infective and anti-tumor effects through two general routes, including first, the effects of the inducible soluble factors, and second, the activities of the innate immune cell populations. Several of the CpG-induced Th1-like cytokines and chemokines are known to have therapeutic activity. For example, type I IFN are already used alone for the treatment of chronic hepatitis C virus (HCV) infection and some cancers, and IP-10 also has some anti-infective activity [56–59] and appears to be important in the migration of T cells into tumors, and for the therapeutic effect of IL-12 in mouse models [60–62]. The CpG-induced secondary activation of innate immune cells has therapeutic effects through, for example, NK cell-mediated tumor killing [21] and activation and enhanced polymorphonuclear leukocyte (PMN) migration and bacterial uptake, providing improved resistance to infectious challenge [63–65]. In addition, the pDC-derived IFN-α induces human monocytes to express increased levels of TNF-related apoptosis-inducing ligand (TRAIL) [66].

TLR9-mediated innate immune activation and pDC maturation is followed by the generation of adaptive immune responses. B cells are strongly costimulated if they bind specific antigen at the same time as TLR9 stimulation [17], even if the antigen is presented in the form of an immune complex [67]. This selectively enhances the development of antigen-specific antibodies. CpG stimulation endows both B cells and pDC with strong antigen-presenting capabilities to T cells. When pDC were first studied, they were thought to be responsible primarily for inducing Th2-type immune responses, and to be relatively ineffective at stimulating primary or recall T cell responses [68, 69]. In fact, several studies have suggested that pDC can even promote tolerance, and have shown that CpG stimulation of highly purified pDC induces regulatory T cells (Treg) that suppress naïve T cell responses [70]. However, the pDC function is dramatically altered when they are activated by CpG within a mixed population of cells [71]. In cultured human peripheral blood mononuclear cells (PBMC), both A-class and B-class CpG ODN increased the frequency of $CD8^+$ T cells with a memory phenotype, while only the B-class increased the frequency of $CD8^+$ T cells with a naïve phenotype [72]. In mice, CpG-induced antigen presentation has been shown to occur in a Th1-like cytokine milieu, stimulating the development of Th1 cells, and resulting in the generation of T helper cell-independent primary effector CTLs [73].

3 Enhancing Vaccination by TLR9 Stimulation with CpG ODN

In addition to the direct effects that CpG ODN has on the innate immune system, in the presence of an antigen CpG ODN can promote the induction of strong Th1-biased antigen-specific immune responses. This has led to their use as adjuvants in both prophylactic and therapeutic vaccines in numerous animal models as well as in humans. TLR9 activation for adjuvanting vaccines is easily accomplished through the use of synthetic ODN of about 8 to 30 bases in length that contain a nuclease-resistant phosphorothioate backbone and the appropriate CpG motifs for the species to be treated. The strong Th1 adjuvant activity associated with CpG ODN is most likely due to a combination of factors including:

1. A synergy in the presence of antigen between CpG-mediated TLR9 and B cell receptor stimulation resulting in enhanced B cell stimulation [17]
2. Inhibition of B cell apoptosis resulting in a prolongation of immune responses [18]
3. Enhanced IgG class switch DNA recombination [74, 75]
4. The secretion of Th1-promoting cytokines and chemokines (e.g., IL-12, IFN-α/β, TNF-α, IP-10) from directly and indirectly CpG-activated DC resulting in the generation of a Th1-biased milieu [73, 76]

The utility of CpG ODN as a vaccine adjuvant for inducing antigen-specific humoral and cellular responses has been confirmed in studies using a wide variety of antigens, including peptide or protein antigens, live or killed viruses, DC vaccines, autologous cellular vaccines, and polysaccharide conjugates. In general, CpG ODN are not effective adjuvants for unformulated polysaccharide antigens, but they are quite effective if a protein carrier is conjugated to the polysaccharide [77–79]. Conjugation of CpG ODN directly to the antigen has been used to enhance antigen uptake and reduce antigen requirements [80, 81].

Comparisons of different adjuvants in mouse models have demonstrated CpG ODN to be the strongest of all at inducing Th1-type responses [82–86]. Moreover, CpG ODN accomplish this level of antigen-specific activation without inducing the harsh local inflammatory effects seen with complete Freund's adjuvant (CFA). Nevertheless, CpG ODN show even greater adjuvant activity when formulated or co-administered with other adjuvants [87]. Such formulations are especially important when the antigen is relatively weak. Combinations of CpG ODN with QS21, Titermax, and monophosphoryl lipid A (MPL) also have shown synergistically increased activity in mice [88].

The Th1-type microenvironment generated by CpG ODN stimulates the development and persistence of cytotoxic T lymphocytes (CTL), the activation of antigen-presenting cells (APC), and the production of opsonizing antibodies such as those of the IgG2a isotype in mice. In contrast, murine Th2-type responses associated with the secretion of Th2-type cytokines (IL-4, IL-5) generate IgG1 and IgE antibodies with only weak or no CTLs. The strong cell-mediated immunity associated with Th-1 responses is generally thought to be essential for the control of intracellular pathogens, whereas strong humoral immunity, which can be associated with Th1 or Th2 responses, appears necessary for the control of extracellular pathogens. Th1-type responses have been shown necessary to clear intracellular infection in murine *Leishmania major* and *Schistosoma mansoni* infection models where resistance or susceptibility to infection is linked to Th1 or Th2 responses, respectively [89, 90], and to the control of certain viral infections, such as the hepatitis B virus (HBV) [91]. In addition to infectious disease therapies, the generation of Th1-type responses may be efficacious in therapies against cancer or in the control of asthma/allergy. The potential use of CpG ODN as an adjuvant in the development of novel prophylactic or therapeutic vaccines against infectious disease is discussed below.

4
CpG ODN as Adjuvants in Infectious Disease Vaccines

In recent years, worldwide mass immunization programs have been highly effective in reducing the mortality and morbidity associated with viral infections, such as smallpox, measles, and polio. Despite the success of immunization programs, infectious disease still accounts for more than 13 million deaths annually, and an estimated 30 million children do not have access to immunization [92]. Often this is a direct result of the cost associated with vaccination, the impracticalities of repeated boosting in developing countries, or both factors together. Immunization could not only reduce the mortality associated with infectious disease but also greatly reduce the costs, particularly in developing countries, associated with disability, illness, and loss of work or schooling resulting from disease. In order to further develop the potential of vaccines to combat infectious disease, new or improved vaccines are needed such that the antigen dose or number of required doses is reduced. One possible way to achieve this is through the use of vaccine adjuvants. At present, the only adjuvant licensed for use in humans in most countries is aluminum hydroxide (alum). However, alum generates a Th2-biased immune response to co-administered antigens with diminished Th1-type responses (e.g., re-

duced CTL, T cell proliferation), and is limited only to vaccines delivered by parenteral routes. In order to improve and expand the use of existing vaccines or for the development of new vaccines, safe and effective vaccine adjuvants are needed.

5 CpG ODN Promote Strong Th1-Type Immune Responses

CpG ODN have been shown to be an effective and well-tolerated vaccine adjuvant when co-administered with numerous viral and bacterial antigens in a range of species, including mice [74, 77, 93–95], rats [96, 97], rabbits [98], guinea pigs [99], chickens [100], pigs [101, 102], cattle [103, 104], sheep [105], dogs [106], non-human primates [94, 107, 108], and humans [109–113]. When compared to other adjuvants, CpG ODN appears to induce one of the strongest Th1 responses. For example, in a murine study comparing 19 different immunological adjuvants with keyhole limpet hemocyanin (KLH) conjugate vaccines containing the two human cancer antigens (MUC1 peptide and GD3 ganglioside), CpG ODN induced the most Th1-biased immune responses of any adjuvant as demonstrated by highest levels of IFN-secretion in antigen re-stimulated splenocytes [82]. Likewise when compared to other commonly used adjuvants [CFA, incomplete Freund's adjuvant (IFA), Titermax Gold (CytRx, Norcross), alum, or MPL] for their ability to augment humoral responses to hepatitis B surface antigen (HBsAg) in mice, CpG DNA gave the highest ratio of IgG2a to IgG1 antibodies—an indirect marker for Th1-biased responses—of any adjuvant used alone [114].

6 CpG ODN Can Overcome Th2-Biased Immune Responses

Immune responses generated by CpG ODN adjuvants are of a Th1 type regardless of whether CpG ODN are used alone or in combination with other adjuvants. Indeed, the strong Th1-bias of CpG ODN can dominate the Th2 bias associated with adjuvants such as alum, or IFA [74, 114]. CpG ODN have also been shown to reduce Th2 bias that can be associated not only with the alum component of certain vaccines but also with the antigen itself. For example, the diphtheria–tetanus–pertussis vaccine contains not only alum as adjuvant but also pertussis toxin, both of which strongly promote Th2 responses. Addition of CpG ODN to this vaccine resulted in a shift in immune responses toward a Th1-type [115]. Likewise, at least in mice, CpG ODN can overcome the Th2

bias associated with commercial killed bovine respiratory syncytial virus (K-BRSV) and formalin-inactivated BRSV (FI-BRSV) vaccines [116, 117].

In both the very young and the elderly there tends to be a bias toward Th2-type immune responses. This can arise since in the very young the immune system has not fully developed whereas in the elderly there can be age-related decreases in both innate and adaptive immune efficiency. Studies in mice suggest that it may be possible to overcome this by the use of adjuvants that promote strong Th1-type responses, such as CpG ODN. A number of studies have now demonstrated that CpG ODN can induce strong Th1-type responses in young mice [118, 119]. In addition, CpG ODN has been shown able to re-direct a pre-established Th2-type immune response toward a Th1-type response in both adults and young mice [120–122]. The ability of CpG ODN to stimulate Th1-biased immune responses has been demonstrated in aged mice following immunization by parenteral [123, 124] and mucosal routes [125]. In these studies, the use of CpG ODN as adjuvant resulted in similar Th1-type responses in aged vs young mice.

7
CpG ODN Can Overcome Vaccine Hyporesponsiveness

The potential of CpG ODN to overcome hyporesponsiveness was first demonstrated in orangutans that were hyporesponsive to the commercial HBV vaccine, Engerix-B (GlaxoSmithKline Biologicals, Rixensart), containing alum-absorbed yeast-derived recombinant HBsAg. In this study, co-administration of CpG ODN with Engerix-B greatly enhanced not only the levels of HBsAg-specific antibodies but also the rate at which they appeared [107]. More recently the ability of CpG ODN to overcome hyporesponsiveness has also been demonstrated in a human clinical trial, whereby human immunodeficiency virus (HIV)-positive individuals who were previously non-responsive to the Engerix-B vaccine rapidly developed sero-protective antibody titers when the same vaccine was administered in combination with CpG ODN (CPG 7909) [109]. Moreover, those individuals receiving CpG ODN as adjuvant had their antibody levels maintained at protective levels for up to 3 years after immunization and showed significantly enhanced antigen specific lymphocyte proliferation for at least 2 years compared to those receiving vaccine alone [109].

8
CpG ODN Can Reduce Antigen Dose

The use of CpG ODN as vaccine adjuvant may allow for the use of lower antigen doses. In murine studies, when HBsAg alone or in combination with alum is given to mice by IM injection, each 10-fold decrease in amount of antigen resulted in a similarly reduced levels of plasma HBsAg-specific antibodies [126]. In contrast, when CpG ODN was used as adjuvant alone or in combination with alum, high levels of plasma HBsAg-specific antibodies were detected in plasma even with a 1,000-fold reduction in amount of antigen [126]. Moreover, in a recent phase Ib blinded, randomized, controlled clinical trial, co-administration of CpG ODN as adjuvant (CPG 7909, 1 mg) with a one-tenth dose of a commercial trivalent killed split influenza vaccine (Fluarix) resulted in similar levels of antigen-specific IFN-ɣ secretion from re-stimulated peripheral blood mononuclear cells as were obtained with the full-dose vaccine administered without CpG ODN [111].

9
CpG Synergizes with Other Adjuvants

A synergistic effect has been reported between CpG ODN and a broad range of other injectable adjuvants including emulsions (e.g., Emulsigen, Montanide ISA51 and ISA720, and MF-59), particulate adjuvants (e.g., cationic and anionic microparticles, nanoparticles, virus-like particles), mineral salts (e.g., aluminum hydroxide), saponins (e.g., QS21, Quill A), liposomes and cationic peptides, polycationic antibiotics (e.g., Polymyxin B), and polysaccharides, as well as a number of mucosal adjuvants such as native and genetically detoxified bacterial toxins (e.g., cholera toxin and the *Escherichia coli* heat-labile enterotoxin), liposomes, and microparticles. The synergy between these adjuvants and CpG ODN most likely arises due to various factors depending on the adjuvant used and may include: (1) protection of CpG ODN and/or antigen from enzymatic degradation, thereby enhancing bioavailability of intact ODN (e.g., with liposomes, microparticles); (2) providing a depot effect such that both antigen and CpG ODN are available for an extended period of time (e.g., with emulsions, mineral salts); and (3) enhancing uptake of antigen and/or ODN into antigen-presenting cells (e.g., particulate or lipid-based adjuvants).

In humans, CpG ODN has been used as adjuvant for infectious disease vaccination either in combination with alum [110] or alone [112]. In addition, CpG ODN has been used with Montanide in a cancer vaccine setting [113].

In a randomized, double-blind phase I/II dose escalation study, healthy individuals received three intramuscular (IM) injections (0, 4 and 24 weeks) of an alum-absorbed HBV vaccine (Engerix-B) either in saline or mixed with VaxImmune (a B-class CpG ODN, CPG 7909; Coley Pharmaceutical Group, Wellesley) at doses of 0.125, 0.5, or 1.0 mg [110]. HBsAg-specific antibody responses (anti-HBs) were shown to appear earlier and with a higher magnitude at all time points up to and including 24 weeks in VaxImmune recipients compared to those individuals who received Engerix-B alone. Moreover, a high proportion of individuals who received VaxImmune as adjuvant developed protective levels of anti-HBs IgG within just 2 weeks of the priming vaccine dose and there was a trend toward higher rates of positive CTL responses in the two higher dose groups of VaxImmune compared to controls. In this study, VaxImmune was also shown to enhance the late affinity maturation process, thus increasing the pool of high-avidity antibodies [127]. This CpG-mediated effect was antigen-specific, isotype-specific, and distinct from the influence on anti-HBs production, as avidity did not correlate with anti-HBs IgG titers [127].

In a separate phase I, observer-blinded, randomized study, healthy individuals received two doses 8 weeks apart of a non-alum absorbed commercial vaccine either alone or mixed with another B-class CpG ODN, called 1018 ISS [112]. In this study, a dose-dependent enhanced immunogenicity associated with CpG ODN was also seen albeit at higher doses (0.3, 0.65, 1.0, and 3.0 mg) [112] than the 1 mg or less that was used in the VaxImmune study. However, the two studies were performed at different times in different populations, with different CpG ODN, and in the presence or absence of alum, so comparison of the responses across studies is difficult.

We have previously demonstrated in mice that when used alone, CpG ODN induces less tissue damage than other adjuvants (e.g., Titermax, CFA, IFA, MPL) and that when used in combination with other adjuvants the amount of tissue damage was generally not greater than either adjuvant alone [114]. In sheep, when CpG ODN was combined with a number of different mineral oil, metabolizable oil, and non-oil adjuvants and tested as an adjuvant to a truncated secreted form of bovine herpesvirus (BHV) glycoprotein D (tgD), the inclusion of CpG ODN in these formulations not only increased immune responses overall, but shifted them to a more Th1-type as evidenced by increased serum IgG2a and production of IFN-ɣ by splenocytes. In addition, in this study the inclusion of CpG ODN allowed lower doses of mineral oil-based adjuvants to be used without decreasing the magnitude of immune responses in mice or sheep, but it also significantly reduced tissue damage at the injection site [105].

10
CpG ODN Is an Effective Adjuvant with Different Routes of Administration

The majority of studies published to date using CpG ODN as vaccine adjuvant have evaluated parenteral delivery (e.g., intramuscular, subcutaneous); however, the majority of infectious agents enter the body via the mucosal surfaces of the gastrointestinal, genitourinary, and respiratory tracts, where the primary source of protection is the mucosal immune system. While parenteral administration can be highly effective at inducing systemic immune responses, administration of vaccines at a mucosal surface is generally required for the induction of mucosal immune responses, and can lead to the generation of efficient mucosal and systemic responses [128]. In addition, a number of benefits may also be associated with mucosal vaccines including ease of administration, particularly by the oral route, and a lack of needles, which may reduce needle-stick injury or cross-contamination and reduce the need for highly trained personnel to administer vaccines.

A number of studies have now demonstrated CpG ODN to be an effective mucosal adjuvant with a variety of different antigens, when administered to different mucosal surfaces, such as the respiratory tract (intranasal droplets) [129–132], the genitourinary tract (intravaginal) [133], the gastrointestinal tract (oral, intrarectal) [96, 132, 134, 135], and the ocular mucosa [98]. In most studies using CpG ODN as mucosal adjuvant, mucosal immune responses, characterized by antigen-specific secretory IgA antibodies, have been induced at a number of sites including those distant to the site of administration. For example, following oral or intranasal administration of tetanus toxoid (TT) with CpG ODN, we have been able to detect TT-specific IgA antibodies in lungs, feces, saliva, nasal, vaginal, and gut washes [132]. In a murine challenge model, intranasal immunization of mice using CpG ODN as an adjuvant to inactivated gp120-depleted HIV-1 elicited strong HIV-specific IgG and IgA antibody responses in serum and the genital tract. It also increased production of β-chemokines and IFN-γ in the genital tract, and enhanced protection against intravaginal challenge with a recombinant vaccinia virus expressing HIV-1 gag compared to mice immunized with antigen alone or with control ODN [136]. Likewise, intranasal immunization using CpG ODN as mucosal adjuvant in association with HSV-1 glycoprotein B has been shown to induce strong mucosal and systemic immune responses as well as protection against vaginal challenge with a lethal dose of HSV-2 [130]. While it is generally considered that parenteral administration, in the absence of hormonal supplementation, does not induce strong mucosal responses, it has been reported that transcutaneous immunization can induce both mucosal

and systemic immune responses. For example, transcutaneous immunization of mice in the lower back region using CpG ODN as vaccine adjuvant to the major outer membrane protein (MOMP) of *Chlamydia muridarum* resulted in the production of MOMP-specific IgA and IgG in uterine and vaginal lavage fluid and MOMP-specific IgG in serum, as well as IFN-ɣ secreting T cells in reproductive tract draining caudal and lumber lymph nodes, and enhanced clearance of *C. muridarum* following intravaginal challenge [137].

In the majority of studies published to date using CpG ODN as mucosal adjuvant, CpG ODN has been admixed with single antigens in a simple saline solution. However, we have also demonstrated that CpG ODN is an effective mucosal adjuvant against multiple antigens when administered with a multivalent vaccine, composed of a killed influenza vaccine together with TT and HBsAg [132]. As has been reported with parenteral delivery, a synergy between CpG ODN and a number of other adjuvants has been reported after mucosal delivery including liposomes [138], cholera toxin (CT) [131, 139, 140], the *E. coli* heat-labile enterotoxin (LT) [139, 141], and their genetically detoxified derivatives [142].

11 Conclusions

In summary, TLR9 agonists such as CPG 7909 and other CpG ODN provided a targeted stimulation of pDC and B cells that promoted strong Th1-like innate and adaptive immune responses. CpG ODN have emerged as remarkably strong vaccine adjuvants that offer numerous advantages over alternative adjuvants. The addition of CpG ODN to most vaccines appears likely to induce significantly faster seroconversion with fewer vaccinations, and may allow reduced antigen doses. Results from several human clinical trials reported to date indicate that response rates to vaccination should be dramatically improved by the addition of a CpG ODN, even among immune-compromised populations. In animal models, CpG ODN also have proved to be strong mucosal vaccine adjuvants, although this has yet to be demonstrated in humans. The strong Th1 bias introduced by TLR9 stimulation offers the possibility of enabling therapeutic vaccines, as suggested by early results in mice. The vaccine-enhancing activity of TLR9 agonists demonstrates the central role of TLR9-expressing pDC and B cells in the regulation of protective immunity. Although far more experience in humans will be required before the safety of this approach can be fully determined, results to date suggest that the addition of a CpG ODN to a vaccine will not markedly aggravate the injection site reactions or other aspects of vaccine safety.

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