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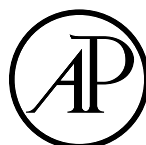

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CHAPTER 1

Dynamic Palmitoylation and the Role of DHHC Proteins in T Cell Activation and Anergy

Nadejda Ladygina,* **Brent R. Martin,**† and **Amnon Altman***

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Abstract Although protein S-palmitoylation was first characterized >30 years ago, and is implicated in the function, trafficking, and localization of many proteins, little is known about the regulation and physiological implications of this posttranslational modification. Palmitoylation of various signaling proteins required for TCR-induced T cell activation is also necessary for their proper function. Linker for activation of T cells (LAT) is an essential scaffolding protein involved in T cell development and activation, and we found that its palmitoylation is selectively impaired in anergic T cells. The recent discovery of the DHHC family of palmitoyl acyl transferases and the establishment of sensitive and quantitative proteomics-based methods for global analysis of the palmitoyl proteome led to significant progress in studying the biology and underlying mechanisms of cellular protein palmitoylation. We are using these approaches to explore the palmitoyl proteome in T lymphocytes and, specifically, the mechanistic basis for the impaired palmitoylation of LAT in anergic T cells. This chapter reviews the history of protein palmitoylation and its role in T cell activation, the DHHC family and new methodologies for global analysis of the palmitoyl proteome, and summarizes our recent work in this area. The new methodologies will accelerate the pace of research and provide a greatly improved mechanistic and molecular understanding of the complex process of protein palmitoylation and its regulation, and the substrate specificity of the novel DHHC family. Reversible protein palmitoylation will likely prove to be an important posttranslational mechanism that regulates cellular responses, similar to protein phosphorylation and ubiquitination.

1. INTRODUCTION

Protein palmitoylation is a reversible and dynamic posttranslational modification characterized by the covalent attachment of a fatty acid, palmitic acid, to proteins, most often to cysteine (Cys) residues (i.e., S-acylation), via a thioester linkage. Like other posttranslational modifications such as phosphorylation and ubiquitination, palmitoylation can regulate the stability, localization, and function of many receptors and intracellular proteins and hence, play an important role in determining the functional outcome of cellular triggering by multiple receptors that are engaged by their respective ligands (Greaves and Chamberlain, 2007; Iwanaga *et al.*, 2009; Linder and Deschenes, 2007; Mitchell *et al.*, 2006; Planey and Zacharias, 2009; Resh, 2006a). Protein palmitoylation also occurs in cells of the immune system, including in T lymphocytes where it has been extensively studied over the years. Palmitoylation of various T cell

proteins, including receptors and intracellular proteins that participate in the complex process of signal transduction initiated by engagement of the antigen-specific T cell receptor (TCR), was found to be important for their proper localization and function (Bijlmakers, 2009; Resh, 2006a). The importance of this posttranslational modification in TCR signaling is also evident from the fact that pharmacological inhibition of protein palmitoylation, or mutation of palmitoylated Cys residues in proteins, can modulate (in most instances inhibit) the activation of T cells. Therefore, identification of palmitoylation substrates in T cells, and elucidation of how this process is regulated are important because they can potentially provide novel drug targets for intervention in immune system diseases and abnormalities, for example, autoimmune diseases.

Despite the fact that protein palmitoylation has been discovered >30 years ago and its importance is well established, progress in this field has been hampered for two main reasons: First, until recently, measuring protein palmitoylation relied on insensitive and cumbersome radioactive assays tied to immunoprecipitation of specific targets, bypassing analysis of global changes in the palmitoyl proteome. Second, the enzymatic regulation of protein palmitoylation and more precisely the identity of the enzymes that palmitoylate or depalmitoylate proteins has remained elusive until recently. In fact, many proteins can acquire *S*-acyl linkage nonenzymatically by thioester exchange with acyl-CoA *in vitro*, so for a long time it was controversial whether palmitoylation was an enzymatic process in cells.

Two recent breakthroughs have recently reinvigorated the study of protein palmitoylation and greatly accelerated research in this area. First, beginning in 2002, several groups identified and molecularly cloned, first in yeast (Lobo *et al.*, 2002; Roth *et al.*, 2002) and later in mammals (Fukata *et al.*, 2004), members of a novel family of palmitoyl acyl transferases (PATs) that specifically catalyze protein palmitoylation on Cys residues. Second, quantitative, highly sensitive, and nonradioactive methods for global identification and profiling of the palmitoyl proteome have recently been developed (Kang *et al.*, 2008; Martin and Cravatt, 2009; Roth *et al.*, 2006). As a result of these two major breakthroughs, it has become possible to analyze the process of protein palmitoylation and its enzymatic regulation at a level of sophistication not heretofore possible, resulting in key recent advances. Still, much of the research in this area is conducted in neuronal and, to a lesser extent, epithelial cells, and little is known about the function, regulation, and physiological substrates of specific PATs in cells of the immune system. The purpose of this chapter is to cover recent developments in the reemerging field of protein palmitoylation. Specifically, the role of protein palmitoylation in T lymphocyte responses that result in productive activation or, conversely, a clinically relevant state of unresponsiveness termed T cell anergy, will be discussed. We will also

briefly review our recent studies that illuminated altered patterns of protein palmitoylation in anergic T cells. The functional relevance of protein palmitoylation in T cell activation (Bijlmakers, 2009; Resh, 2006a) and recent general advances in protein palmitoylation (Greaves and Chamberlain, 2007; Iwanaga *et al.*, 2009; Linder and Deschenes, 2004, 2007; Mitchell *et al.*, 2006; Planey and Zacharias, 2009) have been separately covered in recent reviews. This chapter is meant to bring these two areas together and, hence, provide directions for future studies aimed at analyzing the role of protein palmitoylation in immunity using novel, globally, and mechanistically based approaches.

2. T LYMPHOCYTE ACTIVATION AND ANERGY

Naïve, resting T lymphocytes are triggered to undergo a complex process of biochemical changes and differentiation when the TCR expressed on their surface is engaged by processed antigen bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Depending on the developmental stage of the T cell, the antigen's concentration and avidity, the presence of costimulatory receptors, and the cytokine microenvironment, TCR engagement can result in several distinct functional outcomes such as productive T cell activation, anergy, or cell death. Mechanistic analysis of the differential molecular signaling pathways that lead to these distinct functional states has been a major area of research among T cell biologists since the early 1990s.

2.1. Productive T cell activation and the immunological synapse

The TCR is a complex of integral membrane proteins that participate in the activation of T cells in response to antigen presented by APCs. Clonally distributed subunits of this complex (α and β, or γ and δ) specifically recognize the antigen together with self MHC molecules, while other conserved subunits (CD3-γ, δ, ε, ζ, and/or η) serve to translate the recognition event into a complex series of intracellular signaling events. Productive activation leads to acquisition of defined effector functions such as target cell-killing by cytotoxic T lymphocytes (CTLs) or production of cytokines. Activation also requires engagement of T cell costimulatory receptors, which come in different flavors, by their cognate ligands on the surface of APCs. The major and most extensively studied costimulatory receptor on T cells is CD28, which is triggered by its ligands B7-1/CD80 or B7-2/CD86. TCR and costimulatory receptor engagement promotes stable contact between T cells and APCs, leading to rearrangement

of the T cell actin cytoskeleton and formation of a highly compartmentalized assembly of receptors and intracellular signaling proteins at the contact site, which has been termed the supramolecular activation cluster (SMAC; Monks *et al.*, 1998) or the immunological synapse (IS; Grakoui *et al.*, 1999), by analogy with neurological synapses (Shaw and Allen, 2001). The IS is highly regulated in time and in space, and it consists of several subregions arranged as concentric rings in the mature IS, each of which is characterized by the presence of defined proteins. At the center of the mature IS is the central cSMAC, where the TCR is localized, and surrounding it is the peripheral SMAC (pSMAC), where the integrin LFA-1 and the cytoskeletal protein talin are localized (Monks *et al.*, 1998). This scheme was later revised to include the outer periphery, ring-like structure of the distal SMAC (dSMAC), where the CD45 phosphotyrosine (pTyr) phosphatase and CD43 are localized (Delon *et al.*, 2001; Freiberg *et al.*, 2002).

Subsequent studies revealed for the first time that microclusters (MCs) or ''protein islands'' that contain the TCR, CD4, and other intracellular signaling enzymes and adaptors form all over the T cell–APC interface and move in a dynamic manner (Bunnell *et al.*, 2001, 2002; Douglass and Vale, 2005; Krummel *et al.*, 2000). The development of imaging techniques that allow visualization of the IS and early events of T cell activation in real time, such as total internal reflection fluorescence (TIRF) microscopy, and the use of a supported planar lipid bilayer system for presentation of antigen and costimulatory signals to T cells made it possible to analyze the formation and organization of the IS at a higher level of resolution (Bunnell, 2010; Campi *et al.*, 2005; Yokosuka and Saito, 2010; Yokosuka *et al.*, 2005). It is now established that immediately upon pMHC contact, mature antigen-specific T cells spread and MCs form all over the interface in a step that corresponds to the initiation of Ca^{2+} signaling. After ~1–2 min, these MCs start to translocate in a centripetal manner toward the center of the interface to form the cSMAC. These MCs represent regions of active signaling, and as they move to form the cSMAC, signaling molecules such as ZAP-70 and SLP-76 dissociate from the MCs, and tyrosine phosphorylation can no longer be detected in them. The TCR-containing portion of the cSMAC is now considered to be the site where signaling complexes are proteolytically degraded and internalized (Lee *et al.*, 2003; Vardhana *et al.*, 2010; Varma *et al.*, 2006), and more recent TIRF microscopy revealed that the cSMAC can be divided into two defined subregions, that is, a central, TCR-containing region, and a peripheral region characterized by the presence of CD28 and protein kinase C-θ (PKCθ), which most likely represents a site of sustained, active signaling (Yokosuka and Saito, 2009; Yokosuka *et al.*, 2008). The IS is a dynamic entity where small signaling clusters consisting of the TCR, tyrosine kinases, and adaptor proteins are continuously being formed in the periphery, subsequently moving centripetally toward the cSMAC,

where active signaling is terminated. The organization of the IS likely contributes to the sequestration of signaling molecules into distinct compartments to promote functional interactions.

In addition to the IS, distinct membrane microdomains, that is, lipid rafts are also implicated in TCR signaling and in localization and function of proteins residing proximal to the receptor (He and Marguet, 2008; Jury *et al.*, 2007; Kabouridis and Jury, 2008; Magee *et al.*, 2002; Sedwick and Altman, 2002). Important signaling proteins such as CD4 and CD8 coreceptors, Src-family kinases, and the adaptor protein, LAT, are constitutively localized in lipid rafts due to their palmitoylation (see below), and others are recruited to rafts upon TCR triggering as a result of inducible protein–protein interactions (Bijlmakers, 2009). Lipid rafts have been postulated to function as important platforms to initiate signaling cascades in different cell types (Simons and Toomre, 2000). TCR stimulation induced microscopic lipid rafts to coalesce into large ($\sim$200 nm in diameter) rafts that cluster at the IS (Bi *et al.*, 2001; Burack *et al.*, 2002; Sedwick and Altman, 2002). Although the importance of lipid rafts in TCR signaling is somewhat controversial (Horejsi, 2002; Kenworthy, 2008; Pizzo *et al.*, 2002), it is highly likely that they promote T cell signaling, especially under conditions of suboptimal TCR triggering.

TCR/CD28 signaling proceeds via a complex network of biochemical changes that are initiated by activation of Src-family tyrosine kinases (Lck and Fyn; Kane *et al.*, 2000; Samelson, 2002). Active Lck/Fyn phosphorylates the signaling subunits of the TCR/CD3 complex on multiple cytoplasmic tyrosine residues found in conserved immunoreceptor tyrosine-based activation motifs (ITAMs), leading to recruitment and activation of the ZAP-70 tyrosine kinase via its tandem SH2 domains (Kane *et al.*, 2000; Samelson, 2002). Activated ZAP-70 then phosphorylates a key membrane adaptor protein, linker for activation of T cells (LAT), which in turn serves as a scaffold to recruit and activate, directly or indirectly, many signaling molecules, including enzymes such as phospholipase C-γ1 (PLCγ1), phosphatidylinositol 3-kinase (PI3-K), Itk tyrosine kinase, and the Rho guanine nucleotide exchange factor (GEF) Vav1, and adaptor proteins (e.g., Grb2, Gads, SLP-76; Kane *et al.*, 2000; Samelson, 2002). Together, this TCR-coupled signaling complex activates a number of downstream signaling pathways, including Ca^{2+} mobilization, PKC, Ras, and other small GTPases, mitogen-activated protein kinases (MAPKs), leading to activation of different transcription factors, primarily NFAT, NF-κB, and AP-1, and *de novo* expression of genes that characterize the productively activated T cells. Complete activation then results in T cell proliferation, production of interleukin-2 (IL-2) and other cytokines, and differentiation of distinct subsets of T helper (Th) cells, regulatory T (Treg) cells, or CTLs.

2.2. T cell anergy

The specificity of the T cell response is determined by nature of the antigen. Antigens recognized by the TCR are usually derived from pathogenic cells and organisms, but in some circumstances from the body's own organs and tissues. In healthy individuals, self-antigens fail to initiate a significant immune response because the immune system is tolerant to these antigens. This tolerance is maintained by several mechanisms that have evolved in order to dampen and prevent such self-reactivity. If these tolerance mechanisms are impaired, uncontrolled T cell activation and proliferation can ensue, resulting in harmful autoimmune diseases such as type I diabetes, multiple sclerosis, and rheumatoid arthritis (RA). During thymic development, self-reactive T cells are eliminated in a process of negative selection by activation-induced cell death, and in addition, natural Treg (nTreg) cells develop, which inhibit the activation of escaped self-reactive T cells. Natural regulatory T (nTreg) cells and antigen-induced Treg (iTreg) cells inhibit T cell activation in the periphery (Josefowicz and Rudensky, 2009; Sakaguchi *et al.*, 2008, 2009), but T cell anergy represents another, extensively studied mechanism of peripheral tolerance. T cell anergy, first discovered in 1987 by TCR stimulation of an antigen-specific T cell clone in the absence of CD28 costimulation (Jenkins *et al.*, 1987) is operationally defined as the intrinsic inability (or poor ability) of a previously responsive T cell to respond to TCR restimulation with proliferation and cytokine production, and it can be reversed by addition of exogenous IL-2. The common event in the various anergizing stimuli was proposed to be a lack of costimulatory signal through CD28 (Jenkins *et al.*, 1987, 1990; Quill and Schwartz, 1987; Schwartz, 2003). It is now clear that anergy does not reflect a global failure of TCR signaling but, rather, a selective defect in the activation of a subset of signaling pathways normally induced by TCR and/or costimulatory agonists (Fathman and Lineberry, 2007; Schwartz, 2003). T cell anergy has important clinical and therapeutic implications because it can be associated with the failure to mount effective antitumor T cell responses, in which case strategies that inhibit (or prevent) T cell anergy would be desirable. Conversely, strategies to induce selective alloantigen-specific anergy could be beneficial in allogeneic solid organ and bone marrow transplantation.

Since anergy was first discovered, many groups have worked to elucidate the molecular and biochemical events that are required for the induction and maintenance of the anergic stage. These studies have led to discovery of defined TCR signaling defects in anergic T cells. Earlier studies demonstrated defects in the activation of Ras (Fields *et al.*, 1996), MAPKs (Li *et al.*, 1996), and the transcription factors NF-κB (Sundstedt *et al.*, 1996) and AP-1 (Kang *et al.*, 1992; Sundstedt and Dohlsten, 1998; Sundstedt *et al.*, 1996) in mouse and human anergic T cells, while TCR-induced Ca^{2+} signaling remained relatively intact, the latter observation being consistent with an early report that treatment of primed T cells with

a Ca^{2+} ionophore can, in fact, induce T cell anergy (Jenkins *et al.*, 1987). A molecular basis for this observation was later provided when it was found that activation of Ca^{2+}/NFAT signaling alone in the absence of the NF-κB and AP-1 signaling pathways normally triggered by TCR/CD28 costimulation induces a distinct gene program that leads to anergy induction (Macian *et al.*, 2002). This reflects the binding of anergy-inducing NFAT homodimers (instead of NFAT:AP-1 heterodimers that lead to productive T cell activation) to the promoters of target genes (Soto-Nieves *et al.*, 2009). Later studies demonstrated that genes encoding several E3 ubiquitin ligases are among the targets of anergy induction, and that upregulation of these E3 ligases and the resulting ubiquitination-mediated degradation of key signaling molecules underlies the hyporesponsive state of anergic T cells (Heissmeyer *et al.*, 2004). More recently, we found that anergic T cells display impaired palmitoylation of LAT (Hundt *et al.*, 2006). These findings are discussed in more detail below.

3. PROTEIN PALMITOYLATION (S-ACYLATION)

3.1. Protein acylation

Protein acylation describes the covalent attachment of different fatty acyl chains (such as myristoyl or palmitoyl) to specific residues in proteins. Different lipid modifications provide distinct affinities for membrane association (Shahinian and Silvius, 1995). Fatty acid modification has a variety of effects on signaling, trafficking, protein stability, protein–protein interactions, as well as partitioning to distinct membrane microdomains (Resh, 2006a,b; Smotrys and Linder, 2004). There are three general types of lipid modification: prenylation, N-myristoylation, and palmitoylation. Prenylation, which is found in many small GTPases (e.g., Ras) with a C-terminal CAAX motif, occurs posttranslationally, and consists of the enzymatic linkage of a 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenoid to one or more Cys residues near the C-terminus of proteins via a thioether bond. In N-myristoylation, proteins possessing the N-terminal consensus sequence Met-Gly are cotranslationally processed by N-myristoyltransferases to link the free N-terminal amine of glycine to myristic acid through an amide bond. Although N-myristoylation is irreversible, several N-myristoylated proteins exhibit a "myristoyl switch," where the myristate group is either exposed on the surface of the protein or sequestered into a hydrophobic pocket (Ames *et al.*, 1996). These structural changes facilitate dynamic regulation of membrane association of N-myristoylated proteins without the removal of myristate group.

3.2. Properties and functions of protein palmitoylation

Palmitoylation refers to the posttranslational attachment of the saturated 16-carbon palmitate from its lipid donor, palmitoyl-coenzyme A ester, to Cys residues of proteins. Some secreted signaling proteins are modified at their N-terminal Cys residue by an amide-linked palmitate in a process termed N-palmitoylation. However, the much more common form of palmitoylation consists of thioester-linked covalent attachment of palmitate to internal Cys residues, termed S-acylation or thioacylation. In this chapter, we will only cover this latter form of palmitoylation, and the term palmitoylation will be used as a synonym with S-acylation. To date, no consensus motifs for protein palmitoylation have been found, although some patterns of S-acylation exist, for instance, N-terminal dual S-acylation and myristoylation (e.g., most Src-family tyrosine kinases), C-terminal dual S-acylation and prenylation (Ras proteins), and multiple S-acylations at Cys string motifs (Smotrys and Linder, 2004).

Protein palmitoylation was serendipitously discovered over 30 years ago in the experiments using metabolic [^{3}H]palmitate labeling of virus particles and virus infected cells (Schmidt and Schlesinger, 1979; Schmidt *et al.*, 1979). Originally thought to simply anchor proteins in the membrane, palmitoylation is now known to occur on a wide variety of proteins, including peripherally associated and integral membrane proteins (Smotrys and Linder, 2004), and it is being implicated in the process of protein trafficking between organelles and in the segregation or clustering of proteins in membrane compartments (Mor and Philips, 2006; Plowman and Hancock, 2005). In many cases, palmitoylation adds a hydrophobic membrane anchor directing membrane association (Shahinian and Silvius, 1995). A large number of integral transmembrane (TM) or peripheral membrane proteins are palmitoylated, including G-protein-coupled receptors, the T cell coreceptors CD4 and CD8, members of Ras and Src families, and endothelial nitric oxide synthase (eNOS). Palmitate attachment often causes proteins to partition to submicroscopic membrane microdomains known as lipid rafts (Simons and Toomre, 2000), which are highly enriched with the multiply palmitoylated proteins caveolin and flotillin. Additionally, recent studies in *Saccharomyces cerevisiae* have demonstrated that palmitoylation protects proteins from degradation by preventing their ubiquitination (Valdez-Taubas and Pelham, 2005). It is known that synaptic activity (Kang *et al.*, 2004, 2008) and T cell activation (Bijlmakers, 2009; see below) are both dependent on multiprotein membrane-bound complexes highly enriched in palmitoylated proteins. In each of these examples, palmitoylation is essential for the proper cellular assembly, distribution, and function of important cellular regulatory processes. Hence, changes in the palmitoylation status of proteins can affect their functions and signaling properties. In this chapter, the focus will be on palmitoylation in T cells.

In contrast to other acylation reactions, palmitoylation is a reversible process. The reversible nature of the thioester linkage lends itself to a variety of regulatory scenarios. The dynamic nature of this modification is evidenced by the fact that proteins undergo several palmitoylation/depalmitoylation cycles during their lifetime. Ras proteins, which have been extensively studied in this regard, best exemplify this situation. Ras proteins were among the first proteins reported to undergo dynamic S-acylation (Magee *et al.*, 1987). Lipid anchoring of oncogenic H-Ras is essential for its ability to induce cellular transformation. Deletion of the prenylation site or removal of a single palmitoylation site significantly reduces the protein's oncogenic potential (Willumsen *et al.*, 1996). Oncogenic H-Ras mutants were determined to have shorter palmitate half-lives than the wild-type H-Ras, even though the intracellular levels of palmitoylation did not differ (Baker *et al.*, 2003). Palmitoylation-deficient H-Ras localizes to the Golgi outer membrane, where it is presumably sequestered from growth factor-induced signaling. Further, the half-life of palmitate turnover on inactive GDP-bound H-Ras is decreased by more than 15-fold upon activation, from 150 min to only 10 min (Baker *et al.*, 2003). GTP-bound, activated H-Ras diffuses out of lipid rafts and redistributes throughout the plasma membrane (PM) and cytosol, where it can then be recycled to the Golgi, repalmitoylated, and transported back to the PM and lipid rafts (Prior *et al.*, 2001). Receptor activation of stimulatory G-protein α subunits (Gαs) similarly speeds up palmitate turnover nearly 50-fold (Mumby *et al.*, 1994; Wedegaertner and Bourne, 1994), from a half-life of 90 min to only 2 min. Another example is the neuronal scaffolding protein PSD-95, which is rapidly depalmitoylated and undergoes clustering at the neuronal synapse upon glutamate stimulation (El-Husseini and Bredt, 2002; El-Husseini *et al.*, 2002). In summary, dynamic turnover of palmitoylation may be a common feature of signal transducers to regulate their trafficking between intracellular compartments and the PM, thereby influencing where and when signals are transmitted.

3.3. Palmitoylating enzymes: The DHHC family

Despite the widespread evidence accumulated during the past >30 years that well characterized proteins are palmitoylated, it was only in the last ~9 years that the enzymes responsible for protein palmitoylation were identified. One reason for this delay was earlier suspicions that palmitoylation may be nonenzymatic, as evidenced by palmitoyl-CoA autopalmitoylation of Gαs *in vitro* at the proper Cys residue (Duncan and Gilman, 1996). Years later, however, the mechanism that underlies the transfer of palmitate was uncovered through genetic screens in yeast, which led to the discovery of two related *S*-palmitoyltransferases, from here on referred to as PATs. Erf2 palmitoylates yeast Ras proteins (Lobo *et al.*,

2002), whereas Akr1 modifies the yeast casein kinase, Yck2 (Roth *et al.*, 2002). These two PATs share homology of a Cys-rich domain (CRD) containing a conserved Asp-His-His-Cys (DHHC) motif. Because of the highly conserved DHHC sequence, PATs are also commonly referred to as DHHC proteins. Subsequent searches of genome databases for this DHHC-containing CRD revealed multiple putative PATs in other species. To date, 7, 22, and 23 PATs encoded by *zdhhc* genes have been identified in yeast (Roth *et al.*, 2006), *Drosophila* (Bannan *et al.*, 2008), and mammals (Fukata *et al.*, 2004), respectively. The large number of PAT enzymes may explain why no palmitoylation consensus site has emerged, since each enzyme presumably has different substrates. Indeed, proteomics-based studies have estimated the sizes of the yeast (Roth *et al.*, 2006) or mammalian (Kang *et al.*, 2008; Martin and Cravatt, 2009) palmitoyl proteomes at ~50 and at least ~300 proteins, respectively, consistent with the idea that any given PAT may display preference for a subset of substrates. The DHHC motif is required for PAT activity, since mutation of the Cys residue in this motif abolishes substrate palmitoylation (Fukata *et al.*, 2004, 2006; Lobo *et al.*, 2002). All PATs contain two to six TM domains and are localized in diverse intracellular membrane compartments, including the ER, endosomes, Golgi apparatus, and the PM (Ohno *et al.*, 2006). Further, PATs are expressed differentially across various cell types and tissues (Saitoh *et al.*, 2004; Swarthout *et al.*, 2005; Uemura *et al.*, 2002). The fact that PATs can be found throughout the entire endomembrane system, indicates that different substrates can get modified at different stages of the intracellular life cycle, that is, upon biosynthesis or later in their life. Proteins containing multiple palmitoylation sites could, in fact, be modified by different PATs, possibly at different locations as suggested for the anthrax toxin receptor TEM8 (Abrami *et al.*, 2006).

In the initial characterization of mammalian PATs, all 23 DHHC enzymes were cotransfected with the neuronal scaffold protein PSD-95, and transfected cells were metabolically labeled with [^{3}H]palmitate to assay for enhanced PSD-95 palmitoylation (Fukata *et al.*, 2004). Four candidate PATs (DHHC2, DHHC3, DHHC7, and DHHC15) were capable of enhancing PSD-95 palmitoylation, suggesting that each may function *in vivo* as a PSD-95-specific PAT. This method has been used in numerous studies to identify PATs specific for eNOS (Fernandez-Hernando *et al.*, 2006), SNAP25 (Greaves *et al.*, 2009), G-proteins (Tsutsumi *et al.*, 2009), and many others proteins. However, since this method relies on PAT overexpression, it does not represent a foolproof approach to identify physiological PAT substrates. Indeed, more recent studies have relied on RNAi-mediated PAT knockdown in conjunction with functional signaling assays in order to establish physiological PAT–substrate relationships (Greaves *et al.*, 2010).

A number of studies reported relatively stable physical associations between PATs and potential substrates. In fact, yeast 2-hybrid screens of PAT-interacting proteins identified interactors that were later found to represent true substrates of the relevant DHHC proteins (Fernandez-Hernando *et al.*, 2006; Keller *et al.*, 2004; Li *et al.*, 2010; Nadolski and Linder, 2009; Saitoh *et al.*, 2004; Singaraja *et al.*, 2002; Uemura *et al.*, 2002). Although most enzyme–substrate interactions are transient, and association of PATs with a palmitoyl proteins does not necessarily establish an enzyme–substrate relationship, PAT–substrate interactions likely involve initial autopalmitoylation of the DHHC motif's conserved Cys residue, followed by PAT–substrate association and palmitate transfer from the PAT to its substrate (Hou *et al.*, 2009; Iwanaga *et al.*, 2009). All DHHC proteins autopalmitoylate (Fukata *et al.*, 2004; Huang *et al.*, 2004; Lobo *et al.*, 2002; Mitchell *et al.*, 2006; Roth *et al.*, 2002; Smotrys *et al.*, 2005; Swarthout *et al.*, 2005), and autopalmitoylated PATs may represent covalent enzyme intermediates required for substrate palmitoylation (Hou *et al.*, 2009). Interactions between other regions in PATs and substrates are also important for substrate recognition and palmitoylation (Greaves and Chamberlain, 2010). Some PATs have protein–protein interaction domains (Iwanaga *et al.*, 2009; Mitchell *et al.*, 2006), and regions in palmitoyl proteins distant from their palmitoylated Cys residues, which are required for substrate recognition and palmitoylation, have been identified; further, swapping of these recognition sequences between distinct palmitoyl proteins can confer new substrate specificity patterns (Greaves *et al.*, 2009; Huang *et al.*, 2009; Nadolski and Linder, 2009). Therefore, understanding the molecular basis for PAT–substrate recognition and specificity is of great importance.

There are ample examples that a given PAT can palmitoylate different substrates and, conversely, that a given protein can be palmitoylated by several PATs. This suggests a certain level of redundancy. However, it is becoming clear that natural mutations in, or experimental deletion of, individual DHHC proteins can lead to severe phenotypes or, at a minimum, be associated with human diseases. In fact, aberrant regulation of palmitoylation or depalmitoylation has been implicated in a number of cancer and human diseases. Many of the genes encoding human PATs are associated with cancer and other diseases: DHHC8 with schizophrenia (Mukai *et al.*, 2004), DHHC5 with learning and memory deficits (Li *et al.*, 2010), DHHC13 with osteoporosis, alopecia, and amyloidosis (Saleem *et al.*, 2010), DHHC17/HIP14 with Huntington's disease (Yanai *et al.*, 2006), DHHC15 and DHHC9 with X-linked mental retardation (Mansouri *et al.*, 2005; Yanai *et al.*, 2006), and DHHC2, DHHC9, DHHC17, and DHHC11 with cancer (Ducker *et al.*, 2004; Mansilla *et al.*, 2007; Oyama *et al.*, 2000; Yamamoto *et al.*, 2007). The majority of the demonstrated associations are with cancer, which serves to emphasize

the importance of PATs as potential therapeutic drug targets in human cancers (Karnoub and Weinberg, 2008). Clearly, identification of physiological PAT substrates will provide important information concerning the molecular mechanisms that underlie pathologies associated with PAT mutations. PATs also represent potential drug targets in certain viral infections. Thus, palmitoylation of certain viral proteins directs the association of viral particle with lipid rafts, which is required for the ability of some viruses to infect host cells (Grantham *et al.*, 2009; Higuchi *et al.*, 2001).

3.4. Depalmitoylating enzymes

Protein depalmitoylation appears to be, for the most part, an enzymatic process, but little progress has been made to identify and characterize the responsible thioesterases. Indeed, pervanadate treatment accelerates the depalmitoylation of Lck in Jurkat T cells, and this accelerated turnover is prevented by preincubation with the serine hydrolase inhibitor methyl arachidonyl fluorophosphonate, but not phenylmethylsulfonyl fluoride (Zhang *et al.*, 2010). While the *zdhhc* gene family is relatively large, only two protein palmitoyl thioesterases (PPTs) have been described to be capable of catalyzing the removal of fatty acids from proteins, that is, acyl protein thioesterase 1 (APT1) and palmitoyl protein thioesterase 1 (PPT1; Zeidman *et al.*, 2009), both members of the serine hydrolase enzyme family.

3.4.1. Acyl protein thioesterase 1

APT1 is a ubiquitous cytosolic serine hydrolase (Duncan and Gilman, 1998; Toyoda *et al.*, 1999). This enzyme was initially characterized as lysophospholipase I (LYPLA1; Sugimoto *et al.*, 1996; Wang *et al.*, 1997), but was later shown to have several 100-fold higher activity as a PPT. It is not entirely clear how a cytosolic protein can hydrolyze palmitate from membrane-bound palmitoylated proteins. Some evidence exists that APT1 is itself palmitoylated (Yang *et al.*, 2010), although we have not been able to confirm this result. APT1 depalmitoylates proteins by catalyzing cleavage of the thioester bond between the fatty acyl chain and the protein. Several proteins have been identified as substrates of APT1 *in vitro*, including Ras (Duncan and Gilman, 1998), various heterotrimeric Gαs (Duncan and Gilman, 1996, 1998), eNOS (Yeh *et al.*, 1999), RGS4 (Duncan and Gilman, 1998), SNAP-23 (Flaumenhaft *et al.*, 2007), LAT (unpublished observations), and several viral proteins (Grantham *et al.*, 2009; Higuchi *et al.*, 2001; Schmidt, 1982; Schmidt and Schlesinger, 1979; Schmidt *et al.*, 1979, 1988; Thorp *et al.*, 2006; Yang *et al.*, 1995). siRNA-mediated knockdown of APT1 was shown to decrease synaptic spine volume in cultured neurons (Siegel *et al.*, 2009) and exhibits

activity-dependent local translation at synapses (Banerjee *et al.*, 2009), suggesting that APT1 is important for neuronal development and activity. However, there is still little direct evidence that APT1 is acting as a PPT *in vivo*.

Two APT1 homologues have been cloned: lysophospholipase II (LYPLA2, a.k.a. APT2) and lysophospholipase-like 1 (LYPLAL1). APT2 shares 64% identity with APT1 and has been shown to hydrolyze several lysophospholipid substrates with varying efficiencies. LYPLAL1 is 30% identical to APT1, and is yet to be characterized. There is no evidence that either APT2 or LYPLAL1 can depalmitoylate proteins. Recently, APT1 has been implicated as a PPT responsible for the depalmitoylation of H-Ras in cells (Dekker *et al.*, 2010). Several derivatives of the β-lactone drug tetrahydolipstatin (THL) were synthesized and screened for APT1 inhibition. Serine hydrolases (including APT1) covalently react with β-lactones leaving an esterified active site serine that is slowly hydrolyzed. The most promising β-lactone analog capable of inhibiting APT1, palmitostatin B, was shown to have an *in vitro* APT1 IC_{50} of 670 nM. Application of this inhibitor to cells slowed the subcellular redistribution of microinjected semisynthetic fluorescent Ras proteins in MDCK cells. Further, the subcellular redistribution of a transfected yellow fluorescent protein (YFP) H-Ras fusion was impeded following treatment with 1 μM palmitostatin B. Notably, siRNA-mediated knockdown of APT1 reduced APT1 levels by $>80\%$, yet did not significantly change YFP-H-Ras distribution. Additionally, overnight incubation with 50 μM Palmitostatin B could induce phenotypic reversion of oncogenic H-Ras-transformed MDCK cells. Treatment of cells with such high concentrations of palmitostatin B (50 μM) likely leads to nonselective inhibition of other serine hydrolases. For example, treatment with similar concentrations of THL inhibits more than a dozen serine hydrolases (Hoover *et al.*, 2008).

3.4.2. PPT1 and PPT2

PPT1 was first isolated from soluble bovine brain fractions and found capable of depalmitoylating H-Ras (Camp and Hofmann, 1993). *In vitro*, PPT1 can depalmitoylate diverse palmitoylated proteins and hydrolyze acyl-coenzyme A. Further studies revealed that PPT1 is a lysosomal enzyme (Verkruyse and Hofmann, 1996), indicating it almost certainly cannot be responsible for dynamic deacylation of cytoplasmic proteins. Further, *PPT1* was genetically mapped as the causative gene responsible for infantile neuronal ceroid lipofuscinosis (INCL), a lysosomal storage disease that results in the accumulation of autofluorescent granules. [^{35}S] Cys-labeled lipid thioesters accumulate in immortalized lymphoblasts from patients with INCL, and this accumulation can be reversed by adding recombinant PPT1 to cells (Lu *et al.*, 1996), which is taken up and traffics to lysosomes (Hellsten *et al.*, 1996). PPT1-dependent

neurodegeneration appears to involve ER stress and apoptosis. Thus, current evidence suggests that PPT1 is mainly involved in lysosomal degradation of uncharacterized thioester-containing metabolites.

A homologue of PPT1, PPT2, has been identified. It is also a lysosomal thioesterase but, unlike PPT1, it has substrate specificity for palmitoyl-CoA but not for palmitoyl proteins (Soyombo and Hofmann, 1997). Similarly to *PPT1*, disruption of the *PPT2* gene also causes a type of lysosomal storage disease, albeit with a slower onset and with a milder manifestation (Gupta *et al.*, 2001). The degree of cross-reactivity between PPT1 and PPT2 is unclear. Importantly, however, mice lacking PPT1 or PPT2 display a similar lysosomal storage disease, but an apparently intact immune system (Gupta *et al.*, 2001, 2003). The difference in severity might be explained by other unannotated substrates that are not detected by [^{35}S]Cys labeling. Further, the presence of central nervous system-specific aggregates found in *PPT1*-deficient *Drosophila* mutants cannot be reversed by expressing *Drosophila* PPT2 (Bannan *et al.*, 2008) and endocytosed PPT2 does not reverse the effects of PPT1 deficiency in INCL fibroblasts (Soyombo and Hofmann, 1997). Taken together, it is unlikely that PPT1 or PPT2 functions as protein thioesterases involved in dynamic palmitoylation of cytosolic substrates.

To summarize, APT1 and PPT1 have been shown to deacylate cytoplasmic or lysosomal proteins, respectively, *in vitro*. These enzymes make up the majority of protein thioesterase activity in soluble cellular lysates, but the existence of other membrane-bound activities has been largely ignored. Given the substantial diversity of PATs, it is unlikely that APT1 is solely responsible for all protein depalmitoylation occurring in diverse subcellular compartments. Much more work is required to identify and functionally characterize putative PPTs that function physiologically to regulate the dynamics of palmitoyl proteins.

3.5. Quantitative global analysis of protein palmitoylation

Despite decades of research, the annotation of palmitoylated proteins remains incomplete. Until recently, validation of specific palmitoylated proteins required immunoprecipitation of [^{3}H]palmitate-labeled cells, followed by long film exposures of weeks or months. Understanding the dynamics and regulation of protein palmitoylation requires nonradioactive global approaches to detect and quantify palmitoylation events across the entire proteome. The most established nonradioactive assay is based on the acyl–biotin exchange (ABE) reaction (Berzat *et al.*, 2005; Drisdel and Green, 2004; Schmidt *et al.*, 1988). In this approach, free Cys residues are first alkylated during protein extraction from cells or tissues. Next, thioesters are displaced by treatment with 1 M neutral hydroxylamine, which hydolyzes predominantly thioesters and other weak esters,

exposing previously palmitoylated cysteinyl thiols for capture with a sulfhydryl-biotin. Biotin-linked proteins can then be affinity-purified using streptavidin-coupled beads and digested with trypsin into peptides, leaving the labeled peptides on the affinity beads. Multidimensional protein identification technology (MudPIT; Washburn *et al.*, 2001) is then used to analyze the eluant. The ABE assay was first used globally in yeast, leading to the identification of 50 new palmitoyl proteins (Roth *et al.*, 2006). More recently, the same approach was used in synaptosomes and primary cultured neurons to identify ~200 neuronal palmitoylation candidates, of which >60 were validated as novel palmitoyl proteins (Kang *et al.*, 2008). While many palmitoylation substrates are identified using ABE–MudPIT, the high intrinsic background from the thiol-exchange reaction complicates the identification of low abundance palmitoyl proteins, not to mention that ABE requires large amount of cell lysates and consists of multiple rounds of protein precipitation. Additionally, enzymes with thioester linkages to lipoic acid, phosphopantetheine, and ubiquitin are identified as false positives.

To circumvent the low-fidelity enrichment and slow validation, we sought to develop a nonradioactive palmitoylation probe based on incorporation of a bioorthogonal chemical handle. Notably, PATs show little discrimination among palmitoyl (C16:0), oleoyl (18:1), stearoyl (18:0), and palmitoleoyl (16:1) acyl chains (Lobo *et al.*, 2002), suggesting flexibility in the length of the transferred fatty acyl chain that can be accommodated by these enzymes. Based on this knowledge, we evaluated 17-octadecynoic acid (17-ODYA), a commercially available alkynyl fatty acid originally generated as a low affinity inhibitor of omega-hydroxylases, which are a class of cytochrome p450 enzymes involved in fatty acid metabolism (Shak and Goldstein, 1985; Shak *et al.*, 1985).

Cu(I)-catalyzed, Huisgen's concerted triazole synthesis, more popularly known as click chemistry, is a simple approach to couple alkyne-modified proteins to azide-reporter tags in complex proteomes (Speers and Cravatt, 2004; Speers *et al.*, 2003). This technology allows for the metabolic incorporation of chemical probes within native cellular environments, thus preserving localization, posttranslational modifications, and protein–protein interactions that are essential for profiling endogenous protein states. Click chemistry is then applied to couple the *in vivo*-labeled proteins to azide-containing reporter molecules after cell lysis and homogenization. After incubation with 17-ODYA, cultured cells are lysed and membrane lysates are reacted with rhodamine-azide for fluorescence detection by SDS-PAGE. Dozens of prominent hydroxylamine-sensitive fluorescent bands are visible within a few hours, suggesting that the predominant form of probe incorporation is though S-palmitoylation (Martin and Cravatt, 2009).

In parallel, 17-ODYA-labeled lysates were reacted with biotin-azide for streptavidin enrichment, trypsin digestion, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We identified nearly 300 specifically enriched palmitoylated proteins in Jurkat T cells based on spectral counting thresholds demanding reproducibility, significant average spectral counts, and high contrast over control samples (either incubation with palmitate or 17-ODYA-labeled and treated with hydroxylamine). This number has since been refined to nearly 500 palmitoylated proteins by more sophisticated analysis using newly updated search algorithms previously unavailable at the time of publication (Simon *et al.*, 2009). Essentially all annotated T cell palmitoyl proteins were identified, including all known palmitoylated components of the T cell signaling pathway (CD3, CD4, Lck, LAT, PAG/Cbp, Ras, etc.), more than 10 trimeric G-proteins, more than a dozen small GTPases, and numerous receptors and metabolic enzymes. Hundreds of novel palmitoylated proteins are enriched in this dataset, and the majority of these identifications are for proteins with no annotated function. Many of the identified proteins overlap with identifications from the simultaneous publication of ABE neuronal palmitoylation proteomics from cultured neurons (Kang *et al.*, 2008), further corroborating the accuracy of both approaches. Other groups have reported metabolic incorporation of azido-fatty acids for Staudinger ligation to phosphine-reporters (Hang *et al.*, 2007; Heal *et al.*, 2008; Kostiuk *et al.*, 2008; Martin *et al.*, 2008), but the low efficiency (Agard *et al.*, 2006) and high background (Speers and Cravatt, 2004) has led to general adoption of our methods (Charron *et al.*, 2009; Hannoush and Arenas-Ramirez, 2009; Yount *et al.*, 2010).

False positive data are particularly problematic for interpretation of lower abundance signals from large proteomics datasets. Accordingly, we established a robust protocol to quickly validate proteins enriched in 17-ODYA-treated samples. This method takes advantage of the fact that 17-ODYA-labeled proteins can be visualized by one of multiple platforms, including gel-based readouts (by click chemistry conjugation to rhodamine-azide), which is much simpler and of higher-throughput than LC-MS. Eighteen putative palmitoyl protein cDNAs were subcloned, overexpressed in 293T cells, and labeled with 17-ODYA. Virtually all of the heterologously expressed proteins (16 of 18) were palmitoylated by the endogenous PATs present in 293T cells, allowing simple fluorescent gel-based validation without enrichment. Interestingly, several proteins subject to dual myristoylation and palmitoylation demonstrate some hydroxylamine-resistant labeling, suggesting that 17-ODYA (due to its *in situ* conversion to shorter fatty acyl chains by fatty oxidation pathways) can be used to simultaneously profile both palmitoylation (hydroxylamine-sensitive) and myristoylation (hydroxylamine-resistant) modifications in cells. We believe that combination of modern technologies with functional

genomics methods will allow investigators to identify physiological PAT substrates and exact sites of palmitoylation (Yang *et al.*, 2010). Global profiling approaches will accelerate our understanding of this complex posttranslational modification and unravel new targets and specific sites.

4. PALMITOYLATION IN T LYMPHOCYTES

Protein palmitoylation represents a common lipid modification of neuronal proteins, and it plays an important role in modulating neuronal protein trafficking and the function of neuronal synapses (Huang and El-Husseini, 2005). The neuronal PDZ domain-containing scaffolding protein postsynaptic density-95 (PSD-95) provides a notable example of palmitoyl regulation in neurons. Agonist-induced palmitoylation/depalmitoylation cycles of PSD-95 regulate its lipid raft localization and the clustering of coupled AMPA-type glutamate receptors at excitatory synapses (El-Husseini and Bredt, 2002; El-Husseini *et al.*, 2002). By analogy with neuronal synapses, the term IS has been introduced to describe the contact region between antigen-specific T cells and APCs, where signaling complexes are organized in a spatially and temporally highly regulated manner and where directional cytokine secretion occurs (Grakoui *et al.*, 1999; Monks *et al.*, 1998). The similarity between neuronal synapses and the IS are more than just in name, as both synapses share a protein, agrin, that is important for both neuromuscular and IS formation (Khan *et al.*, 2001). Similar to neuronal synapses, many proteins in the TCR signaling pathway are palmitoylated and accumulate in lipid rafts, which coalesce and cluster at the IS following T cell activation (Bi *et al.*, 2001; Burack *et al.*, 2002).

4.1. Palmitoylated T Cell Proteins

Although it is clear that the palmitoylation status has a marked effect on lipid raft localization and function of T cell signaling molecules such as LAT, Src-family kinases (Lck and Fyn), and others, the importance of lipid raft localization is not fully understood. Similarly, very little is known about the dynamics of palmitate turnover in T cell signaling proteins, and whether this turnover is regulated by receptor signals. However, the recent identification and characterization of the DHHC family, and the establishment of proteomics-based global methods for analysis of the palmitoyl proteome, reviewed above, now provides us with tools to approach these fundamental questions in the context of T cell biology. We will first briefly review some of the important T cell palmitoyl proteins that regulate and participate in TCR signaling. These proteins have also been reviewed recently (Bijlmakers, 2009; Resh, 2006a).

4.1.1. CD4/CD8

CD4 and CD8 are expressed on the surface of T cells and they serve as TCR coreceptors by virtue of two properties: First, the extracellular domains of these coreceptors bind MHC class II and I molecules, respectively, and, thus, participate in TCR recognition of MHC-bound peptide antigens by stabilizing T cell–APC interactions. Second, the cytoplasmic tails of both coreceptors associate with Lck tyrosine kinase and facilitate its activation and its functional coupling to the TCR signaling machinery. While CD4 is expressed as a monomer, in which the extracellular domain is composed of four immunoglobulin-like domains, CD8 is present on most T cells as a disulfide-linked heterodimer of CD8α and CD8β chains, each containing one extracellular immunoglobulin-like domain (Leahy, 1995). However, in NK cells and in some T and dendritic cells, CD8 is expressed as an αα homodimer (Cheroutre and Lambolez, 2008). CD4 is palmitoylated on two juxtamembrane Cys residues (Crise and Rose, 1992) and CD8β (but not CD8α) on a single cytoplasmic Cys residue (Arcaro *et al.*, 2000). As a result, both CD4 and CD8αβ localize in membrane lipid rafts. Although CD4 and CD8αβ palmitoylation is not required for their transport or cell surface expression, this localization enhances, in the case of CD4, raft aggregation, clustering of the TCR and PKCθ at the IS, and tyrosine phosphorylation of signaling proteins, primarily TCR-ζ and ZAP-70 kinase (Balamuth *et al.*, 2004; Fragoso *et al.*, 2003). However, the overall importance of CD4 palmitoylation for its raft localization and coreceptor function is controversial. Inconsistent findings in this regard may reflect the fact that even in the absence of CD4 palmitoylation, it still covalently associates with Lck kinase, which is itself palmitoylated (see below) and, thus, may facilitate the recruitment of CD4 into lipid rafts and TCR signaling complexes. CD4, which serves as an HIV entry coreceptor by virtue of its binding to the HIV surface glycoprotein gp120, and its palmitoylation have also been implicated in facilitating HIV entry, but CD4 palmitoylation is not required for this and, in a more general sense, the importance of lipid rafts for HIV entry is debatable (Bijlmakers, 2009). The CD8αβ heterodimer also contributes to T cell activation and its palmitoylation seems to facilitate this function, at least in mice (Arcaro *et al.*, 2001).

4.1.2. Src-family kinases

Two of the Src-family tyrosine kinases that are expressed in T cells, Lck, and Fyn, play important roles in T cell activation and/or development (Palacios and Weiss, 2004; Perlmutter, 1995). These kinases are composed of a unique N-terminal Src homology-4 (SH4) domain that contains myristoylation and palmitoylation sites, an SH3 domain that can bind specific proline-rich sequences, an SH2 domain that binds specific sites of tyrosine phosphorylation, an SH1 catalytic kinase domain, and a C-terminal

regulatory tail that contains an autoinhibitory tyrosine residue that, when phosphorylated, maintains the kinase in a resting (inactive) state due to its internal association with the kinase's SH2 domain (Paige *et al.*, 1993; Thomas and Brugge, 1997). All members of this family are cotranslationally myristoylated at Gly-2 in a conserved Met-Gly-Cys motif following removal of the N-terminal Met residue. This myristoylation precedes, and is required for, double palmitoylation of most Src-family kinases, including Lck and Fyn, at Cys-3 and Cys-5 (Lck) or Cys-6 (Fyn; Shenoy-Scaria *et al.*, 1993; Yasuda *et al.*, 2000). This lipid modification is necessary to translocate these peripheral membrane kinases to the PM and into lipid rafts and Cys-3 appears to be more critical in this regard (Bijlmakers *et al.*, 1997; Yurchak and Sefton, 1995). Surprisingly, however, despite being constitutively palmitoylated, Lck is not localized in lipid rafts in resting T cells. This may reflect a shift in the dynamics of palmitate cycling on Lck toward depalmitoylation in resting cells, which is reversed following T cell activation in favor of palmitoylation, thereby causing the majority of Lck to undergo lipid raft translocation. Two family members, Src and Blk, reside outside lipid rafts and, instead of being palmitoylated, utilize an N-terminal polybasic sequence to enhance membrane association. Removal of the palmitoylation site(s) in Src-family kinases prevents their membrane (and lipid raft) association and cellular functions, despite normal kinase activity in cell-free assays (Kabouridis *et al.*, 1997; Kosugi *et al.*, 2001).

Lck-deficient mice show dramatic thymic atrophy and a dramatic reduction in the early double-positive ($CD4^+CD8^+$) thymocyte subset; mature, single-positive thymocytes are undetectable and there are only very few peripheral T cells (Molina *et al.*, 1992). These results illustrate the crucial role of Lck in thymocyte development. Lck mutants with amino acid substitutions at the myristoylation or palmitoylation sites are unable to reconstitute TCR-mediated activation in Lck-deficient T cells. These acylation defective mutants do not interact with CD4, and fail to phosphorylate TCR-ζ and activate ZAP-70, thereby preventing propagation of multiple downstream signaling pathways (Kabouridis *et al.*, 1997; Kosugi *et al.*, 2001; Yasuda *et al.*, 2000; Yurchak and Sefton, 1995).

Unlike Lck, the deletion of Fyn has a less severe effect on T cell development. Fyn-deficient thymocytes display reduced TCR-induced Ca^{2+} fluxes and abrogated proliferation, but mature splenic T cells from these mice retain largely normal proliferation despite depressed Ca^{2+} mobilization and IL-2 production (Appleby *et al.*, 1992; Stein *et al.*, 1992). The Fyn tyrosine kinase can be palmitoylated on both Cys-3 and -6, but MS analysis showed that only a minor fraction of Fyn is dually S-palmitoylated, and that the majority is singly palmitoylated only on Cys-3 (Liang *et al.*, 2001). More recent studies revealed that the second palmitoylation site on Cys-6 directly targets newly synthesized Fyn to the PM

(Sato *et al.*, 2009), bypassing the Golgi system. Fyn palmitoylation is highly dynamic, and has a reported half-life of 1.5–2 h (Wolven *et al.*, 1997). Unlike Lck, Fyn is predominantly localized to lipid rafts in resting T cells. Lck was reported to have higher kinase activity outside of lipid rafts, but following activation it is transported to lipid rafts where it activates Fyn (Filipp *et al.*, 2003). These findings led to the suggestion that lipid rafts function to segregate Lck and Fyn in the absence of a T cell stimulatory signal, and allowing them to functionally interact in lipid rafts upon TCR stimulation (Filipp *et al.*, 2003). It appears that Lck and Fyn are at least partially redundant as both are capable of phosphorylating the ITAM motifs in the signaling subunits of the TCR–CD3 complex, but each of these related kinases may also have their unique substrates. The highly distinct effects of Lck *versus* Fyn deletion on T cell development are another indication of their nonredundant functions.

4.1.3. Ras proteins

Ras proteins represent a subgroup of the large family of small GTPases, which is membrane localized. Like other small GTPases, Ras proteins function as molecular switches that cycle between an inactive, GDP-bound and an active, GTP-bound state to regulate cell proliferation, differentiation, migration, and apoptosis (Downward, 1997; Marshall, 1996; McCormick, 1995; Satoh *et al.*, 1992). Activating mutations that generate aberrant, hyperactive (permanently locked in the GTP-bound state) Ras promote cancer and developmental defects. There are three major isoforms, H-, N-, and K-Ras that are ubiquitously expressed. All isoforms contain a C-terminal CAAX motif that targets them for farnesylation (Cadwallader *et al.*, 1994). In addition, H- and N-Ras are reversibly palmitoylated on two or one C-terminal Cys residues, respectively (Magee *et al.*, 1987). Whereas farnesylation targets Ras proteins to endomembranes, that is, the Golgi and endoplasmic reticulum (ER), subsequent palmitoylation (or a polybasic sequence in nonpalmitoylated K-Ras) targets them to the PM (Choy *et al.*, 1999; Hancock *et al.*, 1990). When activated by their respective guanine nucleotide exchange factors (GEFs), Ras proteins act as adaptors that are recruited to the PM and facilitate activation of a wide variety of effectors. Palmitoylation mutants of Ras fail to traffic to the PM and are not associated with the TCR signaling machinery (Rubio *et al.*, 2010). As reviewed earlier, rapid cycles of Ras palmitoylation and depalmitoylation impact its activity and translocation between the PM and the Golgi system (Willumsen *et al.*, 1996), and the half-life of palmitate turnover on inactive GDP-bound H-Ras is accelerated more than 15 times upon activation (Baker *et al.*, 2003). After depalmitoylation, H-Ras is recycled to the Golgi, where it is repalmitoylated and transported back to the PM. Contrary to earlier models, which invoked the PM as the only site from which Ras regulates signaling, later

reports demonstrated that active Ras also localizes in endomembranes, where it can induce downstream signaling (Bivona *et al.*, 2003; Chiu *et al.*, 2002), including in T cells stimulated with low concentrations of activating anti-CD3 antibodies (Bivona *et al.*, 2003; Perez de Castro *et al.*, 2004). Microinjection of semisynthetic fluorescent H-Ras proteins demonstrated that the palmitoylation machinery required for Ras palmitoylation and trafficking does not rely on a single PAT enzyme, and importantly the palmitoyl transferase activity does not distinguish between L- and D- amino acids at palmitoylation sites, questioning the concept of unique substrates for distinct PATs (Rocks *et al.*, 2010).

Following the initial demonstration of TCR-induced Ras activation (Downward *et al.*, 1990), Ras proteins have been extensively shown to play important roles in T cell development and activation (Genot and Cantrell, 2000; Izquierdo *et al.*, 1995). Dominant negative Ras mutants inhibit IL-2 promoter activity in T cell tumor lines (Rayter *et al.*, 1992), and transgenic expression of dominant negative Ras causes a block in thymic development (Swan *et al.*, 1995). Ras is also involved in the activation of the MAPKs Erk and Jnk (Fields *et al.*, 1996; Li *et al.*, 1996) as well as in the transactivation of the AP-1 transcription factor (Kang *et al.*, 1992) in T cells. Recent genetic and biochemical studies have shown that the lipid second messenger diacylglycerol (DAG), liberated by TCR-activated PLCγ1 stimulates Ras-GTP loading by activation of the Ras GEF, RasGRP1 (Dower *et al.*, 2000; Ebinu *et al.*, 2000; Roose *et al.*, 2005; Roosild *et al.*, 2005). Sos is another Ras-activating GEF in T cells, which is recruited to the TCR signaling complex via its constitutive association with the adaptor protein Grb2 and the TCR-induced recruitment of the Grb2-Sos complex to tyrosine-phosphorylated LAT (Koretzky, 1997; Roose *et al.*, 2005; see below).

4.1.4. TM adaptor proteins

Adaptor proteins play important roles in transducing and converting immunoreceptor signals into the cellular responses of hematopoietic cells, for example, differentiation, proliferation, and cytokine expression. Among these adaptor proteins, TM adaptor proteins (TRAPs) represent a unique group of TM proteins that are differentially expressed in a variety of hematopoietic cells, and can influence immunoreceptor signaling either positively or negatively (Horejsi, 2004; Horejsi *et al.*, 2004). The structure of TRAPs is somewhat similar to the immunoreceptor-associated TCR-ζ and CD3-γ chains in that they contain a short extracellular domain and intracellular tyrosine residues (but not ITAMs) that become phosphorylated upon immunoreceptor ligation. This phosphorylation allows the TRAPs to recruit various SH2 domain-containing signaling proteins into proximal signaling complexes. However, unlike TCR-ζ and CD3-γ, TRAPs do not associate with the TCR–CD3 complex. Among

the seven known TRAPs, four are palmitoylated on a cytoplasmic juxtamembrane Cys-X-X-Cys motif and, as a result, are found in membrane lipid rafts: PAG/Cbp, NTAL/LAB, LIME, and LAT. These adaptors also share a short (4- to 17-residue) N-terminal extracellular domain and up to 10 tyrosine residues in the cytoplasmic domain, which are potentially phosphorylated by Src- or Syk-family kinases. Three of these, with the exception of NTAL/LAB, are expressed in T cells. These adaptor proteins have been previously reviewed (Horejsi, 2004; Horejsi *et al.*, 2004) and, therefore, will only be briefly reviewed here, with the exception of LAT, which is discussed in more detail.

4.1.4.1. Linker for activation of T cells LAT was the first TRAP to be isolated based on earlier findings that a ~36-kDa pTyr-containing protein represented a predominant early phosphoprotein in TCR-stimulated T cells (Zhang *et al.*, 1998a). LAT is primarily expressed in T cells, but also in mast and NK cells and in platelets. LAT is a TM protein composed of a short (nine-residue) extracellular domain, a TM domain, and an intracellular domain containing several tyrosine residues that are phosphorylated predominantly by ZAP-70 kinase upon TCR ligation and then bind a number of SH2-containing enzymes and adaptor proteins. Phospho-LAT directly recruits PLCγ1 and the adaptors Gads and Grb2 and, indirectly, other important signaling molecules such as SLP-76, Vav1, Sos, the regulatory subunit (p85) of PI3-K, and Itk tyrosine kinase (Lin *et al.*, 1999; Wange, 2000; Zhang *et al.*, 1999a, 2000). Thus, activated LAT serves as an essential scaffold for the assembly of TCR-coupled signaling complexes that mediate productive T cell activation. However, it is not entirely clear how LAT is recruited to the vicinity of the TCR–CD3 complex and TCR-ζ-associated ZAP-70. One possibility is that the reported association of LAT with CD8 and CD4 (Bosselut *et al.*, 1999) provides a means for recruiting LAT to peptide/MHC-engaged TCRs.

Lat-deficient ($Lat^{-/-}$) mice show a complete block in αβ T cell development at the immature, double-negative (DN) stage, indicating that LAT is essential for pre-TCR signaling (Shen *et al.*, 2009; Zhang *et al.*, 1999b). Analysis of Jurkat T cell lines lacking LAT has revealed its requirement for TCR-mediated Ca^{2+} mobilization, activation of PLCγ1, Vav1, SLP-76, Ras, Erk, and NFAT, and CD69 upregulation (Finco *et al.*, 1998; Lin *et al.*, 1999; Samelson, 2002; Wange, 2000). Reintroduction of LAT into LAT-deficient Jurkat T cells rescued all these defects, indicating LAT is an indispensable adaptor protein that links the TCR and coreceptors to multiple intracellular signaling cascades to promote competent TCR complexation and allow T cell activation. High resolution TIRF microscopy demonstrated that upon TCR stimulation, the majority of LAT formed MCs at the IS (Bunnell *et al.*, 2001; Campi *et al.*, 2005; Seminario and Bunnell, 2008; Yokosuka and Saito, 2010; Yokosuka *et al.*, 2005) in a lipid

raft-independent manner (Douglass and Vale, 2005; Hashimoto-Tane *et al.*, 2010), yet a fraction of LAT resided in mobile, intracellular vesicles beneath the IS (Balagopalan *et al.*, 2009; Purbhoo *et al.*, 2010). These LAT-containing subsynaptic vesicles come in contact with SLP-76 MCs, coinciding with the phosphorylation of LAT on key tyrosines that mediate a new interaction with Gads. Together, LAT and SLP-76 bring PLCγ1 to the PM, where it is phosphorylated and activated. Activation of PLCγ1 elevates several second messengers, including intracellular Ca^{2+} mobilization, which activates NFAT and, consequently, various cytokines that contribute to T cell activation and inflammation.

LAT contains two cytoplasmic Cys residues adjacent to its TM domain, Cis-26 and -29, which are palmitoylated and, hence, target it to lipid rafts (Zhang *et al.*, 1998b). Cys-26 is more critical for this targeting (Zhang *et al.*, 1998b). Although it is clear that LAT palmitoylation is required for lipid raft localization (Lin *et al.*, 1999; Zhang *et al.*, 1998b), the role and importance of LAT palmitoylation in T cell development and activation has been a matter of some controversy. Early studies demonstrated that mutation of these two Cys residues abolished T cell activation (Lin *et al.*, 1999; Zhang *et al.*, 1998b). However, a later study showed that a chimeric protein consisting of the extracellular and TM domains of LAX, a nonpalmitoylated (non-raft-residing) LAT-related TRAP, fused to the cytoplasmic domain of LAT was expressed as a PM integral protein and restored T cell development and activation in *Lat*$^{-/-}$ mice (Zhu *et al.*, 2005), leading to the conclusion that the lipid raft localization of LAT is not essential for its function. The potential resolution of this apparent contradiction comes from studies demonstrating that the primary role of LAT palmitoylation is to induce its sorting from the Golgi compartment to the PM (Hundt *et al.*, 2009; Tanimura *et al.*, 2006). Once it translocates to the PM, its raft localization would be favored because of its palmitoylation. Indeed, recent studies demonstrated that T cells express two pools of LAT localized in the PM and the Golgi compartment and, further, that mutation of Cys-26 and -29 leads to exclusive intracellular (Golgi) localization of LAT (Bonello *et al.*, 2004; Hundt *et al.*, 2009; Tanimura *et al.*, 2003, 2006). Thus, it appears that the TM domain of LAT does not contain sufficient PM-targeting signals. In contrast, the TM domain of nonpalmitoylated LAX apparently contains all the necessary information for PM sorting, which makes it possible for the LAX–LAT chimera to restore T cell development and activation. We extended this finding by demonstrating that even targeting of the LAT cytoplasmic domain to the PM as a peripheral protein by fusing it to the N-terminal membrane-targeting sequence of Src kinase (which is nonpalmitoylated) allowed it restore T cell development and activation on a *Lat*$^{-/-}$ background (Hundt *et al.*, 2009). Therefore, targeting LAT to the PM is sufficient for its function, regardless of specific localization in lipid rafts and, in this context, palmitoylation is only responsible for PM sorting.

However, proper localization and function of LAT also depends on protein–protein interactions mediated by its phosphorylated cytoplasmic domain as evidenced by findings that LAT variants with a tyrosine-mutated cytoplasmic tail are not recruited into signaling clusters (Bonello *et al.*, 2004; Douglass and Vale, 2005).

4.1.4.2. PAG/Cbp, NTAL/LAB, and LIME Protein associated with GEMs (PAG; Brdicka *et al.*, 2000), also known as C-terminal Src kinase-binding protein (Cbp; Kawabuchi *et al.*, 2000) is ubiquitously expressed. It contains a juxtamembrane dicysteine motif, which is palmitoylated and leads to localization of the protein in membrane lipid rafts. PAG/Cbp is constitutively phosphorylated in resting T cells on tyrosine residues in its cytoplasmic domain. As a result, phospho-PAG/Cbp binds the C-terminal Src kinase kinase (Csk) and recruits it to lipid rafts, where it is found in the vicinity of Src-family kinases (Lck and Fyn) and keeps them at a relatively inactive basal state by phosphorylating the C-terminal autoinhibitory tyrosine residues of these kinases. Upon TCR stimulation, PAG/Cbp is rapidly dephosphorylated, causing it to be released from Csk and undergo cytoplasmic localization. Since PAG/Cbp can be phosphorylated by Fyn and, in turn, can negatively regulate Fyn via its association with Csk, this PAG/Cbp-Csk-Fyn system represents a model of negative feedback loop. In addition to this function of PAG/Cbp, it may also regulate crosstalk between lipid rafts and the actin cytoskeleton via binding of its C-terminus to the PDZ domain of the cytoplasmic adaptor protein, ezrin-radixin-moesin (ERM)-binding protein-50 (EBP50), which binds the actin-associated ERM proteins. A nonpalmitoylated PAG/Cbp mutant, which localized in the PM but not in lipid rafts, was still tyrosine phosphorylated and associated with Csk, Fyn, and EBP50 but, nevertheless, unlike wild-type PAG, it did not block proximal TCR signaling (Posevitz-Fejfar *et al.*, 2008).

Given that LAT is not expressed in B cells, a search for a LAT-like adaptor that may couple the antigen-specific B cell receptor (BCR) to downstream signaling pathways led to the discovery of non-T cell activation linker (NTAL; Brdicka *et al.*, 2002), also known as linker for activation of B cells (LAB; Janssen *et al.*, 2003), which, as implied by its name, is mainly expressed in non-T hematopoietic cells. Similar to LAT, NTAL/LAB is palmitoylated on a juxtamembrane dicysteine motif and is phosphorylated on tyrosine in response to BCR or Fc receptor ligation. Although NTAL/LAB can partially reconstitute some missing TCR signaling functions in *Lat*-deficient T cells, it is most likely not the functional equivalent of LAT in B cells since, unlike LAT, tyrosine-phosphorylated NTAL/LAB does not recruit PLCγ1 and, therefore, is not coupled to the Ca^{2+} signaling pathway.

Lck-interacting membrane protein (LIME; Brdickova *et al.*, 2003; Hur *et al.*, 2003) is expressed in T cells, but unlike other TRAPs that are

phosphorylated on tyrosine by immunoreceptor stimulation, LIME is phosphorylated only after antibody-mediated ligation of the CD4 or CD8 coreceptors, or HIV gp120 binding to CD4. When phosphorylated by Src-family kinases, LIME binds Lck, Fyn, and Csk via their SH2 domains. However, relatively little is known about the biological significance of these associations and, more generally, about the physiological functions of this adaptor protein in T cells. One possibility is that phosphorylated LIME binds the SH2 domain of Src-family kinases, thereby preventing the intramolecular association between the SH2 domain and the Csk-phosphorylated autoinhibitory C-terminal tyrosine residue of these kinases. In this scenario, Src-family kinases will be retained in the ''open'' active conformation in the LIME–Lck–Csk complex, consistent with the finding that, paradoxically, the LIME-associated fraction of Lck is more active than the total Lck pool despite being phosphorylated on the C-terminal autoinhibitory tyrosine residue (Brdickova *et al.*, 2003). A more recent study implicated LIME, which is also expressed in splenic B cells, as the functional LAT homologue in BCR signaling, based on findings that phosphorylated LIME recruited similar signaling proteins to those recruited by LAT in T cells, and that siRNA-mediated reduction of LIME expression inhibited BCR-mediated activation of MAPKs, Ca^{2+} flux, PI3-K, NFAT, and NF-κB (Ahn *et al.*, 2006). The impact of mutating the palmitoylated Cys residues in LIME, as well as in NTAL/LAB, on their cellular localization and function has not been studied in detail.

4.2. Alterations in T cell protein palmitoylation and functional consequences

As reviewed elsewhere in this chapter, mutation of palmitoylated Cys residues has often been used to analyze the importance of protein palmitoylation in T (and other) cell signaling. However, pharmacological intervention with protein palmitoylation has also been employed in similar studies, and further, alterations in the palmitoylation of signaling proteins have been reported to occur in T cells from diseased individuals.

Webb *et al.* (2000) screened a number of palmitate analogs for their ability to inhibit the palmitoylation of Fyn, and identified 2-bromopalmitate (2BP) as a palmitoylation inhibitor. However, 2BP also inhibited the myristoylation of Fyn. In Jurkat T cells, 2BP blocked constitutive localization of the endogenous palmitoylated proteins Fyn, Lck, and LAT to isolated detergent-resistant membranes (DRMs), generally considered to represent the biochemical equivalent of lipid rafts. This resulted in impaired TCR signaling as evidenced by reductions in tyrosine phosphorylation, Ca^{2+} release, and activation of the MAPK, Erk1 (Webb *et al.*, 2000). Despite the long-held knowledge that 2BP nonspecifically alkyates numerous membrane proteins (Coleman, 1992), it has found widespread

use as a pan-PAT inhibitor in different cellular systems. Recent high throughput cell-based screening assays have identified new classes of PAT inhibitors (Ducker, 2006). Independent validation verified one of these compounds as a moderate affinity ($IC_{50} > 10$ μM) noncovalent inhibitor of multiple PAT enzymes (Jennings, 2009). Another palmitate analog, 13-oxypalmitic acid was also found to inhibit the palmitoylation of Lck and displace it from lipid rafts and from the GPI-anchored protein, CD48, in T cells without affecting its PM localization. These changes were associated with reduced TCR-induced tyrosine phosphorylation and MAPK activation (Hawash *et al.*, 2002).

Webb *et al.* also evaluated the ability of long chain polyunsaturated fatty acids (PUFAs), that is, arachidonic and eicosapentanoic acids, to inhibit protein palmitoylation, and reported that these PUFAs displaced Fyn from DRMs in non-T cells by inhibiting its palmitoylation (Webb *et al.*, 2000). Stulnig *et al.* (1998) later extended a similar analysis of the biological effects of PUFA treatment to T cells, based on the fact that PUFAs exert an immunosuppressive effect involving, among others, inhibition of T cell activation. They found that culturing Jurkat T cells in PUFA-supplemented medium led to the displacement of Lck and Fyn from the DRM fraction, which was associated with impaired anti-CD3-induced Ca^{2+} mobilization. The same treatment also displaced LAT from T cell lipid rafts, resulting in impaired TCR-stimulated tyrosine phosphorylation of LAT and one of its effectors, PLCγ1 (Zeyda *et al.*, 2002). The same group reported in a more recent study that PUFA treatment also inhibited the formation of a mature IS in human T cells stimulated with superantigen (Staphilococcal enterotoxin E)-pulsed APCs, as evidenced by impaired recruitment of some (F-actin, talin, LFA-1, and CD3) but not other (PKCθ) proteins to the IS (Geyeregger *et al.*, 2005). This treatment also inhibited the SEE-stimulated tyrosine phosphorylation of Vav1 (a hematopoietic cell-specific GEF for Rho GTPases) and the upregulation of CD69 expression, a T cell activation marker. Glucocorticoids were also reported to displace two palmitoyl proteins, LAT and PAG/Cbp from T cell lipid rafts and to decrease the amount of palmitic acid and other saturated fatty acids in lipids isolated from the lipid raft fraction (Van Laethem *et al.*, 2003), Hence, reduction in the palmitoylation of T cell signaling proteins may represent an additional mechanism underlying the well-established immunosuppressive effect of glucocorticoids on T cell activation and immune responses in general.

The T lymphocytes that reside in the synovium of the inflamed joints in patients with RA display severe hyporesponsiveness upon antigenic stimulation, which is probably due to their constant subjection to high levels of oxidative stress. Synovial fluid T lymphocytes from RA patients were found to display severely impaired phosphorylation of LAT, which was associated with its displacement from the PM (and, presumably,

from lipid rafts). The membrane anchorage of LAT, and consequently the phosphorylation of LAT and the TCR-induced activation of synovial fluid T lymphocytes, was restored after supplementation of the intracellular glutathione levels with the antioxidant, *N*-acetyl-cysteine (Gringhuis *et al.*, 2000). Further, mechanistic and biochemical analysis of the effect of alterations in the T cell redox potential on LAT localization and function revealed that LAT is extremely sensitive to intracellular redox balance alterations, and that cellular glutathione depletion *in vitro* displaced LAT from the PM, altered its conformation, and inhibited T cell signaling (Gringhuis *et al.*, 2002). Mutation of redox-sensitive Cys residues within LAT resulted in LAT mutants, which remained membrane-anchored and restored TCR-mediated signal transduction under conditions of chronic oxidative stress. The palmitoylation status of these LAT mutants and their lipid raft localization were not analyzed in this study, but it is tempting to speculate that oxidative stress may interfere with S-acylation, resulting in impaired signal transduction that depends on intact palmitoylation of various signaling proteins.

In summary, several experimental manipulations were found to displace palmitoylated T cell signaling proteins from lipid rafts and/or the PM, resulting in impaired T cell activation. In some, but not all, cases, these treatments reduced the palmitoylation of the signaling proteins, but it is clear that they also altered the overall lipid composition of the rafts. Hence, these experimental treatments are definitely not selective for S-acylation. These studies thus point out the limitations of 2BP and other nonspecific electrophiles as selective probes for inhibition of PAT activity and highlight the pressing need for more selective inhibitors of PAT enzymes.

4.3. Defective LAT palmitoylation in anergic T cells: A role for DHHC proteins?

In 2006, we discovered that anergic T cells display a selective defect in the palmitoylation of LAT (Hundt *et al.*, 2006). Specifically, TCR signaling events downstream of LAT, that is, PLCγ1 phosphorylation and PI3-K recruitment to CD28, were impaired in anergic T cells, whereas upstream events (TCR-ζ and ZAP-70 phosphorylation) remained intact. LAT recruitment to the IS and its localization in the DRM (lipid raft) fraction were defective in anergic T cells (Hundt *et al.*, 2006), reflecting intracellular (Golgi) retention of LAT (Hundt *et al.*, 2009). These defects resulted from impaired palmitoylation of LAT, and were selective since the DRM localization and palmitoylation of another signaling protein, Fyn kinase, was intact. This LAT defect was independent of Cbl-b, which is known to be upregulated in anergic T cells and play an important role in anergy induction (Macian *et al.*, 2002), and did not reflect enhanced LAT

degradation. The impaired LAT phenotype fulfilled two conventional criteria of T cell anergy: first, it was reversed by addition of exogenous IL-2, and second, it was relatively stable in that it lasted for at least 48 h after removing the anergy-inducing stimulus. Importantly, the same LAT defects were observed in anergic T cells induced by two independent protocols, that is, intravenous injection of TCR-transgenic mice with a high dose of soluble antigenic peptide (Falb *et al.*, 1996), or treatment of *in vitro* antigen- or anti-CD3/CD28-primed TCR-transgenic T cells with ionomycin (Jenkins *et al.*, 1987; Macian *et al.*, 2002). These results identify LAT as the most upstream target of anergy induction and, moreover, suggest that changes in the amount of LAT in the IS and DRMs determined by altered palmitoylation contribute to the induction of T cell anergy. Although we do not yet know whether the selective hypopalmitoylation of LAT is the *cause* of T cell anergy, this is an intriguing possibility given the fact that downstream signaling events known to be impaired in anergic T cells such as Ras (Fields *et al.*, 1996), MAPK (Li *et al.*, 1996), NF-κB (Sundstedt *et al.*, 1996), and AP-1 (Kang *et al.*, 1992; Sundstedt and Dohlsten, 1998; Sundstedt *et al.*, 1996) activation (Fathman and Lineberry, 2007; Schwartz, 2003), are dependent on intact LAT function (Finco *et al.*, 1998; Lin *et al.*, 1999; Ouellet *et al.*, 2003).

In seeking the mechanism that underlies LAT hypopalmitoylation in anergic T cells, we considered an imbalance in the dynamic and reversible process of LAT palmitoylation/depalmitoylation. Indeed, our preliminary pulse-chase analysis revealed that the half-life of palmitate on LAT was substantially shorter than the corresponding protein half-life. Impaired LAT palmitoylation in anergic T cells could potentially result from alterations in the expression and/or regulation of PATs that palmitoylate LAT, and/or thioesterases that depalmitoylate LAT. Given the selectivity of the LAT palmitoylation defect, the ability of APT1 to depalmitoylate nonselectively many different proteins *in vitro*, and the lysosomal localization of PPT1 (where it would not be able to access *de novo* palmitoylated LAT), we focused our attention on the novel PAT family of 23 mammalian DHHC proteins. Our current collaborative efforts focus on the use of the proteomics-based approaches described above to identify LAT-palmitoylating PATs, characterize their substrate specificity, and determine whether a defect in some LAT-palmitoylating enzymes plays a causative role in T cell anergy. In a broader context, we are using the same approaches to explore alterations in the T cell palmitoyl proteome under different conditions of T cell stimulation and differentiation. Our preliminary studies using an antigen-specific T cell hybridoma as a model for anergy induction confirmed recent studies that estimated the size of the mammalian palmitoyl proteome at $\geq$300 members (Kang *et al.*, 2008; Martin and Cravatt, 2009), revealed many of the ''usual suspects'' known to be palmitoylated in T cells (e.g., CD4/CD8, Lck, Fyn, LAT, PAG/Cbp, and several small GTPases), as well as a dozen DHHC proteins, and

confirmed our earlier biochemical documentation (Hundt *et al.*, 2006) of defective LAT palmitoylation in anergic T cells (unpublished observations). Most of the candidate palmitoyl proteins revealed by our analysis to date are unvalidated and, therefore, must be viewed with caution. It is clear, however, that utilizing quantitative proteomics-based approaches provides an extremely sensitive and useful tool to explore the biology of protein palmitoylation and the DHHC family in T and other cells.

5. CONCLUDING REMARKS AND PERSPECTIVE

The dynamic process of protein palmitoylation (S-acylation) is now well established to play important roles in the function, trafficking, localization, and turnover of many proteins in different cell types. Recent studies have led to substantial progress in our understanding of the mechanisms of protein palmitoylation and, to a lesser extent, protein depalmitoylation. The recent development of sensitive and quantitative methods for the global analysis and profiling of the palmitoyl proteome and the discovery of the diverse DHHC protein family of palmitoylating enzymes open up new avenues of research, and the promise of rapid progress. Given the preponderance of palmitoyl proteins that reside in the neuronal synapse, it is not surprising that the majority of recent studies on the DHHC family and the palmitoyl proteome were conducted in neuronal cells. Nevertheless, it is clear that palmitoylation also plays a critical role in the functions of hematopoietic and immune system cells. Palmitoylation of receptors and intracellular signaling proteins, both enzymes and adaptors, is critical for proper TCR signaling, and alterations in protein palmitoylation have been associated with impaired T cell activation and, possibly, pathological T cell responses. However, the enzymatic regulation of reversible protein palmitoylation in signaling pathways initiated by the TCR and other immune recognition receptors remains essentially unexplored.

At the same time, important challenges remain in the general field of protein palmitoylation:

(a) Recent global analysis of the palmitoyl proteome has revealed that it contains several hundred mammalian proteins, many of which are unannotated. It will be essential to validate putative novel palmitoyl proteins, annotate the exact sites of palmitoylation, and elucidate the functional relevance of newly identified palmitoylations.
(b) The degree of redundancy versus substrate specificity among members of the DHHC family is not entirely resolved. Many additional studies are necessary in order to explore the molecular basis of PAT–substrate recognition and specificity. Proteomics as well as genetic (i.e., PAT knockdown or knockout), approaches will be extremely useful in this regard.

(c) Mutations in *zdhhc* genes have been shown to be associated with human diseases, and in particular, the number of *PAT* genes that are associated with cancer is remarkably high. Elucidating the relevance of DHHC proteins to diseases and the underlying disregulated signaling pathways is extremely important since it may lead to the discovery of novel drug targets. The development of selective PAT (and perhaps PPT) inhibitors is an exciting future endeavor, given the widespread disease associations and the clear phenotypes that manifest upon mutation of individual members of the DHHC family.
(d) Exploration and elucidation of extrinisic and intrinsic factors that regulate the localization and activity of PATs, and the palmitoylation cycle in general, is another important future task. In this regard, receptor signals have been shown to affect palmitate turnover on cellular proteins (El-Husseini and Bredt, 2002; El-Husseini *et al.*, 2002; Mumby *et al.*, 1994; Wedegaertner and Bourne, 1994), and adaptor proteins that associate with some PATs are required for their function (Swarthout *et al.*, 2005).
(e) Additional studies are needed in order to determine how palmitoylation of proteins protects them from ubiquitination-dependent degradation (Valdez-Taubas and Pelham, 2005), and whether this is a more general mechanism that regulates protein stability.
(f) Progress in identifying and characterizing PPTs has been slow relative to DHHC proteins. Although a few PPTs have been discovered, their substrate specificity, biological relevance, and whether they depalmitoylate proteins in cells are open questions. Additional PPTs likely remain to be discovered.

All of these open questions also apply to the cells of the immune system, including T lymphocytes. For example, it will be interesting to determine whether reversible protein palitoylation regulates the development of T cells, their differentiation into distinct functional subsets, and their effector activities. With the new tools available, we can now begin to analyze the palmitoyl proteome, its enzymatic regulation, and the substrate specificity of the DHHC family in T and other hematopoietic cells at a level of sophistication that until very recently was not possible. These new possibilities bring the promise of exciting and novel new discoveries in the basic scientific, and potentially clinical, arenas of studies on the immune system.

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CHAPTER 2

Transcriptional Control of Natural Killer Cell Development and Function

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Abstract

Natural killer (NK) cells play an important role in host defense against tumors and viruses and other infectious diseases. NK cell development is regulated by mechanisms that are both shared with and separate from other hematopoietic cell lineages. Functionally, NK cells use activating and inhibitory receptors to recognize both healthy and altered cells such as transformed or infected cells. Upon activation, NK cells produce cytokines and cytotoxic granules using mechanisms similar to other hematopoietic cell lineages especially cytotoxic T cells. Here we review the transcription factors that control NK cell development and function. Although many of these transcription factors are shared with other hematopoietic cell lineages, they control unexpected and unique aspects of NK cell biology. We review the mechanisms and target genes by which these transcriptional regulators control NK cell development and functional activity.

1. NATURAL KILLER CELLS

Natural killer (NK) cells are lymphocytes that function within the innate immune system to provide host protection against infection diseases and cancer. Initially discovered for their ability to kill tumors, NK cells were soon appreciated for their ability to recognize and attack virus-infected cells. NK cells play important roles in antiviral immunity and have been shown to protect both mice and humans from herpesviruses, including cytomegaloviruses (CMV), zaricella zoster virus (VZV), Epstein-Barr virus (EBV), and herpes simplex virus (HSV). Additionally, NK cells have been implicating in protecting the host from poxviruses, influenza virus, hepatitis viruses, and HIV-1 (reviewed in Lanier, 2008a; Lee and Biron, 2010). In addition to their role in host defense, NK cells can reject allogeneic hematopoietic stem cell transplants and are involved in regulating pregnancy, autoimmunity, and inflammation, as well as shaping the nature of the adaptive immune response.

NK cells recognize pathogen-infected and transformed cells through a sophisticated array of activating and inhibitory receptors that regulate their functional responses (reviewed in Lanier, 2008b). The activating NK receptors can recognize ligands encoded by microbial pathogens, host-encoded ligands that are induced by cellular stress, or self-ligands that are constitutively expressed on normal, healthy cells. NK cells are restrained from attacking normal cells expressing these self-ligands by their inhibitory receptors, many of which bind to self-major histocompatibility complex (MHC) class I proteins, but also other self-cell surface glycoproteins and extracellular matrix proteins (Kumar and McNerney, 2005). Thus, if MHC class I is absent on the host cells, as a consequence of infection or transformation, NK cells can respond due to interactions between the

unrestrained activating receptors productively signaling when binding to self-ligands on the MHC class I-deficient cell, a phenomenon referred to as ''missing-self'' recognition (Karre *et al.*, 1986).

Humans and mice have evolved numerous activating and inhibiting receptors that are shared between species, such as NKG2D (CD314), 2B4 (CD244), CD94, and CD16. A subset of these shared receptors is encoded by genes located within the natural killer complex (NKC) on chromosome 6 (Lanier, 2005). In rodents, a set of inhibitory and activating receptors known as the *Klra (Ly49)* gene family is located within the NKC. In primates, a structurally unrelated set of inhibitory and activating receptors known as the killer cell immunoglobulin-like receptor (KIR) family is located not within the NKC but on chromosome 19 (Lanier, 2005). An in-depth discussion of the *cis*-acting elements controlling NK cell receptor gene expression is beyond the scope of this review, and we direct the reader to the following reviews (Anderson, 2006; Veinotte *et al.*, 2003).

The *Klra (Ly49)* and *KIR* genes share an unusual expression pattern whereby an individual NK cell expresses a subset of possible receptors. Both *Klra* and *KIR* genes contain promoters that are bidirectional that have been proposed to be responsible for regulating this receptor expression pattern (Anderson, 2006; Davies *et al.*, 2007; Saleh *et al.*, 2004). The reverse promoter activity correlates with no gene expression, whereas the forward promoter activity correlates with receptor gene expression. Competition between different sites for transcription factor binding is thought to be responsible for initiating the pattern of receptor expression found on each individual NK cell (Anderson, 2006; Davies *et al.*, 2007; Saleh *et al.*, 2004).

The silencing of specific *KIR* genes is well correlated with methylation of CpG dinucleotides within their promoters (Chan *et al.*, 2003, 2005; Gomez-Lozano *et al.*, 2007; Santourlidis *et al.*, 2002; Trompeter *et al.*, 2005). Silenced *KIR* genes can be reactivated and expressed by NK cells through treatment with the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine (Chan *et al.*, 2003, 2005; Gomez-Lozano *et al.*, 2007; Santourlidis *et al.*, 2002; Trompeter *et al.*, 2005). Although KIR expression is reactivated with this treatment, little change in histone modification occurs (Chan *et al.*, 2005). Likewise, treatment of NK cells with chemical inhibitors of histone deacetylases does not activate silenced *KIR* genes (Santourlidis *et al.*, 2002; Trompeter *et al.*, 2005). Collectively, these data indicate that stochastic KIR receptor gene expression is regulated by DNA methylation and not through histone modifications.

The intracellular integration of positive and negative signals regulates the immune responsiveness of NK cells. The activating and inhibitory receptors expressed by human NK cells and their signaling functions are comprehensively described in recent reviews (Lanier, 2005, 2008b).

When stimulated either through engagement of their activating receptors by cell surface ligands on target cells or by their cytokine receptors,

NK cells rapidly produce cytokines and chemokines, including IFNγ, TNFα, GM-CSF, Rantes, MIP1α, MIP1β, IL-10, and others. Mature NK cells constitutively express receptors for type I interferons, IL-2, IL-12, IL-15, and IL-18, which can induce their proliferation and cytokine production and augment their lytic activity. Recognition of target cells via activating receptors expressed on NK cells results in target cell lysis, primarily through the delivery of cytotoxic granules containing perforin and granzymes (Lieberman, 2010). Alternative killing mechanisms used by NK cells are dependent on TNF, Fas ligand (FasL), and Trail (Lieberman, 2010).

Although NK cells, like B cells and T cells, arise from a common lymphoid progenitor (Kondo *et al*., 1997), NK cell receptors are germline-encoded and not recombined like the antigen receptor gene loci encoding for the T cell receptor (Lanier *et al*., 1986; Tutt *et al*., 1987) and B cell receptor. Consequently, deficiencies in recombination-activating gene (RAG)-1 or RAG-2 do not intrinsically affect NK cells (Mombaerts *et al*., 1992; Shinkai *et al*., 1992), and NK cells are present in normal numbers in Scid mice (Dorshkind *et al*., 1985), which are deficient in B cells and T cells.

2. NK CELL DEVELOPMENT

NK cells are bone marrow-derived and share a common lymphoid progenitor with T and B cells (Kondo *et al*., 1997). Fetal thymus and liver contain bipotent T/NK cell progenitors that have the potential to develop into NK cells (Carlyle *et al*., 1997; Douagi *et al*., 2002; Ikawa *et al*., 1999; Sanchez *et al*., 1994; Spits *et al*., 1998). Bone marrow ablation, however, leads to NK cell dysfunction, whereas the absence of the spleen or thymus through disease or removal does not result in reduced numbers or impaired NK cells, suggesting that the bone marrow may be the primary site of NK cell development (Herberman *et al*., 1975; Kiessling *et al*., 1975; Kumar *et al*., 1979; Ramos *et al*., 1996; Schwarz and Hiserodt, 1990; Seaman *et al*., 1978; Sihvola and Hurme, 1984; Sirianni *et al*., 1983). Requirements for fetal and adult NK cell development may differ, as has been shown for B cell development (Hardy and Hayakawa, 2001). A small population of NK cells in adult mice has been identified that develops within the thymus and may have functional differences compared with conventional bone marrow-derived NK cells (Vosshenrich *et al*., 2006).

In vitro differentiation of NK cells from bone marrow progenitor cells requires the cytokines and growth factors: stem cell factor (SCF), Flt3 ligand, IL-15, and IL-7 (Mrozek *et al*., 1996; Williams *et al*., 1997). C-kit (CD117) and Flt3 (CD135) are expressed on developing NK cells and are receptors for SCF and Flt3 ligand, respectively. Bone marrow and splenic NK cells are reduced and/or defective in mice lacking these receptors,

demonstrating their requirement for proper NK cell development (Colucci and Di Santo, 2000; McKenna *et al.*, 2000).

NK cell development is critically dependent upon IL-15. The IL-15 receptor comprises of three components, the unique IL-15Rα chain, the IL-2R/IL-15R β chain (CD122), and the common γ chain (CD132), which is shared with a variety of other cytokine receptors. IL-15 works in a unique manner whereby IL-15 is transpresented by the IL-15Rα chain, which is expressed on the surface of dendritic cells and macrophages (Mortier *et al.*, 2009), to responsive cells expressing CD132 and CD122 (Dubois *et al.*, 2002; Koka *et al.*, 2003). Mice lacking IL-15 or any component of its receptor have profound reductions in peripheral NK cell numbers and a block in bone marrow NK cell differentiation (DiSanto *et al.*, 1994; Gilmour *et al.*, 2001; Kennedy *et al.*, 2000; Lodolce *et al.*, 1998; Suzuki *et al.*, 1997; Vosshenrich *et al.*, 2005). IL-15 is rate-limiting for the development of mature NK cells because the absolute number of NK cells is reduced by roughly half in mice that are heterozygous for a null allele of IL-15, and transgenic overexpression of IL-15 in mice results in a dramatic increase in the number of NK cells generated (Fehniger *et al.*, 2001). Signal transducers and activators of transcription (Stat) 5a and Stat5b are crucial mediators of IL-15 receptor signaling and are phosphorylated upon IL-15 receptor engagement, allowing for translocation into the nucleus and activation of target genes. Similar to defects in IL-15 responsiveness, deletion of genes encoding Stat5b or both Stat5a and Stat5b results in defective and lowered levels NK cells or a lack of NK cells, respectively (Imada *et al.*, 1998; Moriggl *et al.*, 1999).

Contrary to the requirement for *in vitro* differentiation cultures, IL-7 and its specific receptor component, the IL-7Rα chain (CD127), are not required for *in vivo* development and function of NK cells derived from bone marrow progenitors. Mice or humans lacking these molecules have normal numbers of functional NK cells (He and Malek, 1996; Moore *et al.*, 1996; Puel *et al.*, 1998; Vosshenrich *et al.*, 2006). However, the small subset of NK cells that develop from the thymus are missing in IL-7Rα-deficient mice and thus dependent on IL-7 (Vosshenrich *et al.*, 2006).

In addition to cytokines and growth factors, bone marrow stromal cells are necessary for acquisition of recognition receptors and the complete differentiation of NK cells derived from *in vitro* culture systems (Roth *et al.*, 2000, 2007; Williams *et al.*, 1999, 2000). Thus, receptor–ligand interactions between molecules expressed on NK cells and stromal cells are crucial for normal NK development and function. NK cells and bone marrow stromal cells express the Tyro3 receptor family and their ligands, respectively (Caraux *et al.*, 2006). Deletion of all three Tyro3 receptor family members (Axl, Tyro3, and Mer) resulted in altered NK cell development, altered expression of NK cell recognition receptors, defective cytokine production, and impaired target cell cytotoxicity (Caraux *et al.*,

2006). Membrane-bound lymphotoxin (LTαβ) expressed by developing NK cells is thought to interact with stromal cells expressing lymphotoxin β receptor (LTβR), thereby enhancing IL-15 production and explaining the NK cell development defects found in mice deficient for LTαβ or LTβR (Iizuka *et al.*, 1999; Ware, 2005; Wu *et al.*, 2001).

A scheme for differentiation stages of developing bone marrow NK cells has been refined based on cell surface markers (Kim *et al.*, 2002; Rosmaraki *et al.*, 2001; Williams *et al.*, 2000). NK cell precursors (NKPs) are CD122+NK1.1− CD49b (DX5)−, as well as being lineage negative (CD3−, CD19−, CD4−, CD8−, Gr-1−, CD11b−), and are limited to cells differentiating into NK cells (Rosmaraki *et al.*, 2001). Acquisition of NK1.1 (in C57BL/6 mice), as well as CD94 and the NKG2 receptors, marks the beginning of the immature NK cell (iNK) stage (Kim *et al.*, 2002; Rosmaraki *et al.*, 2001). Expression of the Ly49 receptors begins during this stage (Kim *et al.*, 2002; Rosmaraki *et al.*, 2001; Williams *et al.*, 2000). CD51 and TRAIL expressions are upregulated at this stage and then downregulated upon further maturation into mature NK cells when CD49b (DX5) is expressed (Di Santo, 2006; Kim *et al.*, 2002). The ability to kill target cells and produce cytokine is acquired at this stage. CD11b (Mac-1) is also acquired at this stage, but effector functions do not necessarily rely on its expression (Di Santo, 2006). A four-stage model for the development of human NK cells has also been proposed based on *in vitro* and *in vivo* studies (Freud *et al.*, 2006). Similar fractionation has proven enormously useful in studying B and T cell development, and the study of NK cell development has and will continue to benefit from these findings. In the following section, we review the transcriptional factors that control development of the NK cell lineage. We also suggest several reviews to the reader that expand on various topics we have discussed in this section (Colucci *et al.*, 2003; Di Santo, 2006; Spits *et al.*, 1998; Yokoyama *et al.*, 2004).

Although NKp46 was initially considered NK cell-restricted in its expression, a unique NKp46+ CD3− population of cells has been identified in the gut that share numerous cell surface markers with NK cells, but are not within the NK cell developmental lineage (Luci *et al.*, 2009; Sanos *et al.*, 2009; Satoh-Takayama *et al.*, 2008). This population of cells expresses NKp46, c-kit, LTβ, LTα, 2B4, and low levels of CD122, NK1.1 (in some cases none), and NKG2D (Luci *et al.*, 2009; Sanos *et al.*, 2009; Satoh-Takayama *et al.*, 2008, 2010). This population develops in the absence of the thymus and RAG-specific recombination (Luci *et al.*, 2009; Sanos *et al.*, 2009; Satoh-Takayama *et al.*, 2008). Unlike NK cells, these cells do not require IL-15 but do require IL-7, commensal flora, and the transcription factor RORγt for development (Luci *et al.*, 2009; Sanos *et al.*, 2009; Satoh-Takayama *et al.*, 2008). These cells are characterized by the production of IL-22, which NK cells do not secrete, and usually do not produce IFNγ upon stimulation or kill conventional NK-sensitive targets like YAC-1

due to lack of perforin, TRAIL, and FasL expression (Luci *et al.*, 2009; Sanos *et al.*, 2009; Satoh-Takayama *et al.*, 2008). RORγt is also required for lymphoid tissue inducer (LTi) cell and lymph node and Peyer's patch development (Eberl and Littman, 2003; Eberl *et al.*, 2004; Kurebayashi *et al.*, 2000; Sun *et al.*, 2000), and these RORγt+NKp46+CD3− gut cells share locations with LTi cells (Luci *et al.*, 2009; Sanos *et al.*, 2009). The requirements for lymphotoxin in NK cell development is very similar to the mechanism by which LTi interacts with the stroma to induce secondary lymphoid organ formation (Iizuka *et al.*, 1999; Ware, 2005; Wu *et al.*, 2001). Thus, it is tempting to speculate that NK cells are connected with these innate gut cells through a common progenitor.

3. TRANSACTING FACTORS IN NK CELL DEVELOPMENT

3.1. Ikaros

Ikaros is the founding member of the Ikaros transcription factor family (Georgopoulos *et al.*, 1992; Morgan *et al.*, 1997; Nichogiannopoulou *et al.*, 1998). Ikaros is expressed in hematopoietic stem cells, common lymphoid progenitors, developing B and T cells, and mature NK, B, and T cells (Georgopoulos *et al.*, 1992; Morgan *et al.*, 1997; Payne *et al.*, 2003). The Ikaros gene (*Ikzf1*) encodes a variety of protein isoforms generated through alternative splicing that all share a common activation domain and C-terminal zinc finger dimerization domain, although only a subset of the isoforms contain an N-terminal zinc finger DNA-binding domain (Nichogiannopoulou *et al.*, 1998). Deletion of the Ikaros activation and dimerization domains results in mice null for Ikaros (Wang *et al.*, 1996a). These mice lack fetal T cells, B cells, and NK cells and are defective for adult T cells, γδ T cells, and dendritic cells due in part to the inability of hematopoietic stem cells to differentiate into lymphoid progenitors (Allman *et al.*, 2003; Nichogiannopoulou *et al.*, 1999; Wang *et al.*, 1996a; Yoshida *et al.*, 2006). NK cells are undetectable in the spleen (Wang *et al.*, 1996a), and Ikaros-deficient fetal livers were incapable of giving rise to functional NK cells when differentiated *in vitro* (Boggs *et al.*, 1998). Ikaros-deficient lymphoid progenitors do not express Flt3 and express reduced levels of c-kit; Ikaros may control the expression of these genes (Nichogiannopoulou *et al.*, 1999; Yoshida *et al.*, 2006). The altered expression of these receptors most likely contributes to the NK cell defect in these mice as these two receptors are important for NK cell development (Colucci and Di Santo, 2000; McKenna *et al.*, 2000; Wang *et al.*, 1996a).

Different domains of the full-length Ikaros protein repress and activate gene expression through chromatin remodeling and localization to heterochromatin (Cortes *et al.*, 1999). The Ikaros isoforms lacking the

DNA-binding domain can act in a dominant-negative fashion by sequestering full-length Ikaros and other family members through heterodimerization, thus preventing them from binding DNA and activating transcription (Cortes *et al.*, 1999; Nichogiannopoulou *et al.*, 1998). The generation of mice lacking the DNA-binding domain of Ikaros resulted in mice expressing the dominant-negative form of Ikaros (Ikaros DN; Georgopoulos *et al.*, 1994). The phenotype of these mice is more severe than the Ikaros-null mice in that most mice died within 3 weeks of birth (the null mice live until adulthood and can breed) and were completely deficient for T cells, γδ T cells, and dendritic cells, in addition to NK cells and B cells (Georgopoulos *et al.*, 1994; Wang *et al.*, 1996a). This increased severity is presumably due to the ability of dominant-negative Ikaros to bind to other Ikaros family members and prevent them from functioning. It remains to be tested whether these other family members play a role in NK cell development or function.

3.2. Ets-family transcription factors: Ets-1, PU.1, and Mef

Multiple members of the Ets-family transcription factors have been shown to play a role in NK cell development, namely Ets-1, PU.1, and MEF (see Fig. 2.1; Barton *et al.*, 1998; Colucci *et al.*, 2001; Lacorazza *et al.*, 2002). These winged helix-turn-helix transcription factors have been shown to control a variety of biological processes including cellular activation, differentiation, and oncogenesis. Ets-family factors are found in a variety of cell types, usually with overlapping expression with other family members. These factors share a common Ets domain that allows for monomeric DNA-binding to a conserved GGAA central DNA motif.

Ets-1 is a proto-oncogene that is expressed in a variety of tissues including lymphoid cells. Deletion of Ets-1 results in defects in B and T cell differentiation, as well as increased peripheral T cell apoptosis and terminal B cell differentiation (Barton *et al.*, 1998; Bories *et al.*, 1995; Clements *et al.*, 2006; Eyquem *et al.*, 2004a,b; Moisan *et al.*, 2007; Muthusamy *et al.*, 1995; Wang *et al.*, 2005). Of note, the NK cell lineage is the most affected hematopoietic cell type in the Ets-1-deficient mouse as splenic and lymph node NK cell numbers are either low or undetectable (Barton *et al.*, 1998). Bone marrow NK cells, as defined by CD3−DX5+, are undetectable (Barton *et al.*, 1998), suggesting a block in NK cell development at the iNK cell stage or earlier. Ets-1-deficient mice cannot reject the NK cell-sensitive MHC class I-deficient tumor line RMA-S, consistent with a significant reduction in NK cell numbers (Barton *et al.*, 1998). Ets-1-deficient CD3−DX5+ cells in the spleen are incapable of killing NK cell-susceptible target lymphoma lines such as YAC-1 or RMA-S, likely due to the low numbers of NK cells represented in this population and/or defects in the ability of the residual NK cells remaining in this population

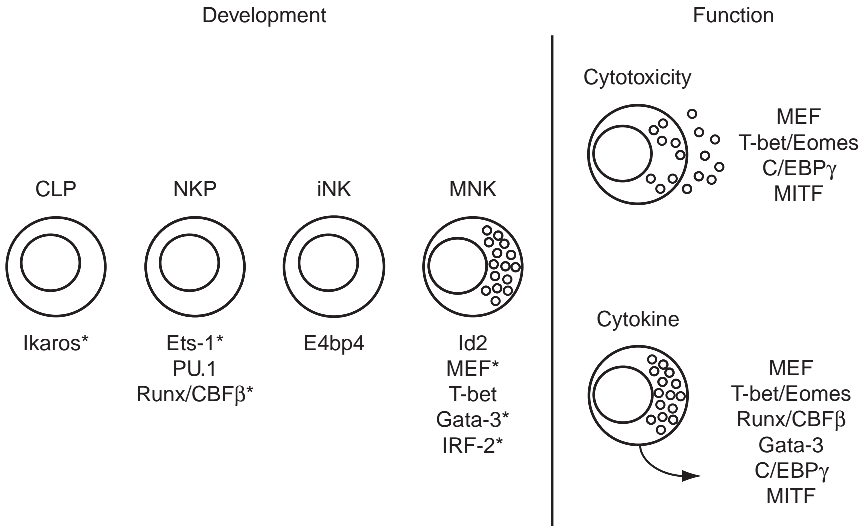

FIGURE 2.1 Requirements for transcription factors in NK cell development and function. On the left side, transcription factors are listed under the first developmental stage in which they play a role. (*) means the exact developmental stage is unclear. This does not exclude a role for each factor in subsequent stages. CLP, common lymphoid progenitor; NKP, NK cell precursors; iNK, immature NK cell; MNK, mature NK cells. On the right side, transcription factors are listed next to the function in which they play a role: cytotoxicity or cytokine production.

to mediate cytolytic function (Barton *et al.*, 1998). Splenocytes from these deficient animals do not show any apparent defects in expression of IL-12, IL-18, or components of the IL-15 receptor (Barton *et al.*, 1998). Stimulation of Ets-1-deficient bone marrow with IL-15 or IL-2 does not rescue the ability to kill NK cell-sensitive target cells, suggesting that either downstream components of these signaling pathways are defective and/or other unrelated yet essential genes are not expressed.

The mechanism and target genes through which Ets-1 controls NK cell differentiation are unknown, but there are potential Ets-1 target candidates that might explain the severe block in NK cell development. For example, Jak3 is an essential mediator of IL-15 receptor signaling and is required for NK cell development; the *Jak3* promoter is regulated by Ets-1 (Aringer *et al.*, 2003; Park *et al.*, 1995). Downregulation of Jak3 in the Ets-1-deficient NK cell lineage would provide an explanation of the severe NK cell defect. Further study of the Ets-1-deficient mice to explore the effects of this mutation on the early developmental stages in the NK cell lineage would provide better insight into the role of Ets-1 in NK cell differentiation.

The MEF transcription factor, encoded by the *Elf4* gene, is expressed in both the myeloid and lymphoid lineages (Lacorazza and Nimer, 2003;

Mao *et al.*, 1999; Miyazaki *et al.*, 1996, 2001). The deletion of *Elf4* in mice did not affect T and B cell development; however, both NK cell and NKT cell numbers were significantly reduced, but still detectable in the periphery (Lacorazza *et al.*, 2002). The remaining peripheral MEF-deficient cells were functionally defective (see Section 4.1). Reconstitution of wild-type recipients with MEF-deficient bone marrow resulted in reduced peripheral NK cell and NKT cell numbers, demonstrating that this defect was cell intrinsic. MEF-deficient splenocytes were not defective for any components of the IL-15 receptor (Lacorazza *et al.*, 2002). Thus, it is unknown what genes are regulated by MEF to control NK cell development. Further characterization of the precise stage where the block occurs during bone marrow development, as well as the effects on the NK cell receptor repertoire, would also be helpful to understand the role of MEF in NK cell differentiation.

PU.1 is expressed in multiple hematopoietic cell types, including the myeloid lineage, B cells, dendritic cells, early developing thymocytes, and the TH2 subset of $CD4^{+}$ T cells (Carotta *et al.*, 2010; Chang *et al.*, 2005; Klemsz *et al.*, 1990; Singh *et al.*, 2007). PU.1 controls these lineages at multiple stages of differentiation. For example, PU.1 controls myeloid lineage cells, in part, by regulating the expression of c-fms (M-CSF receptor), an essential differentiation and growth factor for macrophages and osteoclasts, and the T and B cell lineages through the regulation of IL-7Rα, allowing for full lineage commitment (DeKoter *et al.*, 1998, 2002). After B cell commitment, PU.1 controls genes such as the essential transcription factor EBF (Medina *et al.*, 2004). PU.1 also functions as a tumor suppressor, as the absence or reduction of PU.1 results in the induction of acute myeloid leukemia (Metcalf *et al.*, 2006; Mueller *et al.*, 2006; Rosenbauer *et al.*, 2004). Deletion of PU.1 in mice results in severe defects in the generation of T cells, B cells, monocytes, granulocytes, and dendritic cells (Carotta *et al.*, 2010; McKercher *et al.*, 1996; Scott *et al.*, 1994). PU.1-deficient mice die prenatally or just after birth before NK cells are able to populate the periphery. To enable study of NK cell development and function, PU.1-deficient fetal liver was used to complement alymphoid RAG-2- x γc-deficient mice, allowing for the generation of adult mice (Colucci *et al.*, 2001). NK cell development is perturbed in these chimeric PU.1-deficient mice with reduced numbers of NKPs and iNK cells. The block is not absolute because reduced but detectable numbers of peripheral NK cells were detected (Colucci *et al.*, 2001). Ets-1 is upregulated in PU.1-deficient NK cells, suggesting that Ets-1 may compensate for a lack of PU.1 (Colucci *et al.*, 2001). PU.1 and Ets-1 share about 35% amino acid homology in their DNA-binding domain and have been shown to be nonfunctionally redundant in other cell types (Garrett-Sinha *et al.*, 2001). It is unknown if this lack of redundancy applies within the NK cell lineage.

Expression of the IL-15 receptor components is normal in PU.1-deficient peripheral NK cells; however, the number of NK cells in cell cycle is reduced and PU.1-deficient NK cells respond poorly to IL-2 stimulation (Colucci *et al.*, 2001). Although no direct response to IL-15 by PU.1-deficient NK cells has been measured (Colucci *et al.*, 2001), the IL-2 and IL-15 receptors share common signaling subunits and signal through shared signaling intermediates. This suggests that PU.1 might regulate the ability of NK cells to respond to cytokines and, more specifically, IL-15, explaining the developmental defect and the reduction in peripheral NK cell numbers. Additionally, expression levels of c-kit are down dramatically in PU.1-deficient NK cells (Colucci *et al.*, 2001). C-kit is an important regulator of NK cell differentiation, and the reduction or lack of c-kit expression might contribute to the defect in PU.1-deficient NK cells (Colucci and Di Santo, 2000).

Several other genes important for NK cell development are regulated by PU.1 in other hematopoietic cells (see Fig. 2.2A). Flt3 expression is reduced in PU.1-deficient progenitor cells, and the *Flt3* promoter is a

A

Target genes (columns); Transcription factors (rows)

	Flt3	c-kit	Jak3	IL-7Rα	Id2	S1P5	CD122
Ikaros	?	?					
Ets-1			?				
PU.1	?	?		?			
MEF							
E4bp4					?		
T-bet						✓	✓ ?
Eomes							✓ ?
Runx/CBFβ							✓
C/EBPγ							

B

Target genes (columns); Transcription factors (rows)

	Ly49	KIR	CD45	Dap12	IFNγ	Perforin	Granzyme B	CXCR3
Ikaros								
Ets-1	?							
PU.1	?		?	?				
MEF	?					✓		
E4bp4								
T-bet					✓ ?	✓	✓ ?	✓ ?
Eomes					✓ ?	✓	✓ ?	?
Runx/CBFβ	✓	✓			?	?	?	
C/EBPγ	?							

FIGURE 2.2 Direct control of gene expression in NK cells. The left column of each chart lists the transcriptional regulators and the top row lists target genes expressed in NK cells. (?) means regulation is unclear. (✓ ?) means regulation is highly likely and in some cases, relies partially on studies in other lineages like for T-bet and Eomes. (✓) means the gene in question is regulated by the specified transcription factor in NK cells. (A) Genes primarily involved in NK cell development. (B) Genes primarily involved in NK cell function.

direct target of PU.1 (Carotta *et al.*, 2010; DeKoter *et al.*, 1998; Medina *et al.*, 2004). Although it is unknown if Flt3 expression is reduced in the PU.1-deficient NK cell lineage, it is reasonable to postulate that a reduction or lack of expression of Flt3 in PU.1-deficient NK cells or common lymphoid progenitors is responsible for the decreased numbers of NK cells.

The IL-7Rα chain is required for the generation of the minor population of NK cells derived from the thymus but not for bone marrow-derived NK cells (He and Malek, 1996; Moore *et al.*, 1996; Puel *et al.*, 1998; Vosshenrich *et al.*, 2006). IL-7Rα is not expressed in PU.1-deficient progenitor cells, and PU.1 directly binds and positively regulates *Il7r*-specific *cis*-acting elements in progenitor B cells (DeKoter *et al.*, 2002). IL-7Rα is mostly likely regulated in a similar manner in NK cells as IL-7Rα mRNA is undetectable in peripheral NK cells (Colucci *et al.*, 2001). This suggests that thymic NK cells would be severely perturbed in PU.1-deficient mice, although this has not been examined.

PU.1 is important for B cell and T cell lineage commitment, in part, through the regulation of the *Il7r* and *Flt3* genes as discussed earlier in this section. The requirement for PU.1 is removed after commitment has occurred and differentiation has begun. For example, thymocytes shut off expression of PU.1, while IL-7Rα continues to be expressed in earlier stages of T cell development and then in peripheral T cells. Similarly, conditional deletion of PU.1 after commitment by B cell progenitors allows for fairly normal B cell maturation (Polli *et al.*, 2005). Thus, by analogy, PU.1 might play a significant role in the initiation, but not in the maintenance of NK cell lineage differentiation and gene expression.

3.3. E4bp4

E4-binding protein 4 (E4bp4), encoded by the *Nfil3* gene, contains a basic leucine zipper (bZIP) motif that binds DNA upon dimerization and is related to the PAR family of transcription factors due to similar basic and bZIP domains. E4bp4 has been implicated in controlling a variety of cellular processes including the mammalian circadian clock and IL-3-dependent suppression of apoptosis (Cowell, 2002). This transcription factor is broadly expressed in hematopoietic and nonhematopoietic cell lineages and highly expressed in NK cells, NKT cells, macrophages, and dendritic cells, but not in T cells and B cells (Gascoyne *et al.*, 2009; Kamizono *et al.*, 2009). The targeted deletion of the mouse *Nfil3* gene results in a severe block in NK cell development with peripheral NK cells being almost undetectable (Gascoyne *et al.*, 2009; Kamizono *et al.*, 2009). This defect is dose-dependent, as NK cell numbers are reduced, but detectable, in *Nfil3*+/− mice. The other lymphoid and myeloid populations are normal in number and function in E4bp4-deficient mice, and the mice appeared normal and viable suggesting that the defect is quite

specific to NK cells. *In vivo* killing of MHC class I-deficient targets is completely defective in these mice most likely due to the large reduction in NK cell numbers. The few remaining NK cells in the spleen and bone marrow are unable to kill YAC-1 target cells or produce IFNγ (Gascoyne *et al.*, 2009; Kamizono *et al.*, 2009). The block in bone marrow NK cell development in E4bp4-deficient mice occurs between the NKP and the iNK stages, coinciding with the increase of E4bp4 expression beginning in the iNK stage in wild-type mice (see Fig. 2.1). Expression of CD11b, a marker for NK cell maturation, is severely decreased on bone marrow NK cells. This NK cell differentiation defect is cell intrinsic, as differentiation was not rescued when E4bp4-deficient bone marrow was transplanted into irradiated wild-type recipients or tested in an *in vitro* culture system for NK cell development (Gascoyne *et al.*, 2009; Kamizono *et al.*, 2009). There appears to be no proliferation or apoptosis defect in E4bp4-deficient hematopoietic cells, and thus E4bp4 may be working through a different mechanism than what has been described in the IL-3 pathway (Cowell, 2002; Gascoyne *et al.*, 2009).

Overexpression of E4bp4 in bone marrow progenitor cells lacking the IL-15 receptor allowed for the development of NK cells *in vitro,* suggesting that E4bp4 is genetically downstream of IL-15 receptor signaling (Gascoyne *et al.*, 2009). In turn, the overexpression of Id2, an important transcriptional regulator of NK cell development (see Section 3.4), in E4bp4-deficient NK cells can partly rescue the developmental defect of this deletion, suggesting that Id2 is downstream of E4bp4 (Gascoyne *et al.*, 2009). It is unknown if IL-15 receptor signaling molecules or Id2 are directly controlled by E4bp4, and the target genes of E4bp4 in NK cells remain to be identified. E4bp4 is capable of both positively and negatively regulating gene expression, and thus it is unclear by which mechanisms E4bp4 controls NK cell development.

3.4. Id proteins and repression of E-box proteins

E proteins play a role in a variety of differentiation processes in animals and are essential for both B cell and T cell development (Kee, 2009). There are four E proteins in mice and humans: E12 and E47, which are derived from alternative splicing of the *Tcf3* gene (E2A), and HEB and E2-2. E12 and E47 are the dominant contributors to B and T cell commitment and differentiation, whereas HEB and E2-2 appear to play a more limited role (Bain *et al.*, 1994; Kee, 2009; Zhuang *et al.*, 1994, 1996). These proteins are structurally similar, containing a basic DNA-binding region adjacent to a helix-loop-helix (HLH) dimerization domain, and form homodimers or heterodimers to activate target genes. E proteins can also heterodimerize with any of the four members of the inhibitor of DNA-binding (Id) protein family, Id1-Id4. Id proteins contain an HLH dimerization domain, but

lack the basic DNA-binding domain (Benezra *et al.*, 1990; Kee, 2009). Thus, Id proteins can bind E proteins and prevent them from binding to DNA. The expression of E and Id proteins allows for the balanced control of differentiation programs. The overexpression of an *Id1* transgene in the B cell lineage perturbs B cell development within the mouse and results in a B cell phenotype similar to mice lacking *Tcf3* (E2A; Bain *et al.*, 1994; Sun, 1994; Zhuang *et al.*, 1994). Similarly, overexpression of either Id2 or Id3 inhibits the generation of T cells in an *in vitro* fetal thymic organ culture (FTOC; Heemskerk *et al.*, 1997; Schotte *et al.*, 2010). Conversely, Id proteins promote the development of NK cells. Id2 and Id3 are expressed in NKPs, whereas Id2 is the predominant Id protein expressed in mature NK cells (Boos *et al.*, 2007; Ikawa *et al.*, 2001; Schotte *et al.*, 2010; Yokota *et al.*, 1999). The deletion of Id2 causes a block in adult bone marrow, defective NK cell differentiation using fetal thymus, and a drastic reduction of peripheral NK cells (Boos *et al.*, 2007; Ikawa *et al.*, 2001; Yokota *et al.*, 1999). Similarly, the overexpression of Id2 or Id3 promotes the differentiation of NK cells from progenitor cells in human FTOC (Heemskerk *et al.*, 1997; Schotte *et al.*, 2010). The block in bone marrow NK cell development in Id2-deficient mice occurs after the NKP and iNK stages and before the mature NK cell stage (Boos *et al.*, 2007). In mice, the *in vitro* differentiation of NK cells from Id2-deficient bone marrow cells in the presence of IL-15 was severely defective, whereas synergy was noted between IL-15 and Id2 when human NK cells were generated *in vitro* by overexpression of Id2 (Schotte *et al.*, 2010; Yokota *et al.*, 1999). Id2-deficient bone marrow transferred into wild-type recipients resulted in poor NK cell differentiation, demonstrating that the Id2-specific defect is cell intrinsic (Yokota *et al.*, 1999).

Splenocytes from Id2-deficient mice demonstrate a severe reduction in NK cells (Yokota *et al.*, 1999); however, T cell-dependent B cell responses are normal. The remaining mature splenic NK cells in Id2-deficient mice are defective in producing IFNγ in response to cytokine stimulation (Yokota *et al.*, 1999). These splenic NK cells are also IL-7Rα-positive, suggesting that they might arise from thymus-derived progenitors rather than bone marrow-derived NK cell progenitors cells (Boos *et al.*, 2007; Vosshenrich *et al.*, 2006). Examination of precursor and mature NK cells within the adult thymus suggests that these IL-7Rα^{+} NK cells develop independently of Id2 (Boos *et al.*, 2007).

Progenitor and mature NK cells express a variety of E proteins, although mostly at lower levels compared to progenitor T cells and B cells (Boos *et al.*, 2007; Schotte *et al.*, 2010). The overexpression of the E protein, HEB, inhibited human NK cell development, whereas the elimination of E47 and E12 in the absence of Id2 resulted in further mouse bone marrow NK cell development, suggesting that the function of the Id proteins in NK cell differentiation is to repress E proteins (Boos *et al.*,

2007; Schotte *et al.*, 2010). Id proteins may function to suppress the gene expression programs of other lineages and allow the development of NK cells. For example, if unchecked, E2A (*Tcf3*) will initiate a B cell-specific gene expression program through the expression of the EBF and Pax-5 transcription factors, which regulate each other in a reciprocal relationship that serves to mutually reinforce B cell development (Singh *et al.*, 2005). Id2 and other Id proteins would repress this outcome and allow the NK cell lineage to develop in conjunction with other positive transcriptional regulators.

Mice lacking E47 and E12, as well as Id2, are still deficient in peripheral NK cells, suggesting two nonexclusive possibilities (Boos *et al.*, 2007): (1) Residual E protein activity remains, most likely from HEB and E2-2. In NKP, this activity is counteracted by additional Id repression supplied by Id3 allowing for NK cell differentiation. Mature NK cells, which do not express Id3, are blocked from full maturation by residual unchecked E protein. It is unknown how much redundancy exists between Id2 and Id3. It will be interesting to determine the effects of the elimination of both these Id molecules on NK cell development. (2) In addition to its traditional binding partners, the E proteins, Id2 can bind other transcription factors, notably retinoblastoma (Rb) protein and PU.1 (Iavarone *et al.*, 2004; Ji *et al.*, 2008; Lasorella *et al.*, 2001). Id2 binds Rb and inhibits its antiproliferative activity (Lasorella *et al.*, 2001). The absence of Id2 would result in reduced proliferation, consistent with the reduced numbers of NK cells seen in Id2-deficient mice. PU.1 is an important regulator of NK cells and might interact with Id2 to regulate the NK cell lineage. The amount of expression of PU.1 plays a pivotal role in directing progenitor cells into a specific lineage: a low level of PU.1 selects for B cell generation whereas high expression of PU.1 induces macrophage development (DeKoter and Singh, 2000). A similar requirement might exist for NK cell development, and Id proteins could serve to dampen or modulate PU.1 activity to allow differentiation to occur.

As mentioned in Section 3.3, the NK cell developmental defect in E4bp4-deficient bone marrow can be overcome by overexpression of Id2, suggesting that E4bp4 may positively control *Id2* gene expression (Gascoyne *et al.*, 2009). A reduction of Id2 mRNA levels was observed in early hematopoietic cells from E4bp4-deficient mice (Gascoyne *et al.*, 2009). It is also possible that E4bp4 downregulates E protein expression in developing NK cells after lineage commitment through its ability to function as a negative regulator.

LTi cells and NKp46+RORγt+ cells in the gut have some features in common with NK cells suggesting that they might belong to a common lineage as discussed in Section 2. Interestingly, Id2 is also expressed in gut NKp46+RORγt+ cells, and Id2-deficient mice are deficient in gut NKp46+RORγt+ cells, LTi cells, and lack lymph nodes and Peyer's

patches (Boos *et al.*, 2007; Georgopoulos *et al.*, 1992; Luci *et al.*, 2009; Sanos *et al.*, 2009; Satoh-Takayama *et al.*, 2008, 2010; Yokota *et al.*, 1999). The removal of the E protein gene, *Tcf3* (E2A), in the context of Id2-deficiency rescues this defect resulting in normal LTi development, lymph nodes, and Peyer's patches (Boos *et al.*, 2007). However, NK cells have a greater need for Id function as the removal of E2A does not completely rescue the Id2 defect (Boos *et al.*, 2007). These findings highlight the similarities between NK cells, gut NKp46+RORγt+ cells, and LTi cells in that Id2 is highly important and that E-box activity must be repressed for these cell types to develop. However, RORγt is not required for the development of NK cells, and based on lineage tracing experiments, RORγt is never expressed in the NK cell lineage (Satoh-Takayama *et al.*, 2010).

3.5. T-bet and Eomes

Two members of the T-box family of transcription factors, T-bet (encoded by the *Tbx21* gene) and Eomes control various aspects of NK cell development (Intlekofer *et al.*, 2005; Szabo *et al.*, 2002; Townsend *et al.*, 2004). T-box family members are involved in a variety of developmental processes and share a common DNA-binding domain called a T-box and a variable C-terminal domain where activation motifs have been mapped (Naiche *et al.*, 2005). T-box transcription factors form homodimers or heterodimers and bind palindromic DNA sequences known as T-box-binding elements (Naiche *et al.*, 2005). T-bet and Eomes expression overlaps considerably in $CD8^+$ T cells, TH1 T cells, and NK cells, although Eomes is expressed in low amounts in TH1 cells (Intlekofer *et al.*, 2005; Pearce *et al.*, 2003; Szabo *et al.*, 2000, 2002; Townsend *et al.*, 2004). T-bet is also expressed in B cells and NKT cells (Szabo *et al.*, 2000; Townsend *et al.*, 2004).

NK cell development is altered in T-bet-deficient mice resulting in increased numbers of NK cells in the bone marrow and reduced peripheral NK cell numbers with the exception of lymph nodes where the frequency of NK cells is also increased (Townsend *et al.*, 2004). These defects remained when T-bet-deficient bone marrow was used to reconstitute wild-type or lymphoid-deficient (RAG2- x γc-deficient) mice but were rescued by expression of T-bet via retroviral transduction (Townsend *et al.*, 2004). Collectively, these findings suggest that the NK cell phenotype in T-bet-deficient mice is cell intrinsic.

Mice deficient for sphingosine-1-phosphate receptor 5 (S1P5), a member of a receptor family that controls lymphocyte trafficking, share a similar NK defect to T-bet-deficient mice in that bone marrow and lymph nodes have increased numbers of NK cells (Jenne *et al.*, 2009; Walzer *et al.*, 2007). S1P5 is expressed in high amounts on wild-type NK cells and significantly reduced on NK cells from T-bet-deficient mice (Jenne *et al.*, 2009). S1P5 is a direct target of T-bet because exogenous

expression of T-bet in transformed cell lines induces expression of S1P5 and T-bet binds within the *S1p5* locus (Jenne *et al.*, 2009). Collectively, these findings indicate that the increased number of NK cells in the bone marrow and lymph nodes in T-bet-deficient mice is at least in part due to the diminished expression of S1P5 and the inability of these NK cells to egress.

T-bet-deficient NK cells are impaired beyond the NK cell egress defect and display increased basal rates of proliferation and apoptosis and constitutive cell surface expression of CD69, an activation marker (Townsend *et al.*, 2004). However, T-bet-deficient NK cells have reduced levels of maturation markers such as CD11b, CD43, B220, and Klrg1 and increased levels of c-kit (CD117), a marker of immature NK cells, although the expression of the Ly49 receptors is normal (Robbins *et al.*, 2005; Townsend *et al.*, 2004). Expression of T-bet via retroviral transduction in T-bet-deficient hematopoietic stem cells transplanted into lymphoid-deficient hosts rescued the CD11b expression on NK cells (Townsend *et al.*, 2004). Although T-bet might regulate CD11b expression, it is more likely that the rescued expression of CD11b indicates that T-bet regulates NK cell maturation.

Eomes-deficient mice die during early embryogenesis rendering it difficult to study NK cell development in the absence of Eomes (Russ *et al.*, 2000). Mice lacking both alleles of *Tbx21* (T-bet) and one allele of *Eomes* resulted in a severe drop in the number of peripheral NK cells (Intlekofer *et al.*, 2005). Similar to gene dosage affects that have been noted in *Tbx21+/−* Th1 cells, *Eomes+/−* mice showed a mild decrease in blood NK cells (Intlekofer *et al.*, 2005; Szabo *et al.*, 2002). Thus, expression of both of these transcription factors is critical for NK cell development.

T-bet and Eomes most likely play crucial roles in regulating CD122 (IL-2Rβ) in NK cells (see Fig. 2.2A). As discussed in Section 2, CD122 is critical for NK cell development and mice lacking this molecule are severely defective in peripheral NK cells (Suzuki *et al.*, 1997). Multiple T-box binding sites are located within the *Il2rb* promoter, and both Eomes and T-bet bind to the promoter *in vivo* in T cells and transformed NK cell lines (Beima *et al.*, 2006; Intlekofer *et al.*, 2005). Expression of T-bet or Eomes in TH2 cells, which do not normally express either factor, induces CD122 expression, and either T-bet- or Eomes-dependent transactivation is direct as shown through the use of estrogen receptor-Eomes or T-bet fusion proteins (Intlekofer *et al.*, 2005; Matsuda *et al.*, 2007). Parallel studies in $CD8^+$ T cells, which share function and expression of CD122, T-bet, and Eomes with NK cells, may prove informative. $CD8^+$ T cells have mildly reduced CD122 levels in *Eomes+/−* mice, whereas *Tbx21−/−Eomes+/−* $CD8^+$ T cells have severely reduced CD122 levels (Intlekofer *et al.*, 2005). These data indicate direct regulation of CD122 by both T-bet and Eomes in $CD8^+$ T cells and most likely in NK cells. Control of CD122 by these two

T-box transcription factors would explain the NK cell defect seen in *Tbx21−/−Eomes+/−* mice.

It would appear from studies of $CD8^+$ T cells that although both factors contribute to CD122 expression, Eomes-dependent control is dominant (Intlekofer *et al.*, 2005). However, NKT cells do not express Eomes, and CD122 expression is severely reduced on developing NKT cells in T-bet-deficient mice (Townsend *et al.*, 2004). Ectopic expression of T-bet in *Tbx21−/−* thymocytes rescues CD122 expression on NKT cells, demonstrating that T-bet controls CD122 expression in some circumstances (Townsend *et al.*, 2004). Examination of bone marrow NK cell development in mice lacking Eomes alone or in combination with T-bet needs to be performed.

3.6. Runx proteins

The Runx proteins are involved in a variety of biological processes including development, oncogenesis, bone formation, hematopoiesis, and immune function and are expressed in a variety of hematopoetic cells (de Bruijn and Speck, 2004; Hart and Foroni, 2002; Wheeler *et al.*, 2000). There are three Runx proteins expressed in mammalian cells, Runx1 (PEBP2αB, CBFα2), Runx2 (AML3, PEBP2αA, CBFα1), and Runx3 (AML2, PEBP2αC, CBFα3), each of which contains a conserved 128 amino acid motif known as a Runt domain. The Runt domain allows for both DNA binding and heterodimerization with the binding partner, CBFβ. The binding of CBFβ with Runx1, 2, or 3 increases the affinity of this complex (known as the core binding factor—CBF) for DNA. CBF can both activate and repress gene targets by complexing with other transcription factors, as well as recruiting histone acetyltransferases and histone deacetylases (Hart and Foroni, 2002; Wheeler *et al.*, 2000). The deletion of the *Cbfb* gene results in embryonic lethality at midgestation (Wang *et al.*, 1996b), precluding any study of the role of CBFβ in NK cells. The use of a hypomorphic *Cbfb* allele allows for the generation of mice expressing CBFβ at 15% of wild-type levels, delaying mortality until soon after birth (Talebian *et al.*, 2007). NK cells are undetectable in the fetal thymi or in NK cell differentiation cultures using fetal liver from these CBFβ reduced mice (Guo *et al.*, 2008). Competitive bone marrow chimeric mice generated with wild-type bone marrow and fetal liver from mice expressing CBFβ at 100%, 30%, and 15% wild-type levels resulted in increasingly reduced detection of NK cells in both the spleen and bone marrow from these animals. CBFβ levels at 15% of wild-type resulted in the absence of NK cells suggesting a critical threshold requirement for CBFβ between 30% and 15% of wild-type expression levels (Guo *et al.*, 2008). The Runt domain by itself can function as a dominant-negative by binding to both the DNA consensus sequence and CBFβ more strongly than any

full-length Runx proteins (Sato *et al.*, 2005). The expression of this Runt domain dominant-negative via retroviral transduction preceding *in vitro* NK cell differentiation resulted in a reduction of NK cells, as well as a complete absence of CD122 on more than half of the remaining NK cells (Ohno *et al.*, 2008). Runx binding sites have been located within the *Il2rb* promoter, and Runx proteins bind the promoter *in vivo* (Ohno *et al.*, 2008). Thus, Runx proteins control the expression of CD122, which would at least partly explain the severe defect in NK cell development seen in mice with reduced levels of CBFβ.

Expression of the Runt domain dominant-negative protein as a transgene driven by the *Cd2* promoter allows for examination of the role of the Runx proteins in immature NK cells and mature NK cells because CD2 is expressed beginning in the immature NK cell stage (Ohno *et al.*, 2008; Rosmaraki *et al.*, 2001). Peripheral NK cell numbers are normal in mice expressing this transgene (Ohno *et al.*, 2008), suggesting that Runx proteins may exert their influence on NK cell development early in differentiation and be important for initiation, but not maintenance, of NK cell development.

Collectively, these data show that Runx proteins and their binding partner CBFβ are required for NK cell development; however, it is unclear which specific Runx proteins are necessary for differentiation. Both Runx1 and Runx3 are expressed in the NK cell lineage, and Runx 2 expression levels remain very low throughout all stages of differentiation (Guo *et al.*, 2008; Ohno *et al.*, 2008). Runx3 appears to be the dominant family member with expression levels increasing during NK cell maturation (Guo *et al.*, 2008; Ohno *et al.*, 2008). Both these transcription factors are also expressed in the $CD8^+$ T cell lineage. The use of mice or chimeric mice deficient for Runx1 and/or Runx3 has demonstrated requirements for these proteins in $CD8^+$ T cell development and function, as well as activation and repression of lineage-specific genes (Sato *et al.*, 2005; Taniuchi *et al.*, 2002; Woolf *et al.*, 2003). Similar studies would be very beneficial for expanding our knowledge of the role of Runx1 and Runx3 in NK cell development.

3.7. Gata-3

Within the hematopoietic system, Gata-3 is expressed in the NK cell lineage, as well as common lymphoid progenitors, developing T cells, and TH2 cells (Biassoni *et al.*, 1993; Ho *et al.*, 2009; Rosmaraki *et al.*, 2001). Gata-3-deficient mice die *in utero* necessitating the generation of bone marrow chimeric mice using Gata-3-deficient fetal liver to study NK cell development (Samson *et al.*, 2003). Gata-3-deficient chimeras have normal numbers of splenic NK cells, but reduced numbers of liver NK cells, due to a defect in their ability to migrate from the bone marrow to the liver

(Samson *et al.*, 2003). Decreased expression of CD11b and CD43 in Gata-3-deficient NK cells from the bone marrow and spleen suggests that NK cell maturation is defective, although cytotoxicity against NK cell-sensitive target cells is unaffected (Samson *et al.*, 2003). Gata-3 expression is especially enriched in NK cells derived from the thymus. The absence of Gata-3 eliminates this NK cell population indicating that Gata-3 is required for thymic NK cell development (Vosshenrich *et al.*, 2006)

3.8. IRF-2

The interferon regulatory factor (IRF) family of transcription factors consists of nine members in human and mice and control aspects of Toll-like receptor (TLR) signaling, hematopoietic differentiation, and oncogenesis, in addition to expression of interferons (IFNs) and IFN-inducible genes (Tamura *et al.*, 2008). All IRFs contain an N-terminal DNA-binding domain that binds a consensus recognition sequence. Although most IRFs contain a protein interaction domain that allows for homodimerization and heterodimerization, IRF-1 and IRF-2 lack this domain (Tamura *et al.*, 2008).

Bone marrow and peripheral NK cell numbers are significantly reduced in IRF-2-deficient mice (Lohoff *et al.*, 2000; Matsuyama *et al.*, 1993; Taki *et al.*, 2005). Although NK cell-mediated allogeneic bone marrow rejection and *in vivo* NK cell-dependent tumor killing in IRF-2-deficient mice are severely defective (Lohoff *et al.*, 2000; Taki *et al.*, 2005), this killing defect is due to the reduction in NK cell numbers because cytotoxicity is normal when comparing equal numbers of enriched wild-type and IRF-2-deficient NK cells (Taki *et al.*, 2005). IRF-2-deficient peripheral NK cells are mildly defective in making IFNγ in response to IL-12 stimulation (Taki *et al.*, 2005).

Chimeric mice generated by the transplantation of IRF-2-deficient bone marrow into irradiated wild-type recipient mice, as well as the converse, demonstrated that the defect was cell intrinsic and not due to a deficient bone marrow microenvironment (Lohoff *et al.*, 2000; Taki *et al.*, 2005). IRF-2-deficient bone marrow is reduced in its ability to generate NK cells in the presence of IL-15 *in vitro*, although expression of components of the IL-15 receptor is normal (Lohoff *et al.*, 2000). The majority of bone marrow NK cells from IRF-2-deficient mice express DX5 and a relatively normal repertoire of recognition receptors such as the Ly49 and NKG2 family of receptors, suggesting that they are mature. However, the frequency of NK cells expressing maturation markers such as CD11b was reduced but increased for the immature marker, CD51. IRF-2-deficient bone marrow cells display higher levels of apoptosis explaining why the peripheral NK cells appear to be more immature and express lower levels of the Ly49 receptors (Taki *et al.*, 2005).

3.9. Indirect players: IRF-1, Bcl11b

Mice deficient for IRF-1, a member of the IRF transcription factor family, are severely deficient in peripheral NK cells (Matsuyama *et al.*, 1993; Ogasawara *et al.*, 1998; Ohteki *et al.*, 1998; Taki *et al.*, 1997). This reduction in NK cells explains the inability of these mice to reject NK cell-sensitive tumors and the inability of IRF-1-deficient splenocytes to kill YAC-1 targets (Duncan *et al.*, 1996; Taki *et al.*, 1997). Transfer of IRF-1-deficient bone marrow into irradiated recipients generated peripheral NK cells in the recipient mice. Conversely, transfer of wild-type bone marrow into IRF-1-deficient recipients resulted in a severe reduction in NK cells (Ogasawara *et al.*, 1998). Thus, the requirement for IRF-1 is not NK cell intrinsic but rather for support from the bone marrow microenvironment. IRF-1-deficient bone marrow is defective in expressing IL-15, an essential cytokine for NK cell development explaining the NK cell lineage defect in these mice (Ogasawara *et al.*, 1998; Ohteki *et al.*, 1998). This NK cell defect can be rescued *in vitro* by culturing IRF-1 bone marrow with IL-15 (Ogasawara *et al.*, 1998; Ohteki *et al.*, 1998).

Bcl11b is a zinc finger protein that can act as both a transcriptional repressor and activator and is expressed predominantly within the T cell lineage in the hematopoietic system (Albu *et al.*, 2007; Inoue *et al.*, 2006; Kastner *et al.*, 2010; Wakabayashi *et al.*, 2003 and references therein). Bcl11b-deficient mice do not survive past the 1st day after birth (Wakabayashi *et al.*, 2003). There is a severe defect in αβ T cell development, as well as increased apoptosis, within the CD4, CD8 double-negative stage thymocyte compartment in both fetal and neonatal Bcl11b-deficient mice (Inoue *et al.*, 2006; Wakabayashi *et al.*, 2003). Generation of mice lacking Bcl11b in the T cell compartment using deficient fetal liver cells to generate bone marrow chimeras or through T cell-specific deletion of floxed *Bcl11b* alleles resulted in similar defects in T cell development (Albu *et al.*, 2007; Wakabayashi *et al.*, 2003). These mice did not die at such a young age, suggesting that Bcl11b has functions outside of its role in T cell development (Albu *et al.*, 2007; Wakabayashi *et al.*, 2003). Remarkably, recent findings show that early double-negative thymocytes lacking Bcl11b have a propensity to express a variety of NK cell lineage molecules including Id2, T-bet, Eomes, CD122, E4bp4, NKp46, NKG2A/E/C receptors, perforin, and IFNγ after *in vitro* culture, as well as the potential to develop into myeloid cells under the appropriate culture conditions (Ikawa *et al.*, 2010; Li *et al.*, 2010a,b). These reprogrammed NK like cells harbored *Tcrb* rearrangements demonstrating that they originated from the T cell lineage, although they efficiently killed NK-sensitive targets (Li *et al.*, 2010b). This reprogramming also occurs *in vivo* resulting in NK like cells that protected the host from a tumor challenge (Li *et al.*, 2010b). Bcl11b has been shown to bind to *cis*-acting elements of essential

T cell-encoded genes such as *Zbtb7b* (Thpok) as well as NK cell-encoded genes such as *Id2* (Kastner *et al.*, 2010). Together, these findings suggest that Bcl11b plays an essential role in specifying T cell fate by both upregulating T cell-specific genes and suppressing NK cell genes.

4. TRANSACTING FACTORS IN MATURE NK CELL FUNCTION

Here, we discuss transcription factors that regulate the effector functions of mature peripheral NK cells (see Fig. 2.1). The severe defects in NK cell development and reduction in NK cell numbers in mice lacking Ikaros, Ets-1, E4bp4, and Id2 make it difficult to study the role these transcription factors may play in the function of mature NK cells.

4.1. Ets-family transcription factors: PU.1 and MEF

NK cell development is not completely blocked in MEF-deficient mice and allows for the generation of peripheral NK cells. MEF-deficient splenic NK cells are defective in making IFNγ after stimulation of mice with poly I:C (Lacorazza *et al.*, 2002). MEF-deficient NK cells are able to bind to target cells; however, they are unable to kill target cells, whereas MEF-deficient $CD8^{+}$ T cells can still kill targets, albeit at reduced levels. Expression of perforin, an essential component of the cytotoxic granules that NK cells and $CD8^{+}$ T cells use to kill targets, is dramatically reduced in MEF-deficient NK cells explaining the lack of cytotoxicity (Lacorazza *et al.*, 2002). Two binding sites for Ets-family factors have been identified within the mouse and human *Prf1* (perforin) gene promoters (Koizumi *et al.*, 1993; Lacorazza *et al.*, 2002; Lichtenheld and Podack, 1992; Yu *et al.*, 1999; Zhang and Lichtenheld, 1997). MEF binds to these sites *in vitro* and *in vivo* and can transactivate the promoter, whereas Ets-1 or PU.1 cannot (Lacorazza *et al.*, 2002). Thus, MEF specifically regulates perforin in NK cells (see Fig. 2.2B). By contrast, in T cells, MEF is partially redundant with another transcription factor for perforin expression because MEF is not essential for killing of targets by cytotoxic T cells (Lacorazza *et al.*, 2002).

Ets-family binding sites are critical for Ly49 receptor promoter function and are capable of binding MEF protein (Presnell *et al.*, 2006; Saleh *et al.*, 2004); however, Ly49 receptor expression was not examined in MEF-deficient NK cells (Lacorazza *et al.*, 2002). The regulation of IFNγ, the Ly49 receptors, or other genes important for NK cell function by MEF is not understood and would benefit from further study.

Similarly to MEF, PU.1 is not absolutely required for NK cell development; bone marrow chimeric mice in which the lymphoid compartment is deficient for PU.1 contain peripheral NK cells (Colucci *et al.*, 2001).

PU.1-deficient NK cells are defective in their proliferative ability: Fewer circulating PU.1-deficient NK cells from blood are in cell cycle, and this defect remains when the cells are stimulated with IL-12. PU.1-deficient NK cells do not expand in response to IL-2, although they are viable. However, PU.1-deficient NK cells are normal in their ability to kill YAC-1 tumors (Colucci *et al.*, 2001). It is not reported if PU.1-deficient NK cells can produce IFNγ.

There are a number of PU.1 target genes that play important roles in NK cell function and are defective in PU.1-deficient mice (see Fig. 2.2B). B220 is a heavily glycosylated isoform of the tyrosine phosphatase, CD45. Expression levels of B220 are decreased dramatically in PU.1-deficient NK cells (Colucci *et al.*, 2001), which may indicate that CD45 expression is downregulated in PU.1-deficient NK cells. In progenitor cells and myeloid cells, PU.1 is required for CD45 expression and can transactivate and bind to the *Cd45* promoter (Anderson *et al.*, 2001; Medina *et al.*, 2004), suggesting that PU.1 may regulate CD45 expression in the NK cell lineage. CD45-deficient NK cells are functionally defective and have altered expression of specific Ly49 receptors, such as Ly49D and Ly49A, but are normal for YAC-1 killing, similar to PU.1-deficient NK cells (Hesslein *et al.*, 2006; Huntington *et al.*, 2005; Mason *et al.*, 2006). Thus, defective CD45 expression would partially explain the phenotype of PU.1-deficient NK cells.

DAP12, encoded by the *Tyrobp* gene, a critical signaling adaptor for a large number of mouse and human NK cell and myeloid receptors (Lanier, 2009), is downregulated in PU.1-deficient myeloid cells (Henkel *et al.*, 2002; Weigelt *et al.*, 2007). PU.1 binds the *Tyrobp* promoter *in vitro* and *in vivo* and the PU.1 binding sites are critical for *Tyrobp* promoter activity (Weigelt *et al.*, 2007). PU.1 likely regulates DAP12 in NK cells, although DAP12 expression levels have not been examined in PU.1-deficient NK cells. The cell surface expression of the DAP12-associated activating receptor, Ly49D, is severely reduced in PU.1-deficient NK cells. Ly49D surface expression is dependent on DAP12 and thus the lack of Ly49D expression is consistent with a reduction in expression of DAP12.

Evidence of PU.1 expression in NK cells is contradictory. Colucci and colleagues reported PU.1 expression in IL-2-cultured NK cells and in splenic NK cells by Western blot and RT-PCR analysis, respectively (Colucci *et al.*, 2001). In contrast, Nutt *et al.* (2005) did not detect GFP expression in NK cells isolated from the spleens of a PU.1-specific GFP reporter mouse strain. As discussed in Section 3.2, PU.1 may be important for NK lineage commitment, but not for maintenance, similar to T and B cells. Similarly, PU.1 could initiate transcription at gene loci like *Cd45* and *Tyrobp* and then be replaced by other factors for maintenance of expression. These possibilities may be not mutually exclusive and the role of PU.1 may differ from gene to gene. In any case, PU.1 expression in NK cells needs to be definitely determined.

4.2. T-bet and Eomes

The T-box transcription factors, T-bet and Eomes, control genes crucial for the effector functions of NK cells; IFNγ production and cell-mediated cytotoxicity. IFNγ expression levels are either reduced or absent in immune cells from T-bet-deficient mice (Intlekofer *et al.*, 2005; Lugo-Villarino *et al.*, 2003; Sullivan *et al.*, 2003; Szabo *et al.*, 2002; Townsend *et al.*, 2004). The transcriptional control of the *Ifng* locus is well studied, and a variety of regions have been identified as important *cis*-acting elements (Schoenborn and Wilson, 2007). T-box-binding sites have been identified within the *Ifng* locus including the promoter and some enhancers, and T-bet can bind to the locus *in vivo* in NK cells and T cells (Beima *et al.*, 2006; Hatton *et al.*, 2006; Miller *et al.*, 2008; Szabo *et al.*, 2000; Townsend *et al.*, 2004). Deletion of T-box-binding sites within a critical distal element that bind T-bet *in vivo* eliminated its ability to enhance transcription of the *Ifng* locus (Hatton *et al.*, 2006). Ectopic expression of T-bet in cells that do not express T-bet such as transformed cell lines or TH2 cells results in transactivation of IFNγ reporter constructs, chromatin changes in the *Ifng* locus, and increased endogenous IFNγ mRNA and protein levels (Afkarian *et al.*, 2002; Mullen *et al.*, 2001, 2002; Pearce *et al.*, 2003; Szabo *et al.*, 2000). However, IFNγ expression in T-bet-deficient NK cells is reduced but not eliminated (Townsend *et al.*, 2004). This is likely due to expression of other T-box transcription factors, namely Eomes. Ectopic expression of Eomes induces endogenous IFNγ production in the absence of T-bet in TH2 cells and conversely, there is a significant decrease in IFNγ expression in *Tbx21−/−Eomes+/−* $CD8^+$ T cells compared to *Tbx21−/−Eomes+/+* $CD8^+$ T cells (Intlekofer *et al.*, 2005). The joint expression of T-bet and Eomes in both NK and $CD8^+$ T cells makes it reasonable to draw parallels between these two cell types and conclude that both T-bet and Eomes contribute to IFNγ expression in NK cells.

T-bet-deficient NK cells are mildly defective in killing target cells, and expression of perforin is down minimally in *Tbx21−/−* NK cells (Intlekofer *et al.*, 2005; Townsend *et al.*, 2004). However, T-bet has been shown to bind the gene loci of two essential components of cytotoxic granules, granzyme B and perforin, and to induce perforin and granzyme B expression when expressed ectopically (Beima *et al.*, 2006; Lewis *et al.*, 2007; Miller *et al.*, 2008; Pearce *et al.*, 2003; Townsend *et al.*, 2004). Expression of perforin is down significantly in *Tbx21−/−Eomes+/−* NK cells (Intlekofer *et al.*, 2005). Thus, Eomes also plays a role in controlling perforin and granzyme B, similar to control of CD122 and IFNγ. Ectopic expression of Eomes in TH2 cells and transformed cell lines induces perforin and granzyme B expression and Eomes binds to the *Prf1* (perforin) and *Gzmb* (granzyme B) loci *in vivo* similar to T-bet (Beima *et al.*, 2006; Intlekofer *et al.*, 2005; Lewis *et al.*, 2007; Miller *et al.*, 2008; Pearce *et al.*, 2003). Expression of an artificial dominant-negative Eomes thought to inhibit both T-bet and

Eomes activity reduced the ability of CD8$^+$ T cells to kill (Pearce *et al.*, 2003), presumably by downregulating perforin and granzyme B expression. The evidence for Eomes and T-bet-dependent control of the *Prf1* and *Gzmb* loci is quite strong, although these two factors may control additional genes important for cell-mediated cytotoxicity.

In TH1 cells, T-bet is required for expression of the chemokine receptor CXCR3, which is required for proper homing of NK cells to lymph nodes and tumors (Lord *et al.*, 2005; Martin-Fontecha *et al.*, 2004; Wendel *et al.*, 2008). It is unclear whether CXCR3 expression is downregulated in T-bet-deficient NK cells; however, T-bet binds directly to the *Cxcr3* promoter *in vivo* in T cells and NK cells, transactivates CXCR3 reporter constructs, and induces the endogenous gene in cells that do not usually express it (Beima *et al.*, 2006; Jenne *et al.*, 2009; Lewis *et al.*, 2007; Matsuda *et al.*, 2007; Miller *et al.*, 2008). It is unclear what role Eomes plays in regulating the *Cxcr3* gene, although Eomes can transactivate and bind the endogenous locus in transformed cell lines (Lewis *et al.*, 2007; Miller *et al.*, 2008).

Structure–function analysis of T-bet protein has resulted in the isolation of at least three distinct functions from both separate and overlapping portions of T-bet: promoter transactivation, interaction with H3K4-methyltransferases inducing permissive H3K4 dimethyl modifications, and interaction with H3K27 demethylases causing removal of repressive H3K27 trimethylation modifications. These activities operate on the *Ifng* and *Cxcr3* loci and are found in other T-box proteins including Eomes (Lewis *et al.*, 2007; Miller *et al.*, 2008).

A common theme is the similarity between the roles of T-bet and Eomes in NK cell function and gene expression (see Fig. 2.2B). It is unclear how redundant T-bet and Eomes are for one another in regulating genes in NK cells or other cell types. It may be that the total level of T-box transcription factor activity (T-bet and Eomes combined) is critical for some NK cell and/or CD8$^+$ T cell genes, whereas factor specificity is more important in other cases. Regardless of the overlap in T-bet and Eomes function, T-bet expression in NK cells does affect NK cell function (Townsend *et al.*, 2004; Werneck *et al.*, 2008). T-bet-deficient NK cells fail to control tumor burden and metastasis mostly likely due to the combined effects of reduced cytotoxicity, homing ability, IFNγ production, and survival (Werneck *et al.*, 2008). It will be interesting to learn of the specific roles Eomes has in regulating NK cell function.

4.3. Runx proteins

Runx proteins play a role in NK cell function in addition to development as demonstrated through the use of a *Cd2* promoter-dependent transgene expressing the Runt domain dominant-negative protein. This transgene is expressed in immature NK cells and mature NK cells (Ohno *et al.*, 2008). Ly49 receptor gene promoters contain Runx binding sites that are required

for function (Saleh *et al.*, 2004), and conversely, the expression of the Ly49 family of recognition receptors is reduced in NK cells expressing the Runt domain dominant-negative protein (Ohno *et al.*, 2008). The Ly49 inhibitory receptors are expressed at approximately 50–30% of wild-type levels, and the activating receptor Ly49D is expressed at 20% of wild-type levels in these mice (Ohno *et al.*, 2008). In humans, all KIR promoters contain Runx binding sites that predominantly bind Runx3 in NK cells (Anderson, 2006; Gomez-Lozano *et al.*, 2007; Trompeter *et al.*, 2005). Some promoter studies show that mutation of this Runx binding site causes an increase in transcription rates (Gomez-Lozano *et al.*, 2007; Trompeter *et al.*, 2005), whereas another study shows that these Runx sites are important for positive regulation (Presnell *et al.*, 2006). Thus although Runx proteins are important, it is unclear how the Runx proteins control KIR expression (see Fig. 2.2B).

Total peripheral NK cells numbers are normal although the maturation markers CD11b and CD43 are reduced on NK cells from transgenic mice expressing the Runt domain dominant-negative protein (Ohno *et al.*, 2008). Any possible perturbations in NK cell maturation do not adversely affect cytotoxicity of YAC-1 or IFNγ production. In fact, the reduction in Runx activity increased the ability of NK cells to produce IFNγ (Ohno *et al.*, 2008). In T cells, Runx3 has been shown to cooperate with Eomes and T-bet to positively control IFNγ expression (Cruz-Guilloty *et al.*, 2009; Djuretic *et al.*, 2007). Runx3 has also been shown to regulate granzyme B and perforin in T cells (Cruz-Guilloty *et al.*, 2009). The role of Runx3 in controlling *Prf1* and *Gzmb* gene expression in NK cells is unknown, although there is no defect in target cytotoxicity in NK cells expressing the Runt domain dominant-negative protein. These differences in Runx-dependent gene expression between NK cell and T cells should be addressed by examing NK cells harboring deletions of *Runx3* and/or *Runx1* genes.

Runx proteins are known to exert control of gene expression through cooperation with other proteins (Wheeler *et al.*, 2000) as demonstrated with T-bet and Eomes (Cruz-Guilloty *et al.*, 2009; Djuretic *et al.*, 2007). There are multiple studies demonstrating the critical role of interactions of Ets-1 and PU.1 with Runx proteins for controlling a variety of important hematopoietic genes (Goetz *et al.*, 2000; Gu *et al.*, 2000; Petrovick *et al.*, 1998; Wheeler *et al.*, 2000). It is likely that these other unrelated transcription factors (T-bet, Eomes, Ets-1, and PU.1) interact with the Runx proteins to control expression of critical NK cell lineage genes like KIR (see Fig. 2.2B; Presnell *et al.*, 2006).

4.4. Gata-3

Gata-3 is not required for bone marrow-derived NK cells to populate the spleen (Samson *et al.*, 2003); however, Gata-3-deficient splenic NK cells express an altered Ly49 receptor repertoire with the frequency of NK cells

displaying Ly49D expression especially reduced (Samson *et al.*, 2003). Gata-3 had been implicated in regulating NKG2A, but NKG2A levels have not been measured in Gata-3-deficient NK cells (Marusina *et al.*, 2005; Samson *et al.*, 2003). Gata-3-deficient splenic NK cells can kill YAC-1 target cells normally but were reduced in their ability to produce IFNγ (Samson *et al.*, 2003). The requirement for Gata-3 for normal NK cell-specific IFNγ expression is the converse to that found in $CD4^+$ T cell subsets, where Gata-3 expression is not required for IFNγ production and favors differentiation of the TH2 lineage that does not express IFNγ (Ho *et al.*, 2009). Gata-3-deficient NK cells have reduced expression of T-bet and Hlx, a downstream target of T-bet, that have been both shown to induce IFNγ when overexpressed in T cells (Mullen *et al.*, 2002; Samson *et al.*, 2003). These deficiencies in T-bet and Hlx most likely contribute to the IFNγ defect in the absence of Gata-3, although the relationship between Gata-3 and T-bet in NK cells is unclear.

4.5. CEBPγ

CCAAT/enhancer-binding proteins (C/EBPs) comprise a family of transcription factors that share a basic DNA-binding region and a leucine zipper dimerization motif that can homodimerize and heterodimerize with other family members and unrelated transcription factors (Lekstrom-Himes and Xanthopoulos, 1998). This family of transcription factors has been implicated in a variety of processes including hepatocyte function, adipocyte differentiation, and granulocyte maturation. Most C/EBPs contain transactivation domains, although C/EBPγ lacks such a domain allowing it to act as a dominant-negative. In some cases, however, it does not repress and can activate transcription (Kaisho *et al.*, 1999; Lekstrom-Himes and Xanthopoulos, 1998).

C/EBPγ is expressed ubiquitously and C/EBPγ-deficient mice have a high mortality rate within 2 days after birth. Bone marrow chimeras were used to examine the role of C/EBPγ in the hematopoetic cell compartment (Kaisho *et al.*, 1999). In these mice, T and B cell populations were normal and functional. Peripheral NK cell numbers were normal and could be expanded in IL-15, suggesting that NK cell development was normal in C/EBPγ-deficient mice (Kaisho *et al.*, 1999). However, C/EBPγ-deficient splenocytes, as well as cultured C/EBPγ-deficient NK cells, were severely impaired in their ability to kill YAC-1 target cells, although perforin expression in the NK cells was normal (Kaisho *et al.*, 1999). Likewise, C/EBPγ-deficient splenocytes, as well as cultured C/EBPγ-deficient NK cells, were defective in their ability to produce IFNγ after stimulation with IL-12 and/or IL-18. IL-12 and IL-18 receptor expression and activation of Stat4 and JNK, downstream effectors of the IL-12R and IL-18R, were normal (Kaisho *et al.*, 1999). The functional defects of C/EBPγ-deficient

NK cells are intriguing, and more studies are needed to explain these provocative phenotypes. For example, C/EBP family binding sites are critical for *Klra* (Ly49) gene promoter function, and C/EBPγ has been shown to bind to these sites (Kaisho *et al.*, 1999; Saleh *et al.*, 2004). It is intriguing to speculate that Ly49 receptor expression is altered in C/EBPγ-deficient NK cells.

4.6. MITF

Microphthalmia transcription factor (MITF) is a basic-HLH leucine zipper regulator that controls melanocyte, osteoclast, and mast cell differentiation and function (Cheli *et al.*, 2010). The $Mitf^{Mi}$ allele of *Mitf* encodes a mutant protein with a deletion in the basic DNA-binding domain, resulting in defects in DNA-binding and nuclear localization (Ito *et al.*, 2001; Kataoka *et al.*, 2005). $Mitf^{Mi}/Mitf^{Mi}$ mice contain normal numbers of NK cells, suggesting that NK cell development is not perturbed (Ito *et al.*, 2001); however, $Mitf^{Mi}/Mitf^{Mi}$ NK cells are impaired in cell-mediated cytotoxicity and in IFNγ production in response to IL-12 and IL-18 stimulation (Ito *et al.*, 2001; Kataoka *et al.*, 2005; Seaman *et al.*, 1979). Expression of IL-12Rβ2 and IL-18Rα is severely reduced on $Mitf^{Mi}/Mitf^{Mi}$ NK cells explaining the reduction in IFNγ production (Kataoka *et al.*, 2005).

$Mitf^{Mi}/Mitf^{Mi}$ NK cells lack cytotoxic granules and are deficient in their ability to express perforin, explaining the inability of $Mitf^{Mi}/Mitf^{Mi}$ NK cells to kill target cells (Ito *et al.*, 2001). The protein encoded by the $Mitf^{Mi}$ allele prevents transactivation and nuclear factor binding to an MITF-responsive site located within the *Prf1* gene promoter. MITF does not bind the *Prf1* promoter but acts indirectly by preventing nuclear localization of another factor that directly activates perforin expression in NK cells. On careful examination of the DNA that comprises the MITF-responsive site, we realized that this is one of the two sites that is bound by the Ets-family transcription factor, MEF, as discussed in Section 4.1 (Lacorazza *et al.*, 2002). MEF can bind *in vivo* and transactivate the *Prf1* promoter through this site (Lacorazza *et al.*, 2002). Thus, we postulate that the $Mitf^{Mi}$ protein prevents MEF from entering the nucleus and inducing perforin expression in NK cells. MITF has been shown to interact with another Ets-family transcription factor, PU.1, in regulating osteoclast gene expression (Luchin *et al.*, 2001). Thus, there is precedence for MITF–Ets factor interaction. There is likely alternative regulation for perforin in $CD8^+$ T cells, as perforin expression is intact in both $Mitf^{Mi}/Mitf^{Mi}$ and MEF-deficient $CD8^+$ T cells (Ito *et al.*, 2001; Lacorazza *et al.*, 2002). It would be interesting to find out if this inhibition of MEF by $Mitf^{Mi}$ protein is also responsible for the reduced expression of IL-12Rβ2 and IL-18Rα on $Mitf^{Mi}/Mitf^{Mi}$ NK cells.

5. CONCLUSIONS

We are in the adolescence of the field of NK cell transcriptional control. Many players have been identified, but we know very little about how these transcription factors control NK cell genes and NK cell biology. In many cases, there are single papers describing provocative phenotypes, but no additional studies addressing mechanism including synergy and cooperation between transcription factors. Many of these studies were performed several years ago and a simple reexamination of the NK cells deficient in specific transcription factors using more recent tools and reagents would advance the field significantly. The conditional deletion of genes that are embryonic lethal using NK cell-specific Cre mice will prove useful, and the use of gene expression arrays and chromatin immunoprecipitations would likely reveal probable mechanisms. We anticipate exciting years ahead for the field of NK cell transcriptional control.

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NOTE IN PROOF

We would also to direct the readers to a body of work on TCF-1 and its regulation of Ly49A expression (Held *et al.* Immunity. 1999, 11:433-442), as well as its redundant role with LEF-1 in regulating NK cell development (Held *et al.* Eur. J. Immunol. 2003;33:1393-1398 and references therein).

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CHAPTER 3

The Control of Adaptive Immune Responses by the Innate Immune System

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Abstract

The mammalian immune system comprises an adaptive and an innate component. The innate immune system employs a limited number of germ-line-encoded pattern-recognition receptors (PRRs) that recognize invariant pathogen-associated molecular patterns (PAMPs). In contrast, the adaptive immune system depends on the

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generation of a diverse repertoire of antigen receptors on T and B lymphocytes and subsequent activation and clonal expansion of cells carrying the appropriate antigen-specific receptors. Induction of adaptive immunity not only depends on direct antigen recognition by the antigen receptors but also relies on essential signals that are delivered by the innate immune system. In recent years, we have witnessed the discovery of a still expanding array of different PRR systems that govern the generation of adaptive immunity. Here, we review our current understanding of innate control of adaptive immunity. In particular, we discuss how PRRs initiate adaptive immune responses in general, discuss specific mechanisms that shape the ensuing T and B cell responses, and highlight open questions that are still awaiting answers.

1. INTRODUCTION

Defense against microbial assaults is an essential necessity for all living organisms. Consequently, all life forms have evolved strategies that are designed to limit the invasion of the host by microorganisms. Plants, fungi, and lower multicellular organisms rely on a set of strategies that are collectively called innate immunity. While the precise characteristics of the innate immune systems differ between the various species, they all share several central features. Innate immunity, which is genetically fixed and thus invariant, relies on a defined set of receptors and is nonspecific as it targets whole classes of microbes.

The evolution of vertebrates was accompanied by the emergence of adaptive immunity. Both jawed and jawless vertebrates developed immune systems that allowed for combinatorial diversity through the rearrangement of germ-line-encoded gene segments and thus enabled the direct targeting of specific microbial invaders (Herrin and Cooper, 2010; Pancer and Cooper, 2006). Jawless vertebrates based their immune system on an ancient receptor containing leucine-rich repeats (LRRs), which are a common structural feature used throughout innate immunity including modern Toll-like receptors (TLRs) in mammals. Thus, both the lamprey and hagfish, the remaining jawless vertebrates, diversify two germ-line-encoded LRR-containing receptors by adding additional LRR-encoding gene segments through recombination. In contrast, jawed vertebrates evolved members of the immunoglobulin (Ig) superfamily further, which led to the adaptive immune system of modern mammals. All mammals therefore generate combinatorial diversity through the shuffling of gene segments encoding Ig domains.

As a consequence of its ancestral history, the mammalian immune system consists of two parts: the innate immune system and the adaptive immune system. The innate immune system, which employs a small set of

invariant pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), serves as a first line of defense that is rapid and remarkably effective in clearing most invading pathogens. In contrast, the adaptive immune system, which selectively expands antigen-specific clones from an enormous pool of T and B cells harboring unique antigen receptors, serves as a second line of defense that is highly specific and able to form immunological memory.

The deletion of self-reactive T and B cell clones during the development of the cells forms the basis for the discrimination between self and nonself by the adaptive immune system. However, as the existence of the various autoimmune diseases shows, clonal deletion is an imperfect mechanism. More than 2 decades ago, Charles A. Janeway Jr. suggested that the recognition of PAMPs by the innate system delivers essential signals to the adaptive immune system that provide an additional layer of self/nonself discrimination and allows for the distinction between innocuous and pathogenic antigens (Janeway, 1989). It is now universally recognized that innate instruction of adaptive immunity is a critical step that controls the activation, types, and duration of the adaptive immune response. Innate instruction occurs initially during the interaction between antigen-presenting cells (APCs) and T cells. While this interaction is critical for the generation of an adaptive immune response, it is clear that innate instruction of adaptive immunity is a process that occurs at multiple stages throughout the immune response and involves all cell types participating in a particular response. In this chapter, we will provide an overview of our current understanding of innate instruction of adaptive immunity. In addition, we will emphasize aspects that in our view are currently underappreciated and deserve more attention.

2. DIVERSE SETS OF PRRS

While all PRRs are able to detect microbes and induce innate immune responses, they can nonetheless be classified into distinct functional classes that serve different purposes. The first class, consisting of secreted PRRs such as mannose-binding lectin (MBL), is involved in opsonization and complement activation. The second class consists of receptors that induce phagocytosis on dendritic cells (DCs) and macrophages such as the scavenger receptor or mannose-binding receptor (MR). They facilitate the uptake of microbes into the phagosome and the processing of the foreign proteins into antigenic peptides for T cell stimulation. The third class constitutes a group of PRRs that induce the production of antimicrobial peptides, chemokines, and proinflammatory cytokines. Importantly, these PRRs upregulate costimulatory molecules and trigger

the secretion of cytokines that are essential for the generation of the adaptive immune response. Based on their cellular localization, the latter class of PRRs can be further divided into PRRs that monitor the extracellular milieu (TLRs and some C-type lectins) and PRRs that detect intracellular infections (RIG-I-like receptors, NOD-like receptors (NLRs), and DNA sensors). For the purpose of this chapter, we will restrict our discussion to PRRs that are able to induce an adaptive immune response.

2.1. Transmembrane PRRs

Transmembrane PRRs can be located either on the cellular surface or inside phagosomes and endosomes. They comprise two families, TLRs and a subgroup of C-type lectins, which recognize distinct PAMPs and use distinct signaling pathways.

2.1.1. Toll-like receptors

TLRs are by far the best-studied class of PRRs. Many of their ligands are known, as are their signaling pathways and the physiological consequences of their activation. TLRs owe their prominence in part to the fact that they were the first family of PRRs that was discovered. However, they also serve as a paradigm for the innate control of adaptive immunity. TLRs are sufficient for the induction of adaptive immune responses and control them at multiple levels that include the induction, differentiation, and memory formation of both $CD4^+$ and $CD8^+$ T cells and the generation of antibody responses.

The TLR family consists of at least 13 members in mammals. Three of these receptors (TLR3, TLR7, and TLR9) reside in endosomes where they recognize nucleic acids. The remaining TLRs are located on the cell membrane and are activated by a diverse range of PAMPs that include LPS, bacterial lipoproteins, zymosan, and flagellin (Alexopoulou *et al.*, 2001; Hemmi *et al.*, 2000; Hoshino *et al.*, 1999; Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). All TLRs are type I transmembrane glycoproteins and contain a cytosolic domain known as the Toll/IL-1R (TIR) domain that shares homology with the interleukin-1 receptor (IL-1R; Dunne *et al.*, 2003). At least four adaptor molecules are involved in the signal transduction of TLRs (Akira, 2004). MyD88 is the central signaling adaptor for most TLRs and receptors of IL-1 family members. In addition, MyD88 has also been shown to associate with the IFN-γ receptor and TACI, although the mechanisms of these interactions are less clear (He *et al.*, 2010; Sun and Ding, 2006). The TIR domain of MyD88 associates with the TIR domain of TLRs (and IL-1R), which leads to the recruitment and phosphorylation of IRAK-4 and IRAK-1 (Adachi *et al.*, 1998; Burns *et al.*, 1998; Kawai *et al.*, 1999; Li *et al.*, 2002; Medzhitov *et al.*, 1998; Suzuki *et al.*, 2002; Swantek *et al.*, 2000; Wesche *et al.*, 1997). The activated kinases promote the binding

of TRAF6, and this interaction results ultimately in the expression of NF-κ B-dependent inflammatory cytokines such as TNFα, IL-1, and IL-6. Most TLRs rely on MyD88 as the essential signaling adaptor for the induction of proinflammatory cytokines, while TLR3 does not use MyD88 and depends on the adaptor TRIF to induce these cytokines. TLR4 and the endosomal TLRs can also induce type I interferons. TLR7 and TLR9 induce the type I interferon response via MyD88 and IRF7. In contrast, TLR3 and TLR4 (via TRAM) rely on TRIF, which mediates the activation of interferon regulatory factor 3 (IRF3) in order to induce the expression of type I interferons (Hoebe, 2003; Kawai *et al.*, 2001; Yamamoto *et al.*, 2003).

Activation of TLR signaling in APCs results in cytokine production and the upregulation of costimulatory molecules that are necessary for the induction of T cell responses. The original theories of innate control of adaptive immunity postulated a dependence of the upregulation of costimulatory molecules on PRR activation, but the picture has become more complicated in recent years. Indeed, professional APCs such as DCs already express high levels of costimulatory molecules and yet, in the absence of PAMPs, antigen presentation by these APCs leads to tolerance rather than immunity. Further, inflammatory mediators, such as TNFα, can also activate DCs and induce the upregulation of costimulatory molecules. These DCs can support the expansion of $CD4^+$ T cell but fail to induce their differentiation into effector cells (Sporri and Reis e Sousa, 2005). Despite considerable efforts to understand the rules governing activation of T cells, and the relative role of costimulatory molecules and cytokines in this process, the identities of all the signals that are necessary and sufficient for T cell activation remain poorly understood. It appears to be clear, though, that costimulation alone is necessary but insufficient for T cell activation while TLR-driven activation of APCs is sufficient to provide all signals necessary for the induction of T cell responses.

All TLRs can induce a T_H1 response. In addition, many but not all TLRs can also induce a T_H17 response. PAMPs are essential for marking protein antigens as foreign and their recognition by PRRs thus provides a layer of self versus nonself discrimination in addition to the clonal selection of lymphocytes. The physical association of PAMPs with antigens ensures that the antigen and PAMPs end up in the same endosome of the APC and enables the cell to preferentially present the foreign antigens as peptide–MHC class II complexes on the cell surface (Blander and Medzhitov, 2004).

While TLRs have been shown to be necessary and sufficient for the induction of T cell responses in immunizations using TLRs as adjuvant and in the case of various infections, it should be noted that the requirement for innate instruction of adaptive immunity is not impervious to the unintended effects of artificial experimental systems. In particular, we

have found that TLR signaling is not required when T cell precursor frequencies are artificially inflated through the use of TCR-transgenic T cells. $CD4^+$ T cells carrying an ovalbumin-specific TCR transgene are still able to mount a vigorous immune response to an otherwise TLR-dependent immunization after transfer into MyD88-deficient mice. Similar observations were made with TCR-transgenic $CD4^+$ T cells deficient of the IL-6 receptor α chain (Noah Palm, Simone Nish, Dominik Schenten, Ruslan Medzhitov, unpublished). Thus, certain experimental manipulations can alter or eliminate the requirements for innate immune signals, which can affect the interpretation of the results.

2.1.2. C-type lectins

Dectin-1, Dectin-2, and Mincle are members of a growing family of C-type lectins that are expressed by DCs and macrophages and are involved in the induction of adaptive immunity (Kerrigan and Brown, 2010). While Dectin-1 was initially described as a receptor that recognizes an unknown endogenous ligand, it is now mainly recognized as a PRR that is activated by β-(1,3)-glucans such as zymosan. These glucans are a major component of fungal cell walls, and consequently, Dectin-1 is most prominently recognized as a PRR that is specialized on the detection of fungal species such as *Pneumocystis carinii* or *Candida albicans* (Taylor *et al.*, 2007). However, Dectin-1 can also detect a number of mycobacterial species, even though β-glucans are absent from mycobacteria and the ligands mediating this recognition have so far remained elusive (Lee *et al.*, 2009; Rothfuchs *et al.*, 2007; Shin *et al.*, 2008; Yadav and Schorey, 2006). Dectin-2 is also a PRR that detects components of fungal cell walls. However, it recognizes α-mannans and is therefore able to detect fungal hyphae, while Dectin-1 cannot. Indeed, Dectin-2 deficiency renders mice highly susceptible to infections with several strains of *C. albicans*, while Dectin-1-deficient animals only succumb to these infections in a strain-specific manner (Bi *et al.*, 2010; Saijo *et al.*, 2007, 2010; Taylor *et al.*, 2007). Recent studies also showed that Mincle is another C-type lectin involved in the detection of fungal PAMPs, although the exact nature of the ligand has not been identified so far (Yamasaki *et al.*, 2009). In addition, Mincle also recognizes mycobacterial cord factor as well as necrotic cells (Ishikawa *et al.*, 2009; Matsunaga and Moody, 2009; Schoenen *et al.*, 2010; Yamasaki *et al.*, 2008).

Receptor signaling of these C-type lectins is quite different from that of TLRs and is more closely related to that of antigen receptors in lymphocytes. While the details of their signaling pathways differ depending on the particular receptor and cell type (Goodridge *et al.*, 2009), they all contain a noncanonical ITAM motif that recruits the tyrosine kinase Syk as an essential signaling adaptor. Following the activation of Dectin-1 in DCs, Syk then leads to the recruitment of

CARD9 and the subsequent activation of NF-κB via the Bcl10/Malt1 complex. As a result, the activation of Dectin-1 leads to the production of proinflammatory cytokines and chemokines, including IL-1, IL-6, and CCL3. In addition, Syk also activates MAPK and mobilizes Ca^{2+} that activates NFAT, which results in the production of additional cytokines including IL-23 (Gringhuis *et al.*, 2009). Both Dectin-2 and Mincle are thought to induce a similar set of signaling molecules. However, the detailed consequence of the activation of these receptors is still awaiting further investigation.

Like TLRs, activation of C-type lectins is sufficient for the induction of adaptive immunity. As Dectin-1 induces IL-23 but not IL-12, it has been linked more closely to the induction of T_H17 responses, although it appears to be also involved in the generation of T_H1 responses to mycobacteria (Acosta-Rodriguez *et al.*, 2007b; LeibundGut-Landmann *et al.*, 2007; Zenaro *et al.*, 2009). T_H17 cells are thought to direct immune response against extracellular microbes such as fungi, in part by recruiting neutrophils that kill the microbes by phagocytosis, release of antimicrobial peptides, and neutrophil extracellular traps (NETs). In this context, it is therefore still rather mysterious that intracellular mycobacteria are also potent activators of Dectin-1.

2.2. Cytosolic PRR

The extracellular array of PRRs is complemented by cytosolic receptors, some of which can also initiate adaptive immune responses upon infection of the cell by both cytosolic bacteria and viruses. The receptors fall into at least three classes, namely RIG-Iike receptors (RLRs), DNA sensors, and NLRs.

2.2.1. RIG-I-like receptors

RIG-I and MDA5 are both widely expressed cytosolic RNA helicases that are activated by RNA viruses and recognize the 5′-triphosphate moiety and higher-order structures of dsRNA, respectively (Pichlmair *et al.*, 2009; Schlee *et al.*, 2009). Stimulation of RIG-I and MDA5 results in the binding of the RLRs to the signaling adaptor MAVS, which leads to the activation of NF-κB and the induction of a TBK1 and IRF3-mediated type I interferon response. Interestingly, MAVS localizes to both peroxisomes and mitochondria, resulting in the fast and type I interferon-independent expression of antiviral genes by the former pool of MAVS and the delayed and type I interferon-dependent expression of antiviral genes by the latter pool of MAVS (Dixit *et al.*, 2010). Moreover, RIG-I can also trigger an MAVS-independent pathway that involves the signaling adaptor ASC independently of NLRP3 and leads to the production of IL-1β by caspase-1 (see below; Poeck *et al.*, 2010).

In addition to dsRNA viruses, for example, Reovirus, RIG-I and MDA5 also recognize ssRNA viruses that produce dsRNA during their life cycle (Loo *et al.*, 2008). Thus, some ssRNA viruses produce agonists for both RIG-I and MDA5 (Dengue and West Nile virus), while others activate either RIG-I (Vesicular Stomatitis Virus (VSV), Respiratory Syncytial Virus (RSV), Hepatitis C Virus (HCV), and Influenza) or MDA5 (Polio virus; Kato *et al.*, 2005, 2006; Loo *et al.*, 2008; Saito *et al.*, 2008). Recently, another nucleic acid-detecting receptor has been identified, called LRRFIP1 (Yang *et al.*, 2010). This receptor is not an RLR, but is still stimulated by the RNA of VSV. LRRFIP1 activates β-catenin in order to induce IFN-β. As β-catenin is more commonly known to act as a cofactor in the Wnt signaling pathway and therefore thought to regulate cellular functions like proliferation, differentiation, and adhesion, it is rather unusual to find it in an inflammatory context. However, this protein has also been suggested to negatively regulate NF-κB-driven inflammation in bacterial infections (Duan *et al.*, 2007). Thus, β-catenin may play a broader role in the regulation of innate immunity than initially anticipated.

2.2.2. DNA-sensing receptors

The cytosolic DNA of some viruses and bacteria such as herpes simplex virus 1 (HSV-1), vaccinia virus (VV), adenovirus, and *Legionella* is not sensed directly but induces a type I interferon response through the RIG-I/MDA5 pathway by generating dsRNA intermediates from AT-rich DNA upon transcription by RNA polymerase III (Ablasser *et al.*, 2009; Chiu *et al.*, 2009; Delaloye *et al.*, 2009). In addition to these indirect means of detection, several bona fide DNA sensors have also been postulated. One of these factors is DAI, which senses the Z form of dsDNA and triggers a type I interferon response via IRF3. While DAI can respond to human cytomegalovirus (CMV), DAI-deficient mice are still capable to mount a type I interferon response to exogenous B-DNA, suggesting the existence of additional factors that sense cytosolic DNA. Interestingly, LRRFIP1 can also sense both the B and Z forms of DNA in addition to RNA and thus presents an alternative pathway for the activation of a type I interferon response. Indeed, cells with reduced levels of LRRFIP1 exhibit a significantly reduced induction of IFN-β upon infection with *Listeria monocytogenes* (Yang *et al.*, 2010). Recently, the human protein IFI16 and its murine ortholog p204 have also been implicated in the cytosolic recognition of DNA (Unterholzner *et al.*, 2010). Activation of IFI16 via its HIN domain appears to induce both NF-κB and IFN-β upon the introduction of exogenous DNA into the cytosol or infection with HSV-1. This feature seems to separate IFI16 from the related protein AIM2, which is involved in the activation of caspase-1 but not the induction of IFN-β (see below). Finally, it is interesting to note that cytosolic DNA can also be derived from endogenous sources. The level of endogenous cytosolic DNA is

usually kept low by nucleases such as Trex1. However, the failure of this mechanism can lead to the accumulation of the ligands and cause interferon-driven autoimmune diseases (Stetson *et al.*, 2008).

2.2.3. NOD-like receptors

NLRs form a large group of widely expressed intracellular receptors that are characterized by an LRR domain thought to be responsible for ligand binding (even though direct binding of a ligand to an NLR has not been shown so far) and one of four N-terminal domains that mediate the activation of downstream targets. With respect to the control of adaptive immunity, the best-known NLRs are the CARD-domain containing NLRC1 and NLRC2 (NOD1 and NOD2). NLRC1 and NLRC2 are activated by γ-D-glutamyl-meso-diaminopimelic acid (meso-DAP) and muramyl dipeptide (MDP), respectively, which are derived from the bacterial cell wall component peptidoglycan (PGN). The mode by which the ligands gain access to the intracellular NLRC1 and NLRC2 is not well defined. Intracellular bacteria like *L. monocytogenes* are known to escape into cytosol, whereas MDP and meso-DAP from extracellular bacteria seem to be taken up by endocytosis and then transported into the cytosol via the transporters PepT1 and PepT2 (Ismair *et al.*, 2006; Swaan *et al.*, 2008). Upon stimulation, both NLRC1 and NLRC2 recruit RIP2, which in turn results in the activation of MAPK and NF-κB and subsequent production of inflammatory cytokines and costimulatory molecules (in DCs and macrophages; Todate *et al.*, 2001). In addition, NOD2 can also induce type I interferon in response to viral infections by activating the MAVS pathway (Sabbah *et al.*, 2009). Consequently, NLRC1 and NLRC2 have been shown to promote T_H1 and T_H17 responses and antigen-specific antibody responses (Fritz *et al.*, 2007; Kobayashi *et al.*, 2005; Shaw *et al.*, 2009; van Beelen *et al.*, 2007).

Some NLRs can form large multimeric complexes, termed inflammasomes, which are necessary for the activation of caspase-1 (Schroder and Tschopp, 2010). Caspase-1 activation, in turn, is required for the cleavage of pro-IL-1β and pro-IL-18 in order to generate and secrete IL-1β and IL-18. The secretion of active IL-1β and IL-18 therefore appears to be regulated at the level of posttranslational processing, while the transcriptional activation of pro-IL-1β and pro-IL-18 depends on proinflammatory stimuli such as LPS. Several distinct inflammasomes have been identified and are defined by the NLR protein contained in the complex: The NLRC4, NLRP1, and NLRP3 inflammasome (also called IPAF, NALP1, and NALP3 inflammasome, respectively). Following activation, the NLRP1 and NLRP3 inflammasomes activate caspase-1 via the adaptor protein ASC, while the NLRC4 inflammasome can activate caspase-1 directly (although NLRC4 may also require ASC in some cases). Bacterial products, such as flagellin

and anthrax lethal toxin, stimulate NLRC4 and NLRP1. In contrast, the activation of NLRP3 appears to be more complex. The range of stimuli involves microbial products, pore-forming toxins, inorganic crystals, and extracellular ATP (Dostert *et al.*, 2008; Eisenbarth *et al.*, 2008; Hornung *et al.*, 2008; Ichinohe *et al.*, 2009; Kahlenberg *et al.*, 2005; Mariathasan *et al.*, 2006; Muruve *et al.*, 2008; Shi *et al.*, 2003). It is therefore assumed that NLRP3 does not sense these stimuli directly but instead responds to cellular abnormalities resulting from these stimuli, such as membrane damage caused by inorganic crystals. The efflux of potassium may be an important intermediate step in this process, as ATP triggers the release of potassium through the purinergic ion channel P2X7 receptor and the other classes of stimuli might cause a similar efflux of potassium (Ferrari *et al.*, 2006; Kahlenberg *et al.*, 2005). One consequence of P2X7 activation may be the opening of the pannexin-1 pore, which allows the entry of microbial products such as MDP into the cytosol (Kanneganti *et al.*, 2007; Marina-Garcia *et al.*, 2008; Pelegrin and Surprenant, 2006; Pelegrin *et al.*, 2008). However, other models explaining inflammasome activation have also been put forward and include the generation of reactive oxygen species or the disruption of the lysosome and release of its microbial contents (Dostert *et al.*, 2008; Hornung *et al.*, 2008; Sharp *et al.*, 2009).

Recently, another inflammasome has been described that is defined by the PRR AIM2 (Burckstummer *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Roberts *et al.*, 2009). While AIM2 does not belong to the NLR family, it still relies on ASC in order to activate caspase-1. AIM2 responds to dsDNA and therefore has been implicated in the sensing of DNA viruses like VV and mouse CMV as well as the bacteria *Francisella tularensis* and *L. monocytogenes* (Fernandes-Alnemri *et al.*, 2010; Rathinam *et al.*, 2010; Sauer *et al.*, 2010; Warren *et al.*, 2010). Interestingly, the latter microbe, which is also sensed by the NLRP3 inflammasome, needs the expression of the pore-forming toxin LLO for inflammasome stimulation, indicating that the escape from the lysomes is an essential step in the activation process of either NLRP3 or AIM2 by this microbe (Kim *et al.*, 2010; Meixenberger *et al.*, 2010).

An important feature of NLRP3 (and AIM2) inflammasome activation is the requirement of an additional signal that is delivered by LPS and other microbial stimuli or even TNFα and other cytokines that activate NF-κB. It therefore appears that the NLRP3 inflammasome is not able to be activated without a priming signal from another PRR and therefore is unlikely to induce adaptive immune responses on its own. Nonetheless, IL-1β and IL-18 are important cytokines for the instruction of T cell responses and thus inflammasomes play an important role in the innate instruction of adaptive immunity.

3. CELL-TYPE-SPECIFIC PRR DISTRIBUTION AND THE INTERPLAY BETWEEN PRRS IN ADAPTIVE IMMUNITY

Unlike receptors of the adaptive immune system, PRRs are broadly expressed across multiple migratory and nonmigratory cell types. All these cell types can therefore detect the presence of infection and can theoretically contribute to innate control of adaptive immunity. Elucidating the relative contributions of the various cell types that can detect infection through PRRs is and will continue to be a challenging task since so many cell types can respond to the presence of infection in so many different, yet often partially overlapping, ways. In experimental settings, it is possible to create situations that target individual PRRs and studies that specifically address the functions of individual PRRs have contributed tremendously to the understanding of innate instruction of adaptive immunity. However, such conditions never occur in real-life scenarios. Infectious microorganisms contain ligands for multiple different classes of PRRs, can trigger the same PRR in multiple different cell types simultaneously, and have access to various cellular compartments, all of which results in the activation of multiple classes of PRRs during actual infections. Moreover, simultaneous infection by different microbes can fundamentally alter the outcome of the infections (Barton *et al.*, 2007; Gumenscheimer *et al.*, 2007; Humphreys *et al.*, 2008; Jamieson *et al.*, 2010; Navarini *et al.*, 2006). Consequently, a unifying and systematic insight into the interaction between multiple PRRs is still lacking.

While any given class of PRRs utilizes the same basic signaling machinery, it can still trigger distinct responses depending on the cell type that is activated, the ligand that is recognized, and the recent history of the particular responding cell. For TLRs, which can exhibit a particularly broad range of responses, this distinction is aided by the use of different signaling adaptors. Both TLR2 and TLR4 employ MyD88 in order to induce proinflammatory cytokines while TLR4 also recruits TRIF in order to induce type I interferons. In addition, some TLRs induce distinct responses using the same signaling adaptor. Both TLR7 and TLR9 induce a MyD88-dependent type I interferon responses in plasmacytoid DCs (pDC), while the stimulation of these TLRs in other cell types does not induce a type I interferon response (Gilliet *et al.*, 2008). Moreover, B-type CpG DNA induces only proinflammatory cytokines in pDCs, whereas A-type CpG DNA also results in the production of type I interferon. An analogous difference has also been observed recently for TLR2, which can induce a MyD88-dependent type I interferon response in inflammatory monocytes but not in other myeloid cells (Barbalat *et al.*, 2009). Interestingly, the ability of TLR2 to trigger a type I interferon response in the former cell type depends on the particular ligand.

VV induces such a response while Pam_3CSK_4 does not. The mechanisms underlying this distinction are not well understood. However, the signal triggering the release of proinflammatory cytokines is generated by plasma membrane-associated MyD88, while the induction of a type I interferon response originates from an endosomal pool of MyD88 (Barbalat *et al.*, 2009). The latter aspect seems to be a common feature of all type 1 interferon-inducing TLRs including TLR4, which induces proinflammatory cytokines via the plasma membrane-associated MyD88 and type I interferon via the endosomal TRIF (Kagan *et al.*, 2008). In addition to its effects in pDCs, VV can also induce type I interferons in other myeloid cells. However, in these cells TLR2 induces only proinflammatory cytokines while the type I interferon response is dependent on MDA5 (Delaloye *et al.*, 2009; Zhu *et al.*, 2007).

The relative contributions of TLR versus RLR activation to the type I interferon response and induction of adaptive immunity has been studied in experimental settings that include both immunizations and viral infections. Poly:IC is recognized by both TLR3 and MDA5. Protein immunizations using poly:IC as adjuvant revealed that the MAVS signaling pathway is required for the induction of antibody responses, while the TRIF pathway is not required (Kumar *et al.*, 2008). Nonetheless, the TRIF pathway contributes to the generation of the antibody response, as it is even more defective in MAVS/TRIF-deficient compound mutant mice. The two signaling pathways also cooperate in the induction of both $CD4^+$ and $CD8^+$ T cell responses, which are reduced in mice defective in TLR3 or MDA5 signaling and completely abrogated in MAVS/TRIF-deficient compound mutant mice (Kumar *et al.*, 2008; Trumpfheller *et al.*, 2008). These findings are in contrast to the observations made for the adaptive immune responses against lymphoid choriomeningitis virus (LCMV) and Influenza virus. In LCMV, MAVS-mediated signaling contributes to the secretion of IFN-α. However, MyD88-dependent signaling is the major driver of the adaptive immune response. The serum levels of both type I interferons and proinflammatory cytokines are strongly reduced in MyD88-deficient mice, most likely due to the activation of TLRs in pDCs (Jung *et al.*, 2008). Consequently, MyD88 but not MAVS is required for the inductions of a $CD8^+$ T cell response against LCMV. Similarly, both pathways also contribute to the induction of type I interferons upon infection with Influenza virus. The response is completely defective in MyD88/MAVS-deficient compound mutant mice (Koyama *et al.*, 2007). However, the induction of a $CD4^+$ T cell response as well as the antibody response depends on MyD88 signaling but not MAVS signaling. Interestingly, the $CD8^+$ T cell response required neither MyD88 nor MAVS, suggesting either redundancy between the two pathways in this regard or the involvement of additional PRRs. Cooperation between the MyD88 and MAVS pathways has also been implicated in the generation of

adaptive immunity in response to infection with RSV. While the type I interferon response in RSV infections depends entirely on MAVS signaling, both MyD88 and MAVS are involved in the clearance of the virus. The two pathways also synergistically contribute to the generation of antibody responses but neither MyD88 nor MAVS is required for the induction of CD8$^+$ T cell responses (Bhoj *et al.*, 2008). Interestingly, though, NOD2 signaling via MAVS has been identified as a critical factor in the generation of protective immunity to RSV (Sabbah *et al.*, 2009). Together, these examples show that while PRRs can operate in isolation in some experimental settings, they more commonly act in collaboration to control infections, although the relative contributions of individual PRRs to the instruction of adaptive immunity can vary greatly and depends on the specific infection.

A similar cooperation between different classes of PRRs has also been observed for C-type lectins such as Dectin-1 and TLRs in order to achieve a maximal induction of the adaptive immune response. While the activation of Dectin-1 alone is sufficient to instruct an adaptive immune response, Dectin-1 also synergizes with several TLRs to signal the production of proinflammatory cytokines including TNFα in response to both fungal and bacterial infections (Dennehy *et al.*, 2008; Ferwerda *et al.*, 2008; Lee *et al.*, 2009; Netea *et al.*, 2006; Shin *et al.*, 2008; Yadav and Schorey, 2006). Importantly, the interaction between Dectin-1 and TLRs signaling can also alter the adaptive immune response qualitatively as it has been implicated in shifting the balance between the IL-12-dependent T_H1 and the IL-23-dependent T_H17 response in fungal infections (Dennehy *et al.*, 2009; Gerosa *et al.*, 2008). Coactivation of these pathways in DCs and macrophages triggers the production of IL-6, IL-10, and IL-23, and suppresses production of IL-12 as compared to TLR activation alone (Dennehy *et al.*, 2009). It is not entirely clear how this interaction is regulated on the molecular level, although both Syk and MyD88 are necessary (Dennehy *et al.*, 2008, 2009).

4. INNATE CONTROL OF CD4$^+$ T CELL RESPONSES

The activation of PRRs results in the upregulation of costimulatory molecules and the secretion of many cytokines by the APCs, of which multiple are involved in the instruction of T cell responses. In addition to these indirect means of control, T cells also express a number of PRRs themselves, suggesting that PRRs may shape the ensuing T cell response directly upon encounter of the appropriate PAMPs. We will first discuss the CD4$^+$ T cell-intrinsic function of PRR activation and will then review the role of cytokines in the control of CD4$^+$ T cell responses with a particular emphasis on the effects of IL-1 and IL-6.

4.1. Cell-autonomous control of $CD4^+$ T cell responses

$CD4^+$ T cells express several classes of PRRs. Both murine and human $CD4^+$ T cells express most TLRs, even though different studies have come to varying conclusions about the precise pattern of the TLRs expressed in particular $CD4^+$ T cell subsets or activation states. *In vitro* studies showed that stimulation of T cells with some TLR ligands (in particular, TLR2 and TLR9 agonists) has costimulatory effects that lead to enhanced proliferation and secretion of IL-2. Exposure of $CD4^+$ T cells to CpG DNA also induces BCL_{XL}, suggesting that the activation of TLRs in $CD4^+$ T cells enhances survival under some conditions (Gelman *et al.*, 2004). Interestingly, under these conditions, MyD88 not only activates NF-κB but also associates with PI3K in order to phosphorylate Akt and GSK-3 (Gelman *et al.*, 2006). The latter pathway induces IL-2 production and proliferation, while the former pathway provides survival signals. In addition to their role in facilitating the proliferation and survival of $CD4^+$ effector T cells, TLRs can also influence $CD4^+$ $CD25^+$ regulatory T cells (Tregs) directly by dampening their suppressive capabilities (LaRosa *et al.*, 2007). Activation of both TLR2 and TLR9 leads to the expansion of Tregs. However, in the presence of TLR ligands, Tregs transiently express lower levels of FoxP3 and lose their ability to suppress effector T cells and regain this function once TLR stimulation ceases (Liu *et al.*, 2006; Sutmuller *et al.*, 2006). Thus, some TLRs seem to stimulate both $CD4^+$ effector T cells and suppress Tregs concurrently in order to promote the expansion of the effector T cell population. However, this conclusion is based mostly on results from *in vitro* experiments and at the present time, it is unclear to what extent these findings translate into *in vivo* situations. The direct contribution of TLR signaling to $CD4^+$ T cell responses *in vivo* has been mostly analyzed using MyD88-deficient mice. Bone marrow chimeras with a MyD88-deficient T cell compartment display a decreased ability to generate a T_H1 response to *Toxoplasma gondii*, resulting in an increased lethality that was comparable to that of MyD88-deficient mice (LaRosa *et al.*, 2008; Scanga *et al.*, 2002). Likewise, MyD88-deficient naïve $CD4^+$ T cells fail to induce colitis following their transfer into Rag2-deficient mice, whereas wild-type T cells cause disease (Fukata *et al.*, 2008; Tomita *et al.*, 2008). Moreover, when both cell types are cotransferred, MyD88-deficient T cells do not expand as effectively as wild-type control cells. T cell-intrinsic MyD88 signaling is also important for the induction of antibody responses by $CD4^+$ T cells as well as the control of LCMV infections by $CD8^+$ T cells (Gelman *et al.*, 2006; Rahman *et al.*, 2008). These findings are therefore consistent with the notion of a direct TLR-mediated control of T cell responses. However, as these studies mainly employed MyD88-deficient T cells rather than TLR-deficient T cells, it is important to keep in mind that these observations may instead be attributable to defective signaling of

IL-1 family members in these cells (see next section). Indeed, bone marrow chimeras with a T cell compartment lacking specific TLRs were all as resistant to *T. gondii* infection as wild-type mice, suggesting that the defect cannot be pinpointed to a single TLR (Debierre-Grockiego *et al.*, 2007; Hitziger *et al.*, 2005; Minns *et al.*, 2006; Scanga *et al.*, 2002). Nonetheless, a recent study demonstrated that mice with a TLR2-deficient T cell compartment generated weaker T_H17 responses and were more resistant to experimental autoimmune encephalomyelitis (EAE; Reynolds *et al.*, 2010). This study showed clearly that direct T cell-intrinsic TLR2 activation can positively influence the course of the T cell response, at least under some conditions.

Besides TLRs, T cells also express RLRs and NLRs. NOD2-deficient mice are highly susceptible to infections with *T. gondii*, even though this pathogen is devoid of the NOD2 ligand MDP (Shaw *et al.*, 2009), suggesting the existence of additional NOD2 ligands. Importantly, NOD2-deficiency results in an impairment of IFNγ secretion and this defect can be traced to T cell-intrinsic requirement for NOD2 to generate a T_H1 response. Moreover, NOD2-deficient T cells are unable to induce colitis upon transfer into lymphopenic hosts (Shaw *et al.*, 2009). This defect is associated with defective production of IL-2 by the NOD2-deficient T cells, which is reminiscent of the role of TLR stimulation in the induction of T cell responses.

4.2. Indirect control of $CD4^+$ T cell responses

While it seems clear that PRRs can control $CD4^+$ T cell response directly under some conditions, they are best known for their ability to instruct the response indirectly by inducing the upregulation of costimulatory molecules and the secretion of cytokines and/or type I interferons by APCs. It is this interaction between APCs and T cells that shapes the ensuing T cell response. For the purpose of this chapter, we want to focus mainly on the roles of IL-1 and IL-6 in the control of adaptive immunity as the secretion of these two cytokines is a particularly prominent feature of PRR activation and because there is considerable evidence that these two cytokines play critical roles in controlling T cells responses.

4.2.1. The effects on IL-1 on $CD4^+$ T cell responses

Two related genes encode IL-1. IL-1α is widely expressed and contains a leader sequence for the secretion of the protein without further processing. It is usually associated with the plasma membrane of the producing cell and thus acts locally. In contrast, the leaderless IL-1β, whose expression is mostly restricted to APCs and neutrophils, requires caspase-1 for its cleavage from pro-IL-β and subsequent secretion as a systemically acting protein. Despite the fundamental

differences in the regulation of IL-1α and IL-β, their biological activities are thought to be similar.

As IL-1 is a pleiotropic cytokine that acts on many cell types and tissues, it has been difficult to distinguish between its direct and indirect effects on $CD4^+$ T cell responses. Nonetheless, it is clear that IL-1 controls several aspects of T cell responses directly (Dinarello, 2009; Sims and Smith, 2010). IL-1 is involved as costimulator together with antigen in the generation of both a primary as well as a secondary $CD4^+$ T cell response, in part by facilitating IL-2 signaling through the upregulation of the IL-2 receptor α (CD25) and preventing of apoptosis through the activation of NF-κB and PI3K (Ben-Sasson *et al.*, 2009; O'Neill, 2008). Thus, IL-1 serves as a general activator of $CD4^+$ T cell responses, even though the precise roles of this cytokine in this process are not completely understood.

In recent years, IL-1 received considerable attention because of its effects in T_H17 differentiation. Naïve $CD4^+$ T cells express very low levels of the IL-1R but upregulate it following activation though the TCR and CD28 (Chung *et al.*, 2009). T_H17 cells express high levels of the IL-1R, and multiple studies have shown that IL-1 promotes the differentiation of naïve $CD4^+$ T cells into T_H17 cells *in vitro* (Acosta-Rodriguez *et al.*, 2007a; Chung *et al.*, 2009; Kryczek *et al.*, 2007; Wilson *et al.*, 2007). IL-1 signaling in $CD4^+$ T cells is also required for the induction of T_H17 cells *in vivo* and, consequently, $CD4^+$ T cells deficient in IL-1 signaling fail to induce EAE and colitis, which are both T_H17-driven diseases (Chung *et al.*, 2009; Fukata *et al.*, 2008; Sutton *et al.*, 2006; Tomita *et al.*, 2008). The precise function of IL-1 signaling in the differentiation of T_H17 cells is still not well defined. However, a picture has begun to emerge that suggests a function of IL-1 in the early phase of this process (Chung *et al.*, 2009). In particular, IL-1 induces the expression of the transcription factors RORγt and IRF4, both of which are required for T_H17 development, and triggers the mTOR pathway to induce proliferation of T_H17 (Chung *et al.*, 2009; Gulen *et al.*, 2010). In addition, IL-1 appears to synergize with IL-23 to ensure the maintenance of T_H17 cells.

It is still unclear to what extent T_H1 cells express the IL-1R. Some reports do not detect the receptor, while others find evidence for the expression of IL-1R on T_H1 cells, albeit at much lower levels than T_H17 cells (Chung *et al.*, 2009; Guo *et al.*, 2009; Taylor-Robinson and Phillips, 1994). Irrespective of the precise regulation of IL-1R following the activation of $CD4^+$ T cells, however, IL-1 appears to be involved in the generation of T_H1 responses. The exogenous administration of IL-1 enhances T_H1 responses and bone marrow chimeras harboring IL-1R-deficient T cells generate reduced numbers of T_H1 cells during the course of EAE (Ben-Sasson *et al.*, 2009; Chung *et al.*, 2009). Therefore, IL-1 plays a role in the generation of both T_H1 and T_H17 responses. In contrast, the IL-1-related cytokine IL-18 seems to be more exclusively linked to the

generation of T_H1 responses. T_H1 cells express high amounts of IL-18R in a T-bet-dependent fashion and IL-18 synergizes with IL-12 to induce INFγ. In this regard, the function of IL-18 resembles that of IL-1 and IL-33, namely that it reinforces the T_H1 lineage decision like IL-1 does for T_H17 cells and IL-33 for T_H2 cells (Guo *et al.*, 2009). Thus, members of the IL-1 family appear to be involved in both the activation of $CD4^+$ T cells as well as maintaining the subsequent lineage commitment decision.

In addition to its effects on the development of specific $CD4^+$ T effector subsets, IL-1 is also involved in the regulation of the interaction between effector T cells and Tregs. Tregs express the IL-1R themselves (Chaudhry *et al.*, 2009; Mercer *et al.*, 2010). While the effects of IL-1 on Treg function are not clearly understood, it has been suggested that IL-1 enables T cell responses by blocking the suppressive function of Tregs (O'Sullivan *et al.*, 2006). Alternatively, Tregs may deprive $CD4^+$ effector T cells of IL-1 (Chaudhry *et al.*, 2009). Moreover, IL-1 has been implicated in enabling the conversion of induced Tregs (iTregs) into T_H17 cells (Chung *et al.*, 2009).

4.2.2. The effects on IL-6 on $CD4^+$ T cell responses

Similar to IL-1, IL-6 is a pleiotropic cytokine that is intimately involved in the control of T cell responses. The IL-6 signaling complex consists of the specific IL-6Rα chain and the more promiscuous signaling component gp130 that is also a common component of other cytokine receptors such as the receptors for LIF, IL-11, and oncostatin-M. Signals emanating from the IL-6R are transduced mainly by JAK1 and then relayed by Stat3, although Stat1 plays also a role in this process. Importantly, IL-6R signaling also activates the MAPK and PI3K pathways and activation of Akt by the PI3K pathway has been implicated in the role of IL-6 as a survival factor. In addition to the more conventional transmembrane form of IL-6Rα, some cell types (e.g., macrophages and neutrophils) also produce a soluble version of the receptor (sIL-6Rα), which can bind together with IL-6 to gp130 and thus induce a signal in gp130-expressing cells. The physiological relevance of this process, which is referred to as trans-signaling, in T cell biology is not fully understood. IL-6 trans-signaling has been implicated in the advancement of chronic inflammatory diseases (Rose-John *et al.*, 2006). Recent evidence suggests that the expression of IL-6Rα is restricted to $CD62L^+$ naïve and central memory $CD4^+$ T cells, whereas effector T cells downregulate IL-6Rα (Jones *et al.*, 2010). Consistent with this expression pattern, T_H17 effector T cells seem to require IL-6 trans-signaling for the local lineage maintenance in the inflamed tissue, where neutrophils are a major source of sIL-6Rα (Hurst *et al.*, 2001; Jones *et al.*, 2010).

IL-6 positively influences the survival of $CD4^+$ T cells. For example, it induces the expression of Bcl-2 and downregulates FasL, thus protecting the cells from activation-induced cell death (Ayroldi *et al.*, 1998; Dienz

et al., 2009; Kishimoto and Sprent, 1999; Lotz *et al.*, 1988; Teague *et al.*, 1997). The antiapoptotic function of IL-6 has also been implicated in the positive influence of IL-6 on the expansion of activated $CD4^+$ T cells following immunization (Rochman *et al.*, 2005). However, this effect may also reflect another proposed function of IL-6, namely that it renders naïve $CD4^+$ T cells insensitive to the suppressive effects of Tregs (Pasare and Medzhitov, 2003). Thus, T_H1 responses are severely compromised in IL-6-deficient mice immunized with protein in the presence of LPS, but can be restored upon transient depletion of Tregs.

In addition to its role in activation of T_H1 cells, IL-6 is important for the differentiation of T_H17 cells, which also involves TGF-β (Acosta-Rodriguez *et al.*, 2007a; Bettelli *et al.*, 2006; Miossec *et al.*, 2009; Veldhoen *et al.*, 2006). These triggers are thought to lead to the autocrine activation of the cells by IL-21, which expands the cells (Korn *et al.*, 2007; Nurieva *et al.*, 2007; Zhou *et al.*, 2007). Interestingly, the expression of IL-21 is itself controlled by IL-6 (Dienz *et al.*, 2009). IL-23 then stabilizes the differentiation program and induces effector cytokines such as IL-17 (Mangan *et al.*, 2006; Veldhoen *et al.*, 2006; Zhou *et al.*, 2007). IL-6 also influences the balance between T_H17 cells and iTregs. The addition of IL-6 inhibits the TGFβ-driven expression of FoxP3 and promotes the expression of RORγt *in vitro* (Bettelli *et al.*, 2006). Consequently, $CD4^+$ T cells are thought to differentiate into iTregs at higher levels in IL-6-deficient mice upon immunization (Korn *et al.*, 2007). Interestingly, the negative regulation of iTreg induction by IL-6 might involve IL-6 trans-signaling, which has been shown to induce the TGFβ signaling inhibitor SMAD7 (Dominitzki *et al.*, 2007). However, Tregs can express the IL-6Rα themselves, so IL-6 may also act on these cells directly.

In addition to its involvement in T_H17 differentiation, other $CD4^+$ T cell subsets may also rely on IL-6 for their generation. Recently, IL-6 has been implicated in the generation of follicular T helpers (T_{FH}) cells (Nurieva *et al.*, 2008, 2009). T_{FH} cell differentiation is governed by the lineage defining transcription factor Bcl6 (Johnston *et al.*, 2009; Nurieva *et al.*, 2009). Bcl6 is induced by IL-6 *in vitro*, suggesting that IL-6 drives the differentiation of T_{FH} cells (Nurieva *et al.*, 2008, 2009) Consistent with this idea, T_{FH} cells also depend on IL-21 for their development, which is also induced by IL-6 (Nurieva *et al.*, 2008). However, $CD4^+$ T cells carrying a TCR transgene are able to generate T_{FH} cells following their transfer into IL-6-deficient mice, indicating that IL-6 is either not essential or redundant *in vivo* (Poholek *et al.*, 2010).

The effects of both IL-1 and IL-6 on the generation of $CD4^+$ T cell responses are remarkably similar. Both have been implicated in the release from Treg-mediated suppression, the differentiation of T_H17 cells, and in the shift from a tolerant to an inflammatory state, resulting in the conversion of Tregs into T_H17 cells. Hence, IL-1 and IL-6 are

presumably acting in a cooperative fashion on $CD4^+$ T cells, even though their intracellular signaling pathways are nonoverlapping. This notion is further illustrated by the observation that under some conditions, IL-1 induces the expression of IL-6Rα (Chung *et al.*, 2009).

Despite the fundamental involvement of both IL-1 family members and IL-6 on the generation of $CD4^+$ T cell responses, multiple microbes can trigger $CD4^+$ T cell responses that are independent of these cytokines. For example, the T_H1 response to *L. monocytogenes*, *Salmonella typhimirium*, *T. gondii*, LCMV, and HSV-1 requires neither IL-1 nor IL-18 (Kursar *et al.*, 2004; LaRosa *et al.*, 2008; Seibert *et al.*, 2010; Zhou *et al.*, 2009). The $CD4^+$ T cell response to *L. monocytogenes* also does not depend on IL-6 (Simone Nish, Dominik Schenten, Igor Brodsky, Ruslan Medzhitov, unpublished).

These examples are surprising given the apparent importance of IL-6 and members of the IL-1 family of cytokines in the generation of $CD4^+$ T cell responses under various experimental conditions. Most of the studies that identified such requirements assessed the $CD4^+$ T cell response *in vitro* or following protein immunization *in vivo*, although some studies using microbial infections such as Influenza further confirm the importance of these cytokines in the generation of $CD4^+$ T cell responses (Ichinohe *et al.*, 2009; Longhi *et al.*, 2008). On the other hand, the apparent lack of a requirement for IL-1, IL-18, or IL-6 for primary $CD4^+$ T cell responses against a variety of infections suggests that the requirement of these cytokines for the induction of $CD4^+$ T cell responses is not universal. It is therefore possible that during the course of some infections, alternative cytokines are released that for some cellular processes carry similar information as IL-1 or IL-6 do. For example, cytosolic infections may cause the secretion of type I interferons or IL-15 that prevent $CD4^+$ T cells from undergoing apoptosis or render them refractory to Treg-mediated suppression. The latter scenario has indeed been proposed for IL-15 (Ben Ahmed *et al.*, 2009). In addition, we also speculate that seemingly similar $CD4^+$ T cell responses are not necessarily identical. Thus, both Influenza and LCMV may induce the generation of different subsets of T_H1 cells, with the former being IL-1 dependent and the latter IL-1 independent. T_H1 responses are usually identified by the ability of the $CD4^+$ T cells to secrete IFNγ. However, additional cytokines or other factors involved in effector function may be differentially secreted among the subsets and thus more suited to distinguish between different subsets.

Such a concept is currently emerging in the context of T_H17 differentiation, which appears to be more complex as it may not always involve IL-1 or IL-6. Indeed, T_H17 cells can also be differentiated *in vitro* by TGFβ and IL-21 in the absence of IL-6 (Korn *et al.*, 2007). Moreover, IL-6- or TGFβ-independent T_H17 responses can be generated under certain circumstances *in vivo* (Ghoreschi *et al.*, 2010; Korn *et al.*, 2008). Importantly, T_H17 differentiation can be restored in IL-6-deficient mice in

the absence of Tregs, at least following the immunization with Complete Freund's Adjuvant (Korn *et al.*, 2007). Thus, there seems to be a considerable degree of plasticity in the differentiation of T_H17 cells, which may reflect the existence of distinct T_H17 subsets. The existence of distinct T_H17 subpopulations is also supported by the observation that IL-23 appears to drive the differentiation of T_H17 cells that cause disease in the EAE mouse model of multiple sclerosis, whereas TGFβ and IL-6 can induce T_H17 cells that can also secrete the anti-inflammatory cytokine IL-10 (Ghoreschi *et al.*, 2010; McGeachy *et al.*, 2007).

5. B CELL-INTRINSIC CONTROL OF HUMORAL IMMUNE RESPONSES BY PRRS

The role of TLR ligands in B cell activation has been appreciated since the early days of B cell immunology. LPS is the prototypical T-independent type I (TI-1) antigen that induces an IgG3-dominated antibody response. However, the traditional understanding of T-dependent (TD) antibody responses assumed that B cells rely on B cell receptor stimulation and T cell help for their activation and that the latter comes from T cells that have been activated by PRR-exposed DCs. Thus, innate means of self/nonself discrimination were considered to occur solely in DCs. It was therefore surprising that TLR stimulation on B cells is an important contributor to the generation of efficient T-dependent antibody responses. Consistent with the induction of T_H1 by TLRs, IgG2c antibody responses were particularly affected in the absence of LPS-induced TLR signaling (Pasare and Medzhitov, 2005). Since this initial observation, similar findings have been made in other systems and confirmed that B cell-specific TLR activation by LPS, RNA, and CpG DNA is important for the IgG2c response to immunizations, virus-like particles, and several bacterial and viral infections (Barr *et al.*, 2009; Guay *et al.*, 2007; Heer *et al.*, 2007; Jegerlehner *et al.*, 2007; Ruprecht and Lanzavecchia, 2006). Likewise, B cell-specific TLR signals were also required for the induction of antibody-driven autoimmune diseases (Herlands *et al.*, 2008; Leadbetter *et al.*, 2002; William *et al.*, 2005). While these findings supported the notion that B cell-intrinsic TLR-mediated signals enhance or modulate the B cell response, two other experiments suggested that TLRs are required neither specifically in B cells nor in general for the generation of T-dependent antibody responses (Gavin *et al.*, 2006; Meyer-Bahlburg *et al.*, 2007). These findings were initially confusing, yet it seems clear now that the discrepancies are due to the use of native protein antigen in the former group of studies versus haptenated protein antigens in the latter studies (Palm and Medzhitov, 2009).

The study of the role of PRR signals in the generation of antibody responses are complicated by such subtle differences among the immunizing antigens. It is likely that differences in the affinity, avidity, or chemical nature of the immunizing antigen as well as the choice of adjuvant and route of immunization fundamentally influence the outcome of adaptive immune responses. With this in mind, what then might cause the difference in TLR dependence between haptenated and native proteins? One possibility is that the chemical properties of the hapten itself render the protein immunogenic as the hapten is triggering an innate pathway that is redundant to the TLR pathway. The pathway may therefore be part of an antibody response that is directed against harmful xenobiotics (Palm and Medzhitov, 2009). Alternatively, haptenation of proteins creates multivalent epitopes, particularly in the context of commonly used adjuvants like alum and mineral oil. The degree of repetition of epitopes on particles influences the magnitude of a TD antibody response, irrespective of the overall concentration of the particles (Jegerlehner *et al.*, 2002). Interestingly, defects in complement fixation require a higher degree of repetitiveness in order to induce an antibody response of similar magnitude. Thus, complement lowers the activation threshold of the BCR by binding to CD21. It is therefore possible that TLR activation has the analogous effect in that it converts weak antigens with a low degree of repetitive epitopes into highly immunogenic ones by providing a stronger activation signal to the B cells.

In addition to their role in B cell activation, TLRs also shape the nature of the ensuing B cell response. B cell-intrinsic TLR activation has been implicated in generating an IgG2c response that can occur at the expense of an IgG1 response (Jegerlehner *et al.*, 2007; Pasare and Medzhitov, 2005). However, the underlying mechanisms for this effect are less clear. One possibility is a direct influence of TLR on class switch recombination (CSR; Jegerlehner *et al.*, 2007). Consistent with this notion is the finding that B cell-intrinsic TLR signaling activates germ-line transcription of the Ig constant regions. Alternatively, B cell-intrinsic TLR signaling appears also to influence CSR indirectly by facilitating the differentiation into T_H1 cells (or T_{FH} cells), which secrete IFNγ that induces CSR to IgG2c. Such an example was observed during *Salmonella enterica* infections, in which the T_H1 and T_H17 response required B cell-derived IL-6 or IFNγ (Barr *et al.*, 2009). Interestingly, the induction of the primary T cell response was dependent on B cell-intrinsic MyD88 but not BCR signals, whereas the memory T cell response required solely BCR-mediated signals. As many of the studies on the B cell-intrinsic role of TLR signaling involve MyD88-deficient mice, it is important to note that MyD88 signaling has also been implicated in the signal transduction of two additional pathways. First, MyD88 appears to associate with the IFNγ receptor in order to stabilize the mRNA of IFNγ-induced genes (Sun and Ding, 2006). Second, MyD88

seems to regulate CSR during TI and perhaps also TD antibody responses in a TLR- and IL-1R-independent manner that was dependent on the BAFF and APRIL receptor TACI (He *et al.*, 2010). It therefore possible that some of the B cell-intrinsic effects of TLR signaling are in fact mediated by other cytokines like IFNγ, BAFF, APRIL, or even members of the IL-1 family.

6. PATHOLOGICAL CONSEQUENCES OF DEFECTIVE PRR SIGNALING IN HUMANS

Most of our knowledge about the mechanisms of innate instruction of adaptive immunity is derived from the analysis of cell lines and mice. Genetic mutations have provided valuable insights into the function of the human immune system and its interaction with the environment (Fischer, 2007). Mutations affecting innate immunity, in general, and PRR signaling, in particular, are very rare, thus illustrating the general importance of the innate immune system and its signaling pathways. Nonetheless, mutations causing a functional impairment have been identified in genes of several PRR signaling pathways in humans. The most prominent examples are mutations in TLRs and the signaling adaptors MyD88 and IRAK-4 as well as mutations in IL-12 or its receptor (Altare *et al.*, 1998; Casrouge *et al.*, 2006; Fieschi *et al.*, 2003; George *et al.*, 2010; Ku *et al.*, 2007; Misch and Hawn, 2008; Picard *et al.*, 2002, 2003; von Bernuth *et al.*, 2008; Zhang *et al.*, 2007). More recently, human mutations affecting Dectin and NLR signaling have also been identified (Glocker *et al.*, 2009; Hugot *et al.*, 2001; Ogura *et al.*, 2001).

Genetic polymorphisms in most human TLRs have been associated with an increased rate of infections in the afflicted individuals. Patients deficient of MyD88 or IRAK-4 signaling suffer from pyogenic bacterial infections, often due to *S. pneumoniae* or *S. aureus* (Ku *et al.*, 2007; Picard *et al.*, 2003; von Bernuth *et al.*, 2008). Likewise, individuals with defective IL-12 signaling are commonly infected by mycobacteria, presumably because of their inability to generate a T_H1 response (or in some patients, a T_H17 response; de Beaucoudrey *et al.*, 2008). These infections usually strike during childhood and are life-threatening with a high rate of mortality. However, the spectrum of the infections is quite narrow, particularly considering the central importance of MyD88 and IRAK-4 in both TLR and IL-1 signaling. The diseases can usually be managed with proper antibiotic treatment and decline in severity with age, resulting in a largely disease-free state in adulthood, even in the absence of prophylactic treatment (Bousfiha *et al.*, 2010). Similarly, patients deficient of TLR3 signaling due to mutations in the receptor-encoding gene itself, the downstream signaling adaptor TRAF3, or in UNC93B1, which is required for proper intracellular processing of the

endosomal TLRs, suffer from HSV-1-driven encephalitis (HSE) in early childhood that disappears in adulthood (Casrouge *et al.*, 2006; Perez de Diego *et al.*, 2010; Zhang *et al.*, 2007). However, declining susceptibility with age is not a universal feature of PRR signaling defects in humans. Individuals carrying a homozygous mutation in CARD9, a signaling adaptor downstream of Dectin-1, Dectin-2, and Mincle, suffer from recurrent *Candida* infections that are associated with a high rate of mortality, presumably due to an inability to generate antifungal T_H17 responses (Glocker *et al.*, 2009). Moreover, mutations in Dectin-1 have also been associated with an increased rate of mild *Candida* infections (Ferwerda *et al.*, 2009; Plantinga *et al.*, 2009).

The remarkably narrow spectrum of infections and decreased rate of recurrent infections with increasing age in the documented surviving patients with defective TLR and IL-1 signaling can be interpreted as indication that human TLR and IL-1 signaling is not important for the generation of protective adaptive immune responses in general and antibody responses in particular (Bousfiha *et al.*, 2010). However, one should emphasize that the patients with these severe immunodeficiencies presumably would not have survived the bacterial assaults without antibiotic intervention and that the development of a subsequently protective adaptive response was able to occur under the protection of such treatment. Moreover, the patients are also protected by the conditions of improved hygiene of the modernized world. It is questionable whether their resistance to infections could be maintained under the conditions that existed throughout most of human evolution other than the period of the past 50–100 years. Finally, as outlined in this chapter, innate control of adaptive immunity involves multiple PRR systems with overlapping functions. It is therefore not surprising that some replicating microbes trigger these alternative systems in order to generate protective immunity, while others do not. Thus, CARD9-deficient patients fail to contain fungal infections, while MyD88-deficient individuals develop protective immunity if they survive the primary infections. Indeed, there is precedence for this situation in MyD88-deficient mice, which generate a normal adaptive immune response upon infection with *L. monocytogenes* and *S. enterica* or immunization with BCG (Kursar *et al.*, 2004; Seibert *et al.*, 2010; Way *et al.*, 2003). Moreover, MyD88-deficiency in mice does not cause a total loss of antibody responses even in immunizations that contain only TLR ligands as adjuvant. Instead, it reduces mainly the magnitude of antibody responses, which may provide protection in some case or become sufficient following repeated immunizations or infections. In addition, loss of TLR/MyD88-dependent barrier immunity in the gut can lead to commensal overgrowth, which in turn stimulates compensatory antibody production (Slack *et al.*, 2009).

The mutations mentioned so far cause a direct impairment of the immune response and hence an increased susceptibility to infections. However, the relationship between function and infectious consequence does not always appear that linear. Mutations in the *NOD2* gene have been associated with a highly increased risk for the development of colitis (Hugot *et al.*, 2001; Ogura *et al.*, 2001). It therefore seemed to be counterintuitive for loss-of-function mutations of a PRR to result in a disease that is characterized by increased inflammation. However, recent findings might explain this apparent paradox. While NOD2-deficient cells are indeed impaired in their ability to mount an antibacterial response, NOD2-deficient mice are surprisingly efficient in the control of intestinal bacteria and do not display an increased susceptibility to colitis (Kobayashi *et al.*, 2005; Pauleau and Murray, 2003). However, NOD2 appears to restrict TLR2-induced activation of NF-κB (Maeda *et al.*, 2005; Netea *et al.*, 2005). NOD2-deficiency therefore renders the cells more sensitive to the induction of proinflammatory cytokines by TLR2 that leads to an enhanced T_H1 response (Watanabe *et al.*, 2004). Thus, NOD2 may induce disease by altering the signaling strengths of other PRRs.

7. CONCLUSIONS

In the past few years, many of the basic principles of innate control of adaptive immunity have become clearer. Many of the PRRs have been identified, and their basic signaling pathways have been defined as well. In addition, we are currently seeing a dramatic increase of our understanding of the regulation of T and B cell responses by the innate immune system. These advances are shifting the focus to new questions that will feature prominently in the investigation of innate control of adaptive immunity in the next few years. A few intriguing examples of these questions address the identification of microbial antigens by intracellular PRRs, the interplay between different PRRs, and the existence and possible innate instruction of novel $CD4^+$ T cell subsets.

As outlined previously, we still have not fully grasped how the response of a specific PRR differs between various cell types and what this means for the induction of adaptive immunity. The cooperation between different PRRs in the instruction of the adaptive immune response requires further investigation. Thus, it will be interesting to determine to what extent PRRs other than TLRs are truly capable to drive the adaptive immune response by themselves. Moreover, it will be important to analyze how the coactivation of two or more classes of PRRs influences the balance between the various T cell subsets, for example, between T_H1 and T_H17 responses. This will certainly be important in the context of coinfections. Finally, it will be critical to investigate the effects of inflammasome-mediated instruction of adaptive immunity, in

particular, as it appears that these signals can dominate over RLR-driven signals as shown for RSV infections (Bhoj *et al.*, 2008; Sabbah *et al.*, 2009).

The recognition of PAMPs by PRRs marks the microbial origin of antigens. APCs have been shown to require the direct recognition of PAMPs in order to induce adaptive immune responses (Sporri and Reis e Sousa, 2005), in part because PAMPs induce the preferential presentation of antigen among the sea of self-antigens (Blander and Medzhitov, 2004). For extracellular pathogens, this usually involves the recognition of PAMPs that are physically associated with the antigen (either in the form of a microbe or artificially by adjuvants) by membrane-bound PRRs. In intracellular infections, the APCs are often not the infected cells. Thus, the PAMPs and antigens are not physically associated. Innate control of adaptive immunity by cytosolic PRRs therefore faces the conceptual problem of linking the information provided by the PRR to the presented antigen. This puzzle has not been solved yet, although one possibility might involve two simultaneous signals for the activation of the APCs. One could be the release of type I interferons or other cytokines by infected cells, while the other could be derived from the phagocytosis of infected cells by the APCs and the subsequent recognition of the viral nucleic acid by TLRs (Schulz *et al.*, 2005).

As mentioned earlier, the possibility that the various $CD4^+$ T cell subsets themselves might comprise several distinct subsets is a provocative idea. In particular, the distinction of pathogenic versus protective $CD4^+$ T cells, which has been suggested for T_H17 cells in the context of EAE, is very intriguing. It will be important to determine to what extent these subsets do indeed exist and if so, whether they truly reflect distinct differentiation states or merely distinct activation states of the same $CD4^+$ T cell subset. Regardless of the latter questions, though, it is likely that the innate immune system is instrumental in shaping these states.

Despite the tremendous progress made over the past decade, many fundamental questions regarding control of immune responses remain unanswered. The characterization of PRRs has elucidated the mechanisms of immunogenicity. However, it is becoming increasingly clear that additional layers of control may exist that determine the choice of effector class, the magnitude and the duration of the immune response. How these regulatory mechanisms operate in the context of infections is an exciting area for future investigation.

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CHAPTER **4**

The Evolution of Adaptive Immunity in Vertebrates

Masayuki Hirano, Sabyasachi Das, Peng Guo, and **Max D. Cooper**

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Abstract Approximately 500 million years ago, two types of recombinatorial adaptive immune systems (AISs) arose in vertebrates. The jawed vertebrates diversify their repertoire of immunoglobulin domain-based T and B cell antigen receptors mainly through the rearrangement of V(D)J gene segments and somatic hypermutation, but none of the fundamental AIS recognition elements in jawed vertebrates have been found in jawless vertebrates. Instead, the AIS of jawless vertebrates is based on variable lymphocyte receptors (VLRs) that are generated through recombinatorial usage of a large panel of highly diverse leucine-rich-repeat (LRR) sequences. Whereas the appearance of transposon-like, recombination-activating genes contributed uniquely to the origin of the AIS in jawed vertebrates, the use of activation-induced cytidine deaminase for receptor diversification is common to both the jawed and jawless vertebrates. Despite these differences in anticipatory receptor construction, the basic AIS design featuring two interactive T and B lymphocyte arms apparently evolved in an ancestor of jawed and jawless vertebrates within the context of preexisting innate immunity and has been maintained since as a consequence of powerful and enduring selection, most probably for pathogen defense purposes.

1. INTRODUCTION

In order to survive in a competitive environment, organisms must be able to protect themselves from pathogens seeking to exploit their resources, while sparing their own cells from injury. This requirement for selfdefense in the ongoing struggle for survival led inevitably to the evolution of complex immune systems. Biologists have found that simple multicellular life forms, like sponges, have many of the elements used by vertebrates for immune recognition and microbial defense. These ancient defense strategies, collectively known as innate immunity, defend against infection by relatively nonspecific recognition of pathogen patterns (Beutler, 2004; Hoffmann *et al*., 1999; Janeway, 1989; Medzhitov, 2007). In addition to the complexity of their mechanisms for innate immunity, vertebrates have evolved adaptive immune systems (AISs) that allow specific antigen recognition and mounting of a protective response against bacterial, viral, fungal, and parasitic pathogens. An important feature of the AIS is the capacity for memory of specific pathogenic encounters, which allows for the prevention of a second invasion or a more rapid response to a previously encountered pathogen.

Lymphocytes with diverse anticipatory receptors are primarily responsible for the adaptive immune responses, in particular, the developmentally and functionally distinct lineages known as T and B

cells in jawed vertebrates (gnathostomes). During their development in the thymus and hematopoietic tissues, respectively, T and B cells somatically generate diverse repertoires of immunoglobulin (Ig) domain-based antigen receptors, which can be used to recognize a virtually unlimited range of antigens. The cardinal recognition elements of this type of AIS, the immunoglobulin (*Ig*), T cell receptor (*TCR*), and major histocompatibility complex (*MHC*) genes, are present in all of the jawed vertebrates, whereas none of these essential components have been found in jawless vertebrates (agnathans). Instead, the two extant jawless vertebrates, lampreys and hagfish, use variable lymphocyte receptors (VLRs) composed of somatically assembled leucine-rich-repeat (LRR) motifs to recognize antigens and trigger specific immune responses.

The evolution of alternative AIS in the two branches of the vertebrate lineage within the context of preexisting innate immunity over a relatively short period of evolutionary time poses interesting questions. Reconstruction of immune system characteristics in ancestral preadaptive stages could help to solve this fascinating puzzle of evolutionary biology. However, the inability to access life forms of the immediate ancestors of agnathans and gnathostomes necessitates that this reconstruction is conducted via assessment of immune-related molecules in the currently surviving jawless vertebrates (lampreys and hagfish) and invertebrate species. The most obvious intermediate targets for this phylogenetic reconstruction are potential agnathan orthologues for well-defined components of the gnathostome AIS. After brief consideration of innate immunity in metazoan species, we will compare some of the basic features of the alternative AIS in jawed and jawless vertebrates and describe aspects of their concordance and divergence through phylogenetic time.

2. IMMUNE RESPONSE MOLECULES IN INVERTEBRATES AND PLANTS

Common elements deployed for innate immune defense in plants and invertebrate animals offer insight into how and when our AIS evolved. Notably, the two protein families that contain either the LRR motifs or the immunoglobulin superfamily (IgSF) domains are widely employed for immune defense, as well as for a variety of other purposes.

LRR-containing proteins comprise multiples of 20–30 amino acid units to form horseshoe-like solenoid structures in which the concave surface is formed by parallel β sheets and the convex surface by an array of helices (Buchanan and Gay, 1996). The Toll-like receptors (TLRs) are well-defined examples of LRR-containing proteins that function as pattern-recognition receptors (PRRs) which constitute key components of

innate immune systems throughout the animal kingdom. Plants have a large number of Toll-like nucleotide-binding site (NBS)–LRR proteins (Monosi *et al.*, 2004) that function as disease resistance proteins (Fig. 4.1). Sea urchins and amphioxus also may have hundreds of *TLR* genes (Pancer and Cooper, 2006; Rast *et al.*, 2006).

Members of the IgSF also serve important immune defense functions in invertebrates, in addition to their key roles as specific antigen receptors in the AIS of jawed vertebrates. IgSF members with important roles in innate immunity include the Down syndrome cell adhesion molecule (Dscam) in insects (Watson *et al.*, 2005), fibrinogen-related proteins (FREPs) in snails (Zhang *et al.*, 2004), and the variable region-containing chitin-binding proteins (VCBPs) in amphioxus (Cannon *et al.*, 2002) and sea squirt (Azumi *et al.*, 2003). These molecules can undergo considerable diversification through alternative splicing mechanisms or even somatic mutation to generate potential antigen recognition capacity. Although there are no distinctive structural and functional characteristics that

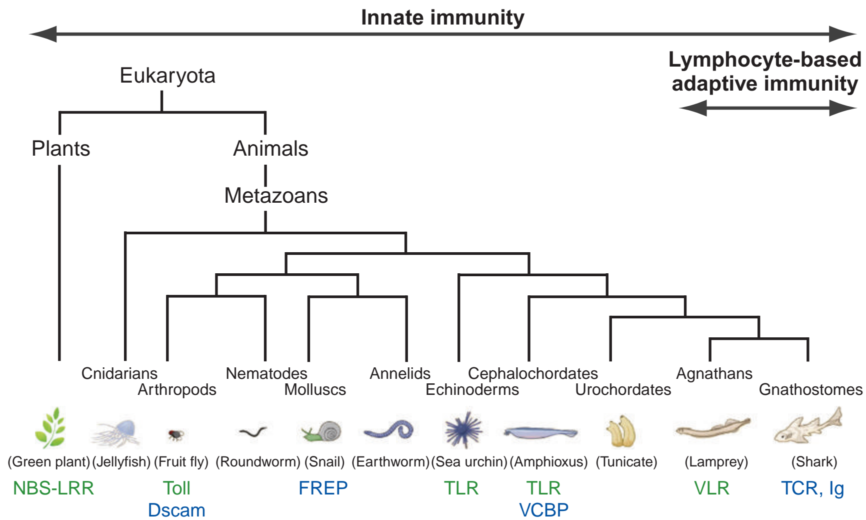

FIGURE 4.1 Hypothetical evolutionary scheme of the emergence of adaptive immunity in conjunction with innate immunity. Families of leucine-rich-repeat (LRR)-based receptors used as immune molecules are indicated in green: nucleotide-binding site–leucine-rich repeat (NBS–LRR), Toll-like receptors (TLRs), and variable lymphocyte receptors (VLRs). Ig-based receptors used in immune defense are indicated in blue: Down's syndrome cell adhesion molecule (Dscam), fibrinogen-related proteins (FREPs), V-type Ig domains and a chitin-binding domain containing proteins (VCBP), T cell receptors (TCR), and immunoglobulins (Ig). One representative for each lineage is named in parentheses.

define these invertebrate immune components as lineal ancestors of the vertebrate *TCR* and *BCR* gene family, these examples of invertebrate IgSF usage illustrate the remarkable versatility of Ig domains. An abundance of LRR motifs and IgSF domains were thus readily available for cooption to provide the basic molecular units for use in the somatic diversification of VLR in jawless vertebrates or of Ig/TCR antigen receptors in jawed vertebrates.

3. EMERGENCE OF LYMPHOCYTES AND GENES CONNECTED WITH MAMMALIAN IMMUNITY IN JAWLESS VERTEBRATES

Phagocytic cells form mobile cellular arms for innate immune defenses in almost all of the metazoan species. However, lymphocytes bearing somatically diversified antigen receptors have been found only in the vertebrates, wherein they play fundamental roles in adaptive immunity. The thymus-derived T lymphocytes and bone marrow-derived B lymphocytes are the cellular pillars of adaptive immunity in the jawed vertebrates. T and B lymphocytes are primarily responsible for cell-mediated immunity and humoral immunity, respectively, and they work together with phagocytic cells and other cell types to mediate effective adaptive immunity. Migratory long-lived lymphocytes expressing anticipatory antigen receptors and having the potential for self-renewal and selective clonal expansion thus represent an evolutionarily innovative type of specialized immunocompetent cells.

Lymphocyte-like cells have not been recognized in invertebrates, but cells with lymphocyte-like morphology that express some lymphocyte-related genes and respond to pathogenic bacteria with an increase in size have been found in amphioxus (Huang *et al.*, 2007). Lymphocyte-like cells that express much of the molecular machinery used by lymphocytes in jawed vertebrates have been characterized in lampreys and hagfish, the most basal vertebrate representatives (Mayer *et al.*, 2002; Nagata *et al.*, 2002; Najakshin *et al.*, 1999; Uinuk-Ool *et al.*, 2002). The latter findings coupled with earlier observations that lampreys and hagfish produce specific agglutinins following immunization with bacteria and foreign red blood cells initially suggested that agnathans could have a lymphocyte-based AIS (Finstad and Good, 1964; Fujii *et al.*, 1979a,b; Linthicum and Hildemann, 1970), although characterization of the agglutinin proteins proved problematic (Litman *et al.*, 1970; Marchalonis and Edelman, 1968; Pollara *et al.*, 1970).

It was anticipated that lampreys and hagfish, as the nearest living phylogenetic relatives of gnathostomes, would have ancestral *TCR*, *Ig*, and *MHC* genes. Transcriptome analysis of lamprey and hagfish

lymphocytes indeed revealed genes orthologous to those that jawed vertebrate lymphocytes use for purposes of cellular migration, proliferation, differentiation, and intracellular signaling, in addition to relatives of genes that gnathostomes use for antigen processing and intracellular transport of antigenic peptides (Mayer *et al.*, 2002; Nagata *et al.*, 2002; Pancer *et al.*, 2004b; Rothenberg and Pant, 2004; Suzuki *et al.*, 2004; Uinuk-Ool *et al.*, 2002). A lamprey *TCR-like* gene with both *Ig V* (variable) and *J* (joining) sequences was identified, but this proved to be a single copy gene encoding both *V*- and *J*-like sequences within one exon and two functional immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic domain (Pancer *et al.*, 2004b; Yu *et al.*, 2009). A *VpreB*-like gene was also found to be expressed by lymphocyte-like cells in lampreys (Cannon *et al.*, 2005), and a family of paired-*Ig*-like receptor genes encoding transmembrane proteins with activating and inhibitory potential, named agnathan paired receptor resembling Ag receptors (APAR), was identified in hagfish (Suzuki *et al.*, 2005). However, *MHC*, *TCR*, *BCR*, and *RAG* orthologues were not found, and their absence fueled the skepticism about earlier reports of adaptive immunity in agnathans. This view was modified dramatically by the identification of the *VLR* genes as key elements for an AIS in lampreys and hagfish (Pancer *et al.*, 2004a, 2005).

4. AIS IN JAWED VERTEBRATES

4.1. Brief overview

The well-described principals of the Ig-based AIS are briefly outlined here primarily for comparative purposes. The two major lineages of clonally diverse lymphocytes that specifically recognize and respond to antigenic determinants of potentially hazardous pathogens and toxins are named T and B lymphocytes, because they are generated in the thymus and the bone marrow or the avian bursa of Fabricius (Cooper *et al.*, 1965; Greaves *et al.*, 1968). The progenitors of T and B lymphocytes are derived from multipotent hematopoietic stem cells (Moore and Owen, 1965; Owen *et al.*, 1965). During early stages in their development, progenitors of T and B lymphocytes begin to rearrange different sets of prototypic variable (*V*), diversity (*D*), and/or joining (*J*) gene segments to generate the antigen-binding regions of the TCRs and B cell receptors (BCRs; Hedrick *et al.*, 1984; Tonegawa, 1983; Yanagi *et al.*, 1984). The recombination-activating genes (*RAG1*/*RAG2*) encode enzymes that initiate V(D)J rearrangement (Schatz *et al.*, 1989). The antigen-binding regions of the different V(D)J combinations are diversified further through splicing variability and the enzymatic addition of nucleotides in the joints created during V(D)J

segment assembly (Dudley *et al.*, 2005). The random nature of this diversification inevitably results in the generation of receptors that recognize self-antigens, necessitating that T and B lymphocytes bearing potentially harmful, self-reactive receptors be eliminated or tolerized in their thymic and bone marrow birthplaces or otherwise inactivated (Goodnow *et al.*, 2005; Jameson *et al.*, 1995; von Boehmer, 2004). The selected populations of long-lived T and B cells then enter the bloodstream to begin their patrol of the body via a migratory route that involves entry into strategically located secondary lymphoid tissues, where they may engage invading pathogens, and their subsequent return to the circulation via lymphatic channels (Gowans and Knight, 1964). The T cells use their TCRs to recognize peptide fragments of antigens presented by accessory cells within cell surface molecules encoded by the *MHC* class *I* and class *II* genes (Bjorkman *et al.*, 1987; Unanue, 1980; Zinkernagel and Doherty, 1974). T cells therefore typically recognize antigens that have been partially digested within specialized antigen presenting cells, primarily dendritic cells, phagocytic cells, and B lymphocytes (Steinman *et al.*, 1999; Storni and Bachmann, 2003). By contrast, the membrane-bound and secreted antibodies made by B lineage cells typically recognize exposed determinants (epitopes) of intact molecules, including surface protein and carbohydrate moieties of invasive microbes. The Ig-based TCR and BCR antigen-binding chains are associated with other transmembrane proteins that can trigger intracellular signaling pathways to induce expression of genes required for immune responses. For most antigen-induced responses, the B cells receive assistance from T cells in the activation process. A wide variety of cell surface molecules and secreted cytokines are used to regulate the homing of the different immune cell participants and to coordinate their cellular interactions (Paul, 2008).

4.2. Immunoglobulin heavy-chain isotypes

The rearranging *Ig* V(D)J genes and their BCR and antibody products are intimately associated with adaptive immune function in all jawed vertebrates, from cartilaginous fish to humans (Flajnik and Kasahara, 2010; Litman *et al.*, 2010). The canonical immunoglobulin (Ig) or antibody molecule is composed of two identical heavy (H) chains and two identical light (L) chains, with the exception of certain antibodies in camelids and nurse shark that lack L chains (Conrath *et al.*, 2003). The type of Ig-H chain defines the class of antibody. The five Ig-H chain types denoted by the α (IgA), δ (IgD), ε (IgE), γ (IgG), and μ (IgM) Greek letters are present in most mammalian species (Klein and Horejsi, 1997), although they may differ in size and composition. Mammalian IgM and IgE heavy chains are composed of four constant region domains, each of which is ~110 amino acids in length and is encoded by separate exons, which most probably

arose from gene duplication during evolution (Lin and Putnam, 1981). Whereas mammalian IgD, IgG, and IgA usually contain only three domains, rodent IgD contains two constant region domains (Flanagan *et al.*, 1984; Tucker *et al.*, 1979; Zhao *et al.*, 2002, 2003).

IgM is produced in both polymeric and monomeric forms by all jawed vertebrates, and its four domain architecture is also conserved from cartilaginous fishes (elasmobranches) to mammals (Flajnik, 2002). Cartilaginous fishes express immunoglobulins that comprise the conventional heavy–light chain isotypes called IgM and IgD. A unique heavy-chain isotype called IgNAR (new antigen receptor), which lacks light chains, has also been identified in sharks (Greenberg *et al.*, 1995; Ohta and Flajnik, 2006). The IgNAR is a disulfide-bonded dimer of two identical polypeptide chains, each of which has one variable and five constant domains (Roux *et al.*, 1998). The IgD-like isotype in sharks, the earliest extant jawed vertebrates, was originally named IgW before its recognition as a homolog of IgD. The IgM and IgD isotypes are thus regarded as ancient isotypes that likely were present in an ancestor predating the emergence of cartilaginous fishes. However, IgD has undergone important structural changes and discontinuous distribution (i.e., being lost in certain birds and mammals) over the course of its evolution in different vertebrate lineages (Lundqvist *et al.*, 2001; Ohta and Flajnik, 2006; Ros *et al.*, 2004). A third heavy-chain isotype, IgZ/IgT, has been found in bony fishes (Danilova *et al.*, 2005; Hansen *et al.*, 2005). Like IgNAR in cartilaginous fishes, the unique IgZ/IgT isotype is thought to be restricted to bony fishes.

The emergence of tetrapods represents a critical time interval during vertebrate evolution in that several new physiological, anatomical, and genetic architectures evolved during this period. Amphibians, which represent the root of tetrapod evolution, have five Ig-H isotypes, IgM, IgX, IgY, IgD, and IgF (Zhao *et al.*, 2006). IgY is found in a variety of birds, amphibians, and reptiles; generally contains four constant region domains; and is regarded as a progenitor of both mammalian IgG and IgE (Mussmann *et al.*, 1996b; Warr *et al.*, 1995). Sequence homology and the common biological properties of IgY and IgE heavy chains suggest that IgY is the immediate ancestor of mammalian IgE, whereas the transition from IgY to IgG involved structural changes that led to formation of a hinge region in IgG molecules. The amphibian IgX molecule is considered as an analog of mammalian IgA primarily because of similarities in the tissue distribution of IgA-positive and IgX-positive B cells (Mussmann *et al.*, 1996a). Although somewhat different from mammalian IgA, IgX is structurally similar to avian IgA and the reptilian IgA-like molecule (Deza *et al.*, 2007). The mammalian IgAs therefore may be descendants of amphibian IgX. The Ig-H isotypes found in the different vertebrate classes are summarized in Table 4.1.

TABLE 4.1 Overview of the immunoglobulin heavy- and light-chain isotypes in vertebrates

	Cartilaginous fish	Bony fish	Amphibians	Reptiles	Birds	Mammals
Ig heavy chain	IgM, IgD, IgNAR	IgM, IgD, IgZ/T	IgM, IgX, IgY, IgD, IgF	IgM, IgY, IgA, IgD	IgM, IgY, IgA,	IgM, IgG, IgA, IgD, IgE
Ig light chain	σ-cart, σ, κ, λ	σ, κ, λ	σ, κ, λ	κ, λ	λ	κ, λ

4.3. Genomic organization of the *IgH* locus

The *IgH* locus in cartilaginous fishes has a cluster type of genomic organization, the most common form of which features closely linked V_H-D_H1-D_H2-J_H-C_H clusters that are dispersed throughout the genome (Litman *et al.*, 1991). The overall length of a cluster is quite large, usually ~18 kb. The presence of germline-joined *Ig* genes is a second characteristic that is unique to the cartilaginous fishes. Approximately half of the germline heavy-chain loci examined thus far are partially (V_HD_H-J_H) or fully ($V_HD_HJ_H$) joined and lack intervening sequences between *V*-domain encoding genes (Litman *et al.*, 1990). The joined *Ig* genes appear to be derived from conventional, unrearranged *Ig* genes as a consequence of RAG-mediated recombinatorial activity in germ cells (Kokubu *et al.*, 1988; Lee *et al.*, 2000), perhaps because the germline-joined clusters have a transcriptional advantage early in the development of cartilaginous fishes (Rumfelt *et al.*, 2001). The *IgH* loci of bony fishes and tetrapods instead contain multiple V_H, D_H, and J_H genes followed by C_H genes. The latter type of organization, known as translocon type of organization, is common to all higher vertebrates. The transformation from cluster type to translocon type of organization of *IgH* locus offers the evolutionary advantage of enhanced antibody diversification.

4.4. Evolution of IgH class switching

On encountering antigens in peripheral lymphoid compartments, B lymphocytes can change the class of antibody expressed from IgM to IgG, IgA, or IgE through a recombination/deletion process termed immunoglobulin heavy-chain class switch recombination (CSR). CSR is a deletional recombination event that occurs via the introduction of DNA double-stranded breaks in two participating switch (S) regions, rejoining

of the broken S regions to each other and deletion of the intervening sequences containing various C_H genes (Chaudhuri and Alt, 2004; Honjo *et al.*, 2002). The B cell protein known as activation-induced cytidine deaminase (AID) is required for the CSR activity; AID is also essential for the initiation of two other Ig diversification processes: somatic hypermutation and gene conversion (Chaudhuri *et al.*, 2007; Honjo *et al.*, 2002; Muramatsu *et al.*, 2007). Somatic hypermutation introduces point mutations, and sometimes small insertions and deletions, into the V_H gene segments during B cell clonal expansion. Chickens and rabbits, which have limited numbers of V_H genes, generate Ig diversity primarily through intrachromosomal gene conversion using upstream variable region pseudo-genes as donor sequences (Knight and Barrington, 1998; Langman and Cohn, 1993; McCormack *et al.*, 1991; Reynaud *et al.*, 1987). AID and somatic hypermutation of *Ig* genes are observed in all jawed vertebrates including the cartilaginous fishes (Conticello *et al.*, 2005), whereas amphibians are the most primitive jawed vertebrates known to use DNA recombination to switch antibody classes. Both of these mechanisms for antibody diversity have been maintained in every vertebrate group that has evolved subsequently (Stavnezer and Amemiya, 2004). CSR has not been demonstrated in bony and cartilaginous fishes, despite their expression of AID. Nevertheless, zebrafish AID can restore normal CSR in AID-deficient mouse B cells, indicating that the AID functional domains required for CSR existed before the emergence of land vertebrates (Barreto *et al.*, 2005). Since the appropriate DNA switch regions are missing in the heavy-chain loci of cartilaginous and bony fishes, the rate-limiting step for class switching therefore may have been the evolution of appropriate DNA switch regions in the translocon type of *IgH* locus organization.

4.5. Evolution of immunoglobulin light chains

The main function of an Ig light (IgL) chain is its contribution to antigen binding and, hence, enhanced antibody variability. The two IgL isotypes in humans, kappa (κ) and lambda (λ) initially were recognized serologically (Korngold and Lipari, 1956). The κ and λ denomination has since been extended from humans to other vertebrate species by comparisons of nucleotide or amino acid sequences. Use of molecular cladistic markers indicates that amphibians have three different IgL isotypes, κ, λ, and sigma (σ; Das *et al.*, 2008). However, the σ IgL isotype appears to be absent in the reptilian, avian, and mammalian lineages. Only the λ IgL isotype is present in the avian species that have so far been examined (Das *et al.*, 2010; Lundqvist *et al.*, 2006), suggesting that *Ig-κ* encoding genes were lost in birds during their divergence from the reptilian lineage. The genes encoding κ and λ isotypes are located in

different genomic regions (Das *et al.*, 2008). In the *κ*-encoding locus of reptiles and other tetrapods, multiple *Jκ* genes are followed by a single *Cκ* gene, whereas the *Jλ* and *Cλ* genes occur as *Jλ*–*Cλ* blocks in the λ-encoding locus, usually being present in multiple copies. However, only one *Jλ*–*Cλ* block is found in birds (Das *et al.*, 2010). The IgL isotype demarcation in lower vertebrates is problematic because the cladistic molecular markers which define κ, λ, and σ isotypes in tetrapods are not well preserved in bony and cartilaginous fishes. However, four primordial IgL isotypes (σ, σ-cart, κ, and λ) can be identified in cartilaginous fishes based on phylogenetic reconstruction (Criscitiello and Flajnik, 2007). The distribution of the IgL isotypes in jawed vertebrates (Table 4.1) thus indicates early divergence of IgL isotypes in jawed vertebrate evolution.

4.6. TCR evolution

The TCR and Ig are the closest relatives to each other among all of the IgSF members. The structure of the antigen-binding portions of TCR and Igs are very similar and both are diversified by RAG-mediated rearrangements. Nevertheless, there are interesting dissimilarities in the genetic structure and evolutionary history of the *TCR* and *Ig* genes. First, the organization of *TCR* genes has changed very little throughout jawed vertebrate evolution (Rast *et al.*, 1997). The basic evolution of T cell development is also conserved for all jawed vertebrates, except for a few species in which the system seems to have degenerated (Flajnik and Kasahara, 2010). Recombination of the *TCR* V(D)J gene segments takes place primarily in the thymus, and each T cell expresses a unique TCR heterodimer that can react with specific peptide fragments (epitopes) bound to a cell-associated MHC molecule. The TCR binding to the MHC-peptide epitope together with the respective binding of the T cell surface glycoproteins CD8 and CD4 to MHC class I or II molecules initiates downstream signaling to induce an immune response. The *TCR* genes in the cartilaginous fishes are highly homologous to mammalian *TCR* genes, and these basal vertebrates also have a well-defined thymus with medullary and cortical regions (Luer *et al.*, 1995). The presence of *MHC* class *I* and class *II* genes further attest a functional TCR recognition system in cartilaginous fishes (Bartl and Weissman, 1994; Kasahara *et al.*, 1992, 1993). Nevertheless, the T cell responses in these elasmobranchs differ in magnitude and other characteristics from those seen in higher vertebrates (Smith and Davidson, 1992).

T cells develop along discrete differentiation pathways characterized by expression of either alpha (α)/beta (β) or gamma (γ)/delta (δ) TCRs. The αβ T cells differ from the γδ T cells with regard to the types of antigen which they recognize (Chien and Jores, 1995). The presence of *α*, *β*, *γ*, and *δ* *TCR* genes in cartilaginous fishes suggests that all four *TCR* loci evolved

very early in the evolution of jawed vertebrates. The TCR β and δ chains are encoded by the rearrangement of variable (*V*), joining (*J*), and diversity (*D*) genes, making them more complex than the α and γ chains which lack *D* gene products. The *TCR α* locus contains many *J* segment genes which may contribute to extensive receptor editing in developing T cells (Guo *et al.*, 2002). The same translocon type of organization for *TCR* genes is found in all gnathostomes (Rast *et al.*, 1997). The *TCR α* locus is closely linked with *TCR δ* locus, whereas the *TCR β* and *TCR γ* loci are located in two different genomic regions. Besides the canonical *TCR* loci, other types of *TCR* genes have been found in marsupials (*TCR mu*) and sharks (*NAR-TCR*; Criscitiello *et al.*, 2006; Parra *et al.*, 2007). Although not orthologues, the TCR mu and NAR-TCR are expressed as atypical TCR isoforms with double variable domains. These unusual types of *TCR* apparently arose independently in the shark and marsupial lineages, possibly via rearrangement between *TCR* and *Ig* loci. Apart from these modifications and the reduced complexity of the avian *TCR* loci (Cooper *et al.*, 1991), the basic principles of T cell development and *TCR* diversification appear to be remarkably well conserved throughout the jawed vertebrate lineages.

4.7. Evolution of RAG1 and RAG2

The acquisition of a mechanism for gene rearrangement to produce clonally diverse Igs and TCRs was critical for the development of adaptive immunity in jawed vertebrates. Discovery of multiple *V*, *D*, and *J* gene segments with specific recombination signal sequences (RSSs) provided insight into the recombinatorial system employed in the *Ig* and *TCR* loci to generate clonal diversity (Hedrick *et al.*, 1984; Tonegawa, 1983; Yanagi *et al.*, 1984). The RAG1/RAG2 proteins recognize the RSSs flanking the *V*(*D*)*J* gene segments to initiate the double-stranded DNA breaks and recruitment of other proteins required for recombination (Chaudhuri and Alt, 2004; Schatz and Baltimore, 1988). *RAG1* and *RAG2* genes are found in all jawed vertebrates that have been examined.

The RAG1 and RAG2 proteins form a transposase that can excise DNA containing the RSSs and reinsert it elsewhere, thus supporting the theory that *RAG1/RAG2* originally were components of a transposable element (Agrawal *et al.*, 1998; Hiom and Gellert, 1997). In this scenario, an ancestral *RAG* transposon consisting of RSSs flanking *RAG1*- and *RAG2*-like genes was mobilized and inserted into an exon of a receptor gene like the *TCR*-like gene in lamprey or agnathan paired antigen receptors (*APAR*) in hagfish. The recipient gene could then be expressed when the inserted transposon was excised by the RAG proteins and the two exonic ends rejoined by repair factors for double strand DNA breaks. This type

of split gene would have a structure analogous to that of the genes for *Ig* light chains and the *TCR* α and γ chains. A second transposon insertion into the same exon could split it again into *V* and *D* gene fragments to yield the tripartite structure characteristic of the Ig heavy chain and the *TCR* β and δ chain variable-region genes (Schatz, 2004). Alternatively, the *D* segment may have arisen through germline recombination events resulting in the formation of signal joints with junctional insertions (Lee *et al.*, 2000; Lewis and Wu, 2000). Duplications of the *V*, *D*, and *J* gene segments and retention of the *RAG1* and *RAG2* genes elsewhere in the genome would then yield the basic recombinatorial immune system of gnathostomes.

The evolutionary origin of the vertebrate *RAG1* and *RAG2* genes is unclear, however, since *RAG1* and *RAG2* orthologues are not found in the genomes of amphioxus, the representative head of the chordate lineage, or in *Ciona intestinalis*, a tunicate representative; neither have *RAG* genes been found so far in the jawless vertebrates. Thus it is possible that the *RAG1* and *RAG2* genes entered the genome of a jawed vertebrate ancestor via horizontal transmission (Schluter *et al.*, 1999).

4.8. Evolution of the MHC

Whereas the BCR and γδ TCR recognize native antigens, αβ TCR recognizes fragmented antigens in the form of peptides bound to MHC class I or class II molecules, a functional interaction that suggests coevolution of the *MHC* and *αβ TCR* genes. Notably, both the antigen-binding receptors and MHC molecules contain similar C1-type Ig-like domains and several of the genes encoding proteins involved in antigen presentation and processing are located in the *MHC* region (chromosome 6 in humans). The latter includes (i) proteasomes and low-molecular-mass polypeptides 2 and 7 (LMP2 and 7), which increase the efficiency of endogenous antigen processing; (ii) transporters associated with antigen processing (TAP) that deliver peptides to the endoplasmic reticulum (ER); (iii) tapasin, which helps peptides bind to nascent class I molecules in the ER; (iv) a molecule known as retinoid X receptor b (RXRB) that regulates the expression of class I protein.

Two rounds of genome-wide duplication occurred during vertebrate evolution, the first of which is thought to have happened in a common ancestor of jawless and jawed vertebrates and the second in an ancestor of the jawed vertebrates (Ohno, 1970; Putnam *et al.*, 2008). Three *MHC* paralogous regions considered to have been derived in this manner have been identified in jawed vertebrates (Flajnik and Kasahara, 2001). These multigene *MHC* paralogous regions are located on chromosomes 1, 9, and 19 in humans. Although jawless vertebrates have orthologous genes for some of the antigen processing molecules, such as a TAP family

member (Uinuk-Ool *et al.*, 2003), they lack recognizable *MHC* class *I* and class *II* genes (Pancer *et al.*, 2004b; Suzuki *et al.*, 2004). These findings together with the capacity for allograft rejection in lampreys and hagfish (Finstad and Good, 1964; Fujii and Hayakawa, 1983; Hildemann and Thoenes, 1969) raise questions about the mechanism for agnathan allorecognition.

5. VLR-BASED AIS IN JAWLESS VERTEBRATES

An alternative AIS that uses LRR-based VLRs as antigen receptors has been recognized only recently in lampreys and hagfish. We summarize here the accumulating evidence indicating that this alternative AIS displays an anticipatory receptor repertoire complexity comparable to that of the Ig-based AIS of gnathostomes.

5.1. VLR discovery and diversity generation in lampreys and hagfish

Since a transcriptome analysis of lymphocyte-like cells from naïve lampreys failed to reveal evidence for an AIS, lampreys were stimulated with an antigen and mitogen mixture to survey the transcriptome of activated lamprey lymphocytes, the intent being to catch lamprey lymphocytes in the act of an immune response. Large activated lymphoblastoid cells sorted by their light scatter characteristics were used to construct a cDNA library, which was subtracted by myeloid and erythrocyte cDNAs (Pancer *et al.*, 2004a). Again no *TCR*, *BCR*, and *MHC* genes were detected, but this transcriptome assessment of the lymphoblasts yielded an abundance of transcripts for highly diverse LRR proteins. These were named VLRs because of their lymphocyte-restricted expression and remarkable sequence diversity. Each *VLR* transcript was found to encode a conserved signal peptide (SP) followed by highly variable LRR modules: a 27–38 residue N-terminal LRR (LRRNT), the first 18-residue LRR (LRR1), variable numbers (up to eight) of 24-residue LRRs (LRRV), one 24-residue end LRRV (LRRVe), one 13-residue connecting peptide LRR (LRRCP), and a 48–65 residue C-terminal LRR (LRRCT; Fig. 4.2). The invariant threonine/proline-rich stalk region contained a glycosyl-phosphatidyl-inositol (GPI) cleavage site, and the phospholipase cleavage of a recombinant VLR from the surface of transduced mouse myeloma cells was indicative of membrane anchorage by GPI linkage.

After the discovery of the lamprey *VLR* that is now called *VLRB*, two hagfish *VLR* homologues, *VLRA* and *VLRB*, were identified through analysis of an expressed sequence tag (EST) database of hagfish

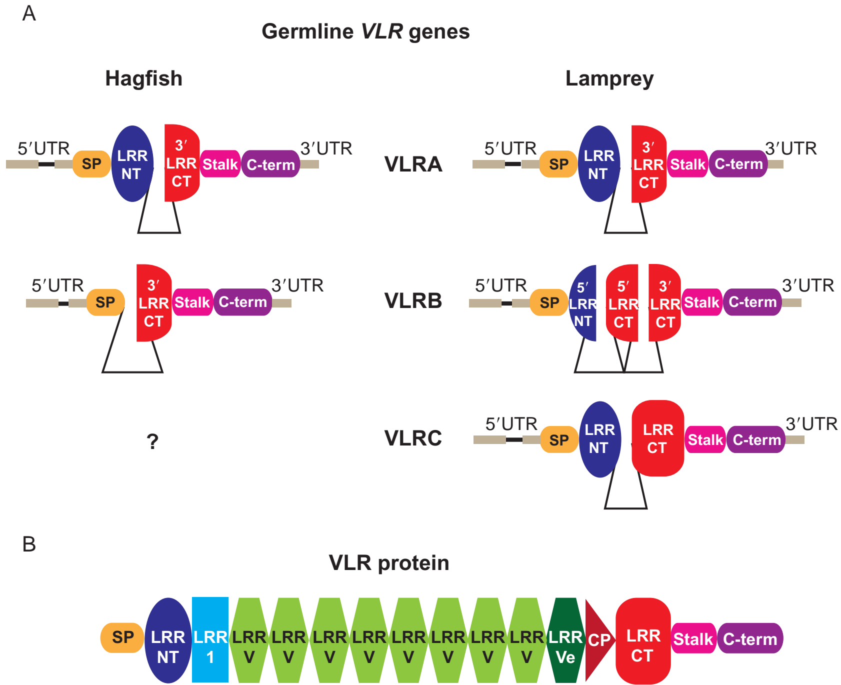

FIGURE 4.2 Organization of *VLR* genes and protein. (A) Germline *VLR* genes in hagfish and lamprey. The incomplete germline *VLR* genes contain regions encoding for portions of the LRRNT and LRRCT separated by noncoding intervening sequences and for the invariant stalk region. (B) Assembled (functional) VLR protein. Mature VLR protein consists of a SP, an LRRNT, an LRR1, up to eight LRRV cassettes, a CP LRR, an LRRCT, an invariant stalk region, and a C-terminal hydrophobic region. SP, signal peptide; CP, connecting peptide.

leukocyte transcripts (Pancer *et al.*, 2005). Lamprey *VLRA* was identified in a subsequent search of the draft sequence database of the sea lamprey genome (Rogozin *et al.*, 2007). All of these germline *VLR* genes are incomplete in that they have coding sequences only for the leader sequence, incomplete amino- and carboxy-terminal LRR subunits and the stalk region (Fig. 4.2). There are two *VLR* exons, with the first exon encoding only a portion of the 5′ untranslated region. The second exon contains the rest of the 5′ untranslated region, a SP, a 5′ portion of the LRRNT, a 3′ portion of the LRRCT, and the stalk region. For hagfish *VLRA* and *VLRB* and for lamprey *VLRA*, the 5′ LRRNT sequence is separated from the 3′ LRRCT sequence by a relatively short noncoding intervening sequence that lacks canonical splice donor and acceptor sites. The lamprey *VLRB* gene is more complex in that it has a 5′ LRRNT coding sequence located between two large intervening

sequences. The germline *VLR* genes are flanked by hundreds of *LRR*-encoding sequences, which can be used as templates to add the missing *LRR* cassettes needed for completion of a mature *VLR* gene. For example, the sea lamprey *VLRA* gene is flanked by >390 *LRR* segments, and *VLRB* is flanked by >450 *LRR* segments (Rogozin *et al.*, 2007).

A gene conversion-like mechanism has been postulated for the complex *VLR* assembly process in which the intervening sequence is replaced in a stepwise, piecewise manner of assembly involving random selection of flanking *LRR* cassettes to serve as templates for adding the necessary sequences to complete a *VLR* gene (Alder *et al.*, 2005; Cooper and Alder, 2006; Nagawa *et al.*, 2007). The assembly process can be initiated at either the 5′ LRRNT or the 3′ LRRCT ends (Fig. 4.3). Short stretches of nucleotide homology (10–30 bp) between donor and acceptor sequences guide the copying of flanking *LRR* segments into the germline gene (Fig. 4.3). Notably, the donor *LRR* sequences are not rearranged during the *VLR* assembly process, in keeping with their lack of RSSs and the absence of *RAG1* and *RAG2* genes in the lamprey. The *VLR* gene is assembled on one allele at a time, and monoallelic *VLR* gene assembly is the rule (Bajoghli *et al.*, 2011; Guo *et al.*, 2009; Kishishita *et al.*, 2010; Nagawa *et al.*, 2007; Pancer *et al.*, 2004a). Analysis of the diversity of *VLR* gene sequences suggests potential repertoires of $>10^{14}$ distinct *VLRA* and *VLRB* receptors, that is, a magnitude comparable to that of the theoretical repertoire of the mammalian B cells (Alder *et al.*, 2005). Although the molecules involved in the *VLR* gene assemblies have not yet been elucidated, two AID-apolipoprotein B mRNA editing catalytic component family orthologues named cytidine deaminase 1 (CDA1) and 2 (CDA2) have been identified in the lamprey. These enzymes are postulated to be key elements in gene conversion-like mechanism for *VLR* assembly (Rogozin *et al.*, 2007). Moreover, *CDA1* expression can be detected only in the VLRA lymphocyte lineage, whereas *CDA2* expression is restricted to the VLRB lymphocyte lineage (Bajoghli *et al.*, 2011; Guo *et al.*, 2009). The currently available data is thus consistent with the hypothesis that CDA1 catalyzes *VLRA* gene assembly and CDA2 plays a similar role in *VLRB* gene assembly.

5.2. Characterization of VLRA$^+$ and VLRB$^+$ cells as T-and B-like lymphocyte populations

The lymphocytes that produce the two types of VLRs have been characterized through the use of antibodies that are specific for the invariant stalk regions of either the VLRA or VLRB proteins. This analysis indicates that lamprey lymphocytes express either VLRA or VLRB receptors, and never both. The VLRA$^+$ and VLRB$^+$ cells therefore represent two separate lymphocyte populations in blood, kidneys, typhlosole, and the

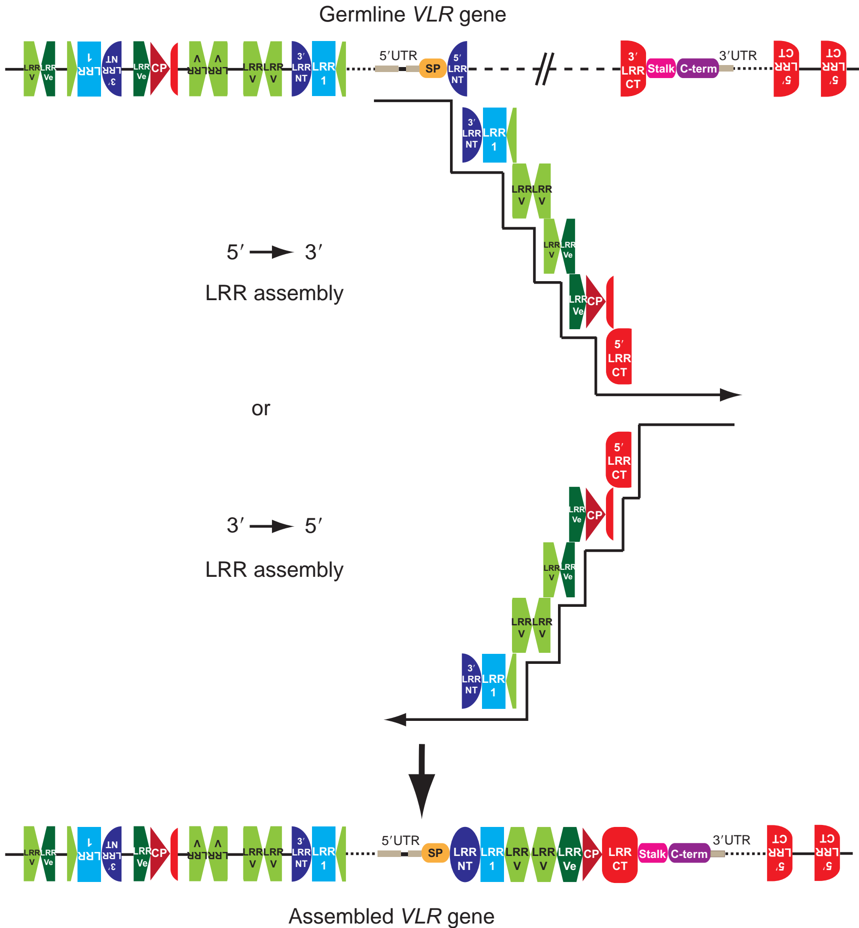

FIGURE 4.3 *VLR* gene assembly by gene conversion-like ("copy choice") mechanism. The germline *VLR* genes are flanked by hundreds of *LRR* cassettes. The noncoding intervening sequence between portions of the *LRRNT* and *LRRCT* is replaced by *LRR* fragments that are sequentially copied from the flanking donor *LRR* sequences. *VLR* gene assembly is initiated at either the *LRRNT* or *LRRCT* end and proceeds in a stepwise manner that is directed by short sequence homology between the donor and acceptor *LRR* sequences for the completion of a mature *VLR* gene.

gill region (Guo *et al.*, 2009). The VLRB$^+$ lymphocytes are the dominant population, except in the gill region. VLRB$^+$ lymphocytes typically outnumber VLRA$^+$ cells, by ~8:1 in the blood and kidneys and by a ~2:1 ratio in the typhlosole. Analysis of purified VLRA$^+$ and VLRB$^+$ lymphocytes indicates that *VLRA* gene assembly is restricted to the VLRA$^+$ lymphocytes and *VLRB* assembly is unique to the VLRB$^+$ cells.

The VLRA and VLRB lymphocytes in lamprey have proven to be remarkably similar to gnathostome T and B lymphocytes in several ways (Guo *et al.*, 2009). The lamprey VLRB-bearing lymphocytes resemble B cells of jawed vertebrates in that they can bind unprocessed cognate antigens and respond to immunization with bacteria, fungi, or foreign erythrocytes with proliferation, lymphoblastoid transformation, and differentiation into plasmacytes that secrete VLRB antibodies that are specific for either protein or carbohydrate epitopes of antigens (Alder *et al.*, 2008; Herrin *et al.*, 2008). The VLRA$^+$ lymphocytes also respond to immunization with lymphoblastoid transformation and proliferation, but they do not secrete their VLRA proteins before or after immunization. Further, unlike the VLRB$^+$ lymphocytes, VLRA$^+$ lymphocytes do not appear to bind native antigens. However, VLRA$^+$ cells vigorously respond to phytohemagglutinin (PHA), a classical T cell mitogen, with lymphoblastoid transformation and proliferation to become the predominant lymphocyte population.

A limited transcriptome analysis for the VLRA and VLRB lymphocyte populations indicates that they have very different gene expression profiles (Guo *et al.*, 2009). The VLRB$^+$ lymphocytes preferentially express mRNA for orthologues of several genes that are preferentially expressed by B cells in jawed vertebrates (Fig. 4.4); these include transcripts for the hematopoietic progenitor homing receptor CXCR4; the herpes virus entry mediator/tumor necrosis factor receptor superfamily member 14 (TNFRSF14) that binds to LIGHT on T cells; two components of the BCR-mediated signaling cascades, spleen tyrosine kinase (Syk) and the B cell adaptor protein (BCAP); the chemotactic inflammatory cytokine IL-8; the IL-17 receptor; and the TLR orthologues TLR2abc, TLR7, and TLR10, the ligation of which induces B cell activation. Conversely, the VLRA$^+$ lymphocytes express genes orthologous to those typically expressed by T cells in the jawed vertebrates; these preferentially expressed genes include ones that encode the GATA2/3, c-Rel, aryl hydrocarbon receptor (AHR) and BCL11b transcriptional factors used for T cell differentiation, the CCR9 chemokine receptor that is involved in thymic homing of thymocyte progenitors, the Notch1 T cell fate-determining molecule, the CD45 tyrosine phosphatase receptor protein that is essential for T cell differentiation, the IL-17 and MIF proinflammatory cytokines and the CXCR2 IL-8 receptor (Fig. 4.4). Activated VLRA$^+$ cells upregulate their expression of *IL-17* and *MIF*, whereas activated VLRB$^+$ cells upregulate their expression of *IL-8*. Coupled with the reciprocal expression of *IL-17R* by VLRB$^+$ cells and *IL-8R* by VLRA$^+$ lymphocytes, these findings suggest the potential for functional interactions between these two lymphocyte populations.

Hagfish *VLRA* and *VLRB* genes appear to be orthologous to lamprey *VLRA* and *VLRB* based on sequence homology. In the hagfish, the *VLRA*

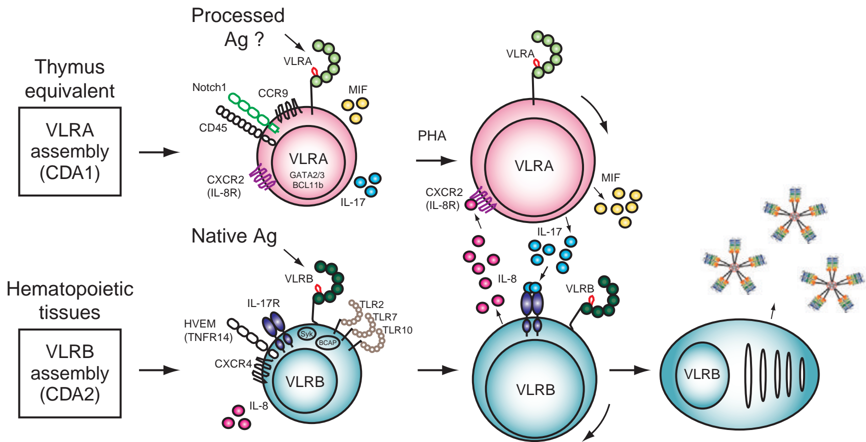

FIGURE 4.4 Model of B- and T-like lymphocytes in lamprey. Antigens (Ag) induce lymphoblastoid transformation of VLRA and VLRB cells, but whether or not receptors of VLRA type see unprocessed antigens is unknown. Antigen-stimulated VLRB cells differentiate into VLRB-secreting plasmacytes. Activated VLRA cells fail to secrete their antigen receptors, but may produce the proinflammatory cytokines, IL-17, and macrophage migration inhibitory factor (MIF). VLRA and VLRB cells express transcripts that encode orthologues for several genes required for respective T cell and B cell development in jawed vertebrates: GATA binding protein 2/3 (GATA2/3), B cell lymphoma/leukemia 11b (BCL11b), C-C chemokine receptor 9 (CCR9), Notch homolog 1, translocation-associated (*Drosophila*) (Notch1), protein tyrosine phosphatase receptor C (PTPRC/CD45), C-X-C chemokine receptor 2 (CXCR2, IL-8 receptor), tumor necrosis factor receptor superfamily member 14 (TNFRSF14), C-X-C chemokine receptor 4 (CXCR4), spleen tyrosine kinase (Syk), B cell adaptor protein (BCAP), IL-8, IL-17 receptor (IL-17R), and TLR orthologues TLR2abc, TLR7, and TLR10. The reciprocal expression of cytokines (IL-17 in VLRA and IL-8 in VLRB cells) and their cytokine receptors (IL-17R in VLRB and IL-8R in VLRA cells) suggest functional interaction between the two types of lymphocytes.

and *VLRB* loci are very distant neighbors on the same chromosome, which could facilitate their function as separate units (Kasamatsu *et al.*, 2007). In keeping with the lamprey data, the analysis of hagfish *VLR* gene assembly at the single-cell level indicates that monoallelic *VLR* assembly is the general rule, although exceptions occur in which a second allele is nonproductively assembled (Kishishita *et al.*, 2010). In each hagfish lymphocyte, only one type of *VLR*, either *VLRA* or *VLRB*, is assembled and transcribed, suggesting that VLRA and VLRB receptors are expressed by separate lymphocyte populations. This conclusion is confirmed at the protein level by immunofluorescence analysis of hagfish lymphocytes with antibodies specific for the invariant stalk regions of VLRA and VLRB proteins (P. Guo *et al.*, unpublished data). Moreover, soluble

VLRA is not detected in hagfish plasma, whereas the VLRB proteins are secreted into the circulation (P. Guo *et al.*, unpublished data). These findings indicate that distinctive T- and B-like lineages are a common feature of the AIS in lampreys and hagfish, in keeping with their monophyletic relationship.

5.3. A third lamprey VLR

Another VLR, designated VLRC, has been identified recently through analysis of the sea lamprey EST database (Kasamatsu *et al.*, 2010). A full-length *VLRC* cDNA clone for the Japanese lamprey, *Lethenteron japonicum*, encodes a protein composed of a 24-amino acid (aa) SP, 36-aa LRR N-terminal capping motif (LRRNT), 25-aa LRR1, 24-aa LRRV, 24-aa LRRVe, 16-aa connecting peptide (CP), 49-aa LRR C-terminal capping motif (LRRCT), and 74-residue 3′ terminus (Fig. 4.2). The SP and 3′ terminus regions are very different from those of the previously known *VLRA* and *VLRB*, but like the other two *VLR* isotypes, the germline *VLRC* gene contains sequences coding for the 5′-UTR, SP, LRRNT, 5′-part of LRR1, 3′-part of CP, the entire LRRCT 3′ terminus, stalk region and 3′-UTR. A notable difference of the germline *VLRC* gene from the *VLRA* and *VLRB* genes is that it only lacks the *LRR* cassette coding sequences. *VLRC* diversity, like *VLRA* and *VLRB* diversity, is generated during its assembly by insertion of diverse *LRR* sequences. However, limited LRRCT diversity is possible because the entire LRRCT fragment is encoded in the germline *VLR* gene and only two potential LRRCT donor sequences, which are almost identical, are located downstream of the *VLRC* gene. The VLRC structure is predicted to be similar to that of lamprey VLRA and VLRB, except that VLRC lacks the thumb-like loop protrusion that is encoded by the variable *LRRCT* inserts of *VLRA* and *VLRB* which are important for antigen recognition (Fig. 4.2). VLRC is expressed exclusively by lymphocytes in the VLRA/VLRB double-negative population, thereby suggesting that the VLRC^{+} cells represent a third lymphocyte lineage. A phylogenetic analysis suggests that lamprey *VLRC* is more closely related to hagfish and lamprey *VLRA* than to *VLRB* (Kasamatsu *et al.*, 2010). The discovery of VLRC thus raises interesting questions about the function of VLRC^{+} lymphocytes, their antigen-binding potential, and their potential role in pathogen responses.

5.4. VLRB antibody characteristics

The VLRB antibodies which are secreted by lamprey plasmacytes are highly variable in size, primarily because of variation in the numbers of constituent LRR units. Under nonreducing conditions they are of relatively high molecular weight (>225 kDa), but under reducing

conditions they are found to be composed of single units varying from 22 to 30 kDa in apparent molecular weight (Alder *et al.*, 2008). This VLRB size heterogeneity is an impediment to structural study, but the production of recombinant monoclonal VLRB antibodies alleviates this technical bottleneck. A mammalian expression system has been developed to generate recombinant Ag-specific VLRB antibodies using cDNA libraries prepared from lymphocytes of immunized lampreys (Herrin *et al.*, 2008). VLRB clones are transfected into HEK-293T cells and the specificity of the secreted multimeric VLRB proteins can be determined by standard antibody screening techniques, such as ELISA, immunofluorescence-based flow cytometry, and agglutination assays. Recombinant VLRB antibodies with exquisite specificity for the human blood group O antigen H-trisaccharide and the BclA major coat protein of *Bacillus anthracis* spores have thus been characterized (Han *et al.*, 2008; Herrin *et al.*, 2008). A high-throughput yeast surface-display system has also been used to isolate recombinant VLRB antibodies for structural and functional analysis (Tasumi *et al.*, 2009). The latter technique facilitates the selection of VLR mutants with high antigen-binding affinity.

EM imaging of recombinant VLRB antibodies indicates a paired chain structure that resembles IgM antibodies without light chains (Herrin *et al.*, 2008). The VLRB antibodies use disulfide bonds at the carboxy terminus of the stalk region to form tetramers or pentamers with 8–10 antigen-binding sites. Analysis of crystal structures of the LRR portions of lamprey and hagfish VLRs reveals the horseshoe or crescent shape that is characteristic of LRR family proteins (Han *et al.*, 2008; Kim *et al.*, 2007). Variable VLR residues on the face of the β-sheets lining the concave surface determine the antigen-binding potential (Han *et al.*, 2008; Herrin *et al.*, 2008). Crystal structural analysis of VLRB antibodies complexed with the H-trisaccharide human blood group O^+ antigen (Han *et al.*, 2008) and with hen egg lysozyme (HEL; Velikovsky *et al.*, 2009) indicates that a highly variable loop in the LRRCT portion of VLRB antibodies also makes an important contribution to the antigen-binding ''pocket.'' The RBC36 recombinant VLRB antibody was shown to bind the H-trisaccharide to residues on the concave surface of the solenoid LRR structure and with the Trp^{204} in the LRRCT loop stacked parallel to the galactose ring (Han *et al.*, 2008). The HEL-specific VLRB uses more of its concave surface to bind this protein antigen (Velikovsky *et al.*, 2009), and most notably, the LRRCT loop inserts into the active catalytic site of HEL. This unusual cleft-binding potential is shared by the heavy-chain-only types of camelid V_H and shark IgNAR antibodies (Chan *et al.*, 2008; Dumoulin *et al.*, 2002; Stanfield *et al.*, 2004; Tasumi *et al.*, 2009), whereas conventional antibodies with Ig heavy and light chains typically bind to planar surface epitopes of protein antigens.

VLRB antibodies have been shown to have other exceptional characteristics. They can discriminate between closely related protein antigens, for example, between the BclA-CTD coat proteins of *B. anthracis* and *Bacillus cereus* T strains that differ in sequence at 14 of 134 amino acids, only 9 of which are solvent exposed (Herrin *et al.*, 2008). VLRB antibodies also display remarkable avidity to antigens with repetitive epitopes because of their multivalency. VLR4, an anti-BclA VLRB antibody, agglutinates *B. anthracis* spores at a thousandfold lower concentration than a corresponding high-affinity mouse IgG antibody against the same antigen (Herrin *et al.*, 2008). Further, VLRB antibodies are resistant to a wide range of pH and temperatures. VLRB antibodies retain their capacity to bind antigens after elution from antigen columns at pH > 11 or after incubation at 70 °C. These characteristics suggest that VLRB antibodies may serve as useful single-chain alternatives to conventional antibodies (Herrin and Cooper, 2010).

5.5. A lamprey thymus equivalent

All of the jawed vertebrates examined so far have been found to have a well-defined thymus early in development, but whether or not lampreys have a thymus has long been debated. Collections of lymphoid cells have been observed in the gill region, but none of the characteristic capsular, stromal, or lymphoepithelial features of the thymus in jawed vertebrates were found in lampreys (Amemiya *et al.*, 2007; Ardavin and Zapata, 1988; Du Pasquier, 2005; Finstad and Good, 1964; Litman *et al.*, 2010). The surprising observation that the lamprey VLRA cells resemble the T cells in jawed vertebrates coupled with their exclusive expression of CDA1 suggested the feasibility of a more molecular-based search for a thymus equivalent in lampreys (Guo *et al.*, 2009). The relatively high concentration of VLRA cells in the gill region favored the pharyngeal region as a likely site for their generation. The limited gene profile analysis indicated that VLRA lymphocytes express several transcription factors, chemokine receptors and Notch 1 that are used in jawed vertebrates for homing of lymphocyte progenitors to the thymus and for their subsequent T cell lineage commitment. Moreover, recent studies had identified a lamprey *FOXN1* orthologue (also known as *Foxn4L*), and *FOXN1* encodes a transcription factor whose epithelial cell expression is essential for the thymopoiesis in gnathostomes (Bajoghli *et al.*, 2009). A lamprey orthologue of *DLL-B*, a *delta-like* gene which is important for the differentiation of lymphocyte progenitor cells into the T cell lineage, was also identified. *In situ* hybridization was therefore used to search for tissue sites of lamprey larvae in which stromal cell expression of *FOXN1* and *DLL-B* and lymphocyte expression of *VLRA* and *CDA1* might coincide. This analysis revealed that expression of all four of the above genes,

which might mark a thymus equivalent, occurs only in the gill filament tips and the neighboring secondary lamellae throughout the gill basket (Bajoghli *et al.*, 2011). *CDA1* expression was restricted exclusively to this discrete gill region, and was unaffected by immunization or stimulation with the T cell mitogen, PHA. Light microscopy and ultrastructural analyses demonstrated that the gill filament tips contain lymphocytes in close proximity with epithelial cells. Moreover, nonfunctional *VLRA* gene assembly was frequently demonstrable in lymphoid cells within the gill filament tips and not in cells located elsewhere. These findings suggest that the gill filament tips and the neighboring secondary lamellae in the lamprey pharyngeal gill region serve as thymus equivalent sites in which VLRA assembly occurs.

Conversely, VLRB lymphocytes, like the B lymphocytes in jawed vertebrates, appear to be generated in the hematopoietic tissues of lamprey larvae, in that most of the lymphoid cells that express the CDA2 enzyme, with or without VLRB proteins, are found in the hematopoietic typhlosole and kidney tissues. These observations indicate that both jawless and jawed T- and B-like lineages undergo spatially segregated development in distinctive tissue microenvironments. The identification of a thymus equivalent in lampreys moreover provides a starting point to begin to address some of the more challenging questions concerning VLRA repertoire development in this jawless vertebrate representative.

6. CONCLUSIONS

Burnet (1968) has suggested that cells of the mammalian immune system could have evolved from the hemocytes in the invertebrates, with the view that such wandering phagocytic cells are ancestral lymphocytes. Phagocytic cells are well known to play a critical role in innate immune defense of the evolutionarily ancient starfish (Metchnikoff, 1891). In teleost fish and frogs, B lymphocytes have also been shown to have phagocytic activity (Li *et al.*, 2006) in keeping with the idea that B cells are the evolutionary derivatives of phagocytes. It is interesting to note also that VLRB cells of jawless vertebrates express many of the TLRs that phagocytes and B cells of jawed vertebrates may express. Cells with cytotoxic capability could also have diverged from primitive phagocytic cells in that a protease for cellular cytotoxicity (Bilej *et al.*, 1998) and allograft rejections have been reported in earthworms (Cooper *et al.*, 1999) and the coelomocytes in sea urchin (phagocytic amebocytes) show cytotoxic activity (Lin *et al.*, 2001). Cytotoxic NK and T cells may have been derived from a common ancestor, given that they share many properties including cytotoxic granule production (Litman *et al.*, 2010; Sun and Lanier, 2009).

The demonstration of cells that resemble lymphocytes in amphioxus (Huang *et al.*, 2007), which is considered to be the representative head of the chordate lineage (Putnam *et al.*, 2008), and the presence of T- and B-like lymphocyte lineages in both jawless and jawed vertebrates may imply that bifurcation of the lymphocyte lineage preceded the emergence of the diverse anticipatory receptors that characterize the alternative lymphocyte-based AIS. This view would be consistent with observations suggesting that B and T cells differentiate from myeloid B progenitors and myeloid T progenitors, respectively (Bell and Bhandoola, 2008; Wada *et al.*, 2008).

A schematic view of the evolution of T- and B-like cells with distinctive anticipatory receptor systems is illustrated in Fig. 4.5. Since *VLR* genes are not found in jawed vertebrates, the LRR-based recombinatorial system of VLRs may have evolved in an ancestor shared only by the extant agnathans. Molecular phylogeny favors a monophyletic origin for lampreys and hagfish (Delsuc *et al.*, 2006; Heimberg *et al.*, 2010; Kuraku *et al.*, 1999; Stock and Whitt, 1992; Takezaki *et al.*, 2003) which share the same type of VLR-based AIS. The VLR recombinatorial system alternatively could have evolved in an ancestor common to the jawed and jawless vertebrates, but the subsequent acquisition of a V(D)J recombinatorial system would have led at least initially to coexistence of the VLR and Ig V(D)J recombinatorial mechanisms for lymphocyte receptor diversification. The random nature of receptor diversification via both mechanisms working in concert inevitably would have resulted in the generation of lymphocytes having receptors with both self- and non-self specificity. Prevention of the predicted autoimmune consequences of such mixed signals for lymphocyte activation therefore theoretically would have necessitated loss of one of the two recombinatorial immune systems. So far, however, there is no evidence for a residual set of *VLR* genes in jawed vertebrates, nor is there evidence for elements of the Ig-based AIS in jawless vertebrates. The VLR- and Ig-based AIS therefore most likely evolved as convergent solutions for the generation of highly diverse anticipatory receptors for specific immunity.

This phylogenetic excursion of immunity indicates that all of the surviving vertebrates, both jawless and jawed, have a lymphocyte-based AIS. This fact alone suggests a strong survival advantage for an AIS. The convergent evolution of two very different types of clonally diverse anticipatory receptors to achieve adaptive immunity in jawless and jawed vertebrates further attests for the survival value of an AIS, although we are unlikely ever to know whether or not other vertebrates fell victim to pathogen-mediated extinction because they failed to acquire a recombinatorial immune system. Our analysis further indicates that the strategy of functionally interactive T and B lymphocyte arms is a fundamental feature of an AIS. The reason for this may be the inherent

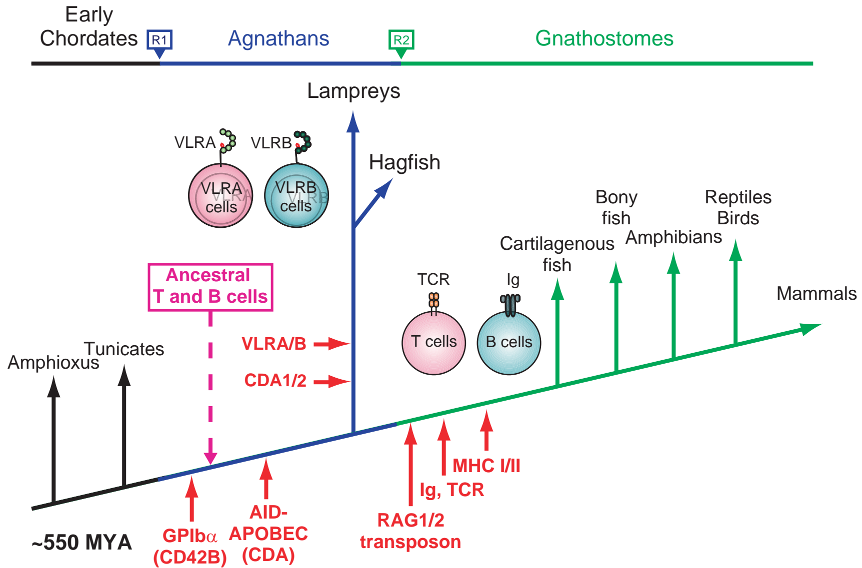

FIGURE 4.5 Hypothetical scheme depicting the evolution of separate T- and B-like lymphocyte lineages and their distinctive types of antigen receptors in vertebrates. Separate lymphocyte populations in the extant agnathans, hagfish, and lamprey, somatically generate diverse leucine-rich-repeat (LRR)-based receptors, VLRA and VLRB, for use in antigen recognition. The ancestral gene for the agnathan *VLRs* is thought to be an orthologue of the mammalian gene for the platelet receptor glycoprotein 1B alpha (*CD42B*). *VLR* gene assembly is postulated to be catalyzed by the AID–APOBEC family cytidine deaminases, CDA1 and CDA2. *VLR* genes have been found only in jawless vertebrates, whereas *RAG1*, *RAG2*, *Ig*, *TCR*, and the *MHC I* and *II* genes are found only in jawed vertebrates. The first round of genome-wide duplication (R1) is thought to have occurred before the split for the jawless and jawed vertebrate lineages and the second round (R2) may have occurred in an extinct basal jawed vertebrate. MYA, million years ago. This figure was modified from the figure of Cooper and Herrin (2010).

threat of autoimmunity that is inevitable with the emergence of an AIS featuring a randomly generated receptor repertoire being expressed by lymphocytes with proinflammatory potential. The two functionally interactive arms of an AIS could be essential to achieve balance and self-regulation. In keeping with this idea, one would anticipate that the repertoire of VLRA and VLRB lymphocytes is selected, beginning within the thymus equivalent for the VLRA cells. Clearly much remains to be learned about the biology of the agnathan T- and B-like cells. At this point, we can only conclude that the amazing complexity of our integrated innate and adaptive immune systems is the result of powerful and enduring selection, most probably to improve the possibility of pathogen defense.

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CHAPTER 5

T Helper Cell Differentiation: More than Just Cytokines

Beata Zygmunt and **Marc Veldhoen**

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Abstract

CD4$^+$ T helper (T_H) cells play a critical role in orchestrating a pleiotropy of immune activities against a large variety of pathogens. It is generally thought that this is achieved through the acquisition of highly specialized functions after activation followed by the differentiation into various functional subsets. The differentiation process of naive precursor T_H cells into defined effector subsets is controlled by cells of the innate immune system and their complex array of effector molecules such as secreted

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cytokines and membrane bound costimulatory molecules. These provide a unique quantitative or qualitative signal initiating T_H development, which is subsequently reinforced via T cell-mediated feedback signals and selective survival and proliferative cues, ultimately resulting in the predominance of a particular T cell subset. In recent years, the number of defined $T_H$cell subsets has expanded and the once rigid division of labor among them has been blurred with reports of plasticity among the subsets. In this chapter, we summarize and speculate on the current knowledge of the differentiation requirements of T_H cell lineages, with particular focus on the T_H17 subset.

1. INTRODUCTION: T HELPER CELLS

The immune system, divided in an innate and adaptive branch, consists of many different cell types which vary in their topographical location as well as in function. T and B lymphocytes constitute the major cellular components of the adaptive immune response. These lymphocytes are instrumental in defending the host against pathogens that are continuously evolving strategies to evade the more static detection mechanisms of the innate immune system. Lymphocytes have the unique ability to rearrange a set of germline genes that encode all elements of the receptor which, after several developmental stages, are expressed on their surface. Stringent selection processes ultimately give rise to one or two functional receptors per lymphocyte that are not only able to recognize virtually any antigen but can also discriminate between the body's self and nonself antigens.

Cell-mediated immune responses are largely controlled by T cells. Possibly, the best understood are cytotoxic T lymphocytes (CTLs) identified via the surface expression of the cluster of differentiation (CD)8 molecule, which can directly kill pathogen infected cells. CD4-expressing T helper (T_H) cells are the second major class of T lymphocytes. Their name reflects the first observations revealing a role of these cells in facilitating B cell antibody production (Hamaoka *et al.*, 1973). Although CD4 T cells may not necessarily be directly involved in combating pathogens, they are of central importance in orchestrating adaptive immunity. In addition to their role in B cell-mediated antibody production, CD4 T cells were also shown to induce delayed-type hypersensitivity (DTH), a cell-mediated immune response (Cher and Mosmann, 1987).

A central question in T cell immunology is how this group of CD4 T cells can coordinate such diverse immunological processes involving many different cell types. Vital for the ability of CD4 T cells to direct such varied immune processes is their capacity to acquire highly

specialized effector functions following activation, accumulating in distinct functional T_H cell subsets. The great variety of functions fulfilled by T_H cells highlights their central role and importance for immune defense as well as immune tolerance. It therefore stands to reason that tight management of T cell lineage differentiation is of central importance for successful immune surveillance. In this chapter, we describe and speculate on recent insights into some of the mechanisms resulting in of T_H cell lineage commitment, with a focus on the new T_H17 subset.

2. T HELPER CELL SUBSET IDENTITIES

The initial activation of CD4 T cells results in the selective production of chemical mediators, cytokines, which are important in the subsequent activation of other cell types in order to fight an infection. The first classification of effector CD4 T lymphocytes in those orchestrating cell- or humoral-mediated responses (Parish, 1971), which resulted in the first description of T helper type 1 (T_H1) and T_H2 (Mosmann *et al.*, 1986) cells, was based on the selective production of two cytokines, interferon (IFN)-γ and interleukin (IL)-4, respectively. Although recent data suggest that a degree of flexibility in T cell lineage commitment exists and cells can gain or lose some characteristics during their lifespan (Murphy and Stockinger, 2010), T_H subsets are still identified by their cytokine profile.

T_H1 cells produce IFNγ, which can activate macrophages and other innate cells and greatly enhance their ability to kill intracellular pathogens (Mosser, 2003). In addition, they support CD8 T cells effector functions and regulate the expression of other mediators in immune and nonimmune cells. Although T_H2 are mainly identified via the production of IL-4, they are also able to secrete IL-5, IL-6, and IL-13 (Paul and Zhu, 2010). The T_H2-mediated response is required for immunity against extracellular pathogens, such as parasites (Anthony *et al.*, 2007). Further, they provide help to B cells, regulating their activation and antibody class-switching.

The dichotomous T_H1–T_H2 paradigm was expanded, but not significantly changed, with the discovery of regulatory T cells (T_{REG}; Sakaguchi *et al.*, 1995). In contrast to T_H1 and T_H2 cells, which are generated in the periphery and require T cell activation, most T_{REG} mature in the thymus, these are referred to as natural (n)T_{REG}. Interestingly, some T_{REG} differentiation may take place in the periphery, inducible (i)T_{REG}, although this may be limited to specialized sites such as the intestine (Belkaid, 2007). Both varieties are associated with the expression of transforming growth factor (TGF)β, IL-10, and IL-35 (Vignali *et al.*, 2008). In contrast to T_H1 and T_H2, these cells play an important regulatory role in dampening immune cell activation and function and are able to alter both T_H1- and T_H2-mediated immune responses (Belkaid, 2007).

The discovery of a fourth CD4 T cell subset, T_H17, facilitated a changing mode in T_H biology, initially uprooting the original T_H1–T_H2 paradigm (Harrington *et al.*, 2005; Park *et al.*, 2005; Veldhoen and Stockinger, 2006; Veldhoen *et al.*, 2006a). T_H17 are characterized by their expression of IL-17A, IL-17F, and IL-22. They can be detected at sites of inflammation early on in the immune response, orchestrating innate immune responses such as additional neutrophil recruitment and activation (Fossiez *et al.*, 1996; Khader *et al.*, 2007; Liang *et al.*, 2007; Lin *et al.*, 2009; Ye *et al.*, 2001). Interestingly, T_H17 cells are enriched at epithelial barrier sites such as the skin, lungs, and the intestine (Denning *et al.*, 2007; Uematsu *et al.*, 2008; Zygmunt *et al.*, 2009). Their topographical location is in line with their proposed function in epithelial barrier immunity, fighting extracellular bacteria and fungi (Happel *et al.*, 2005; LeibundGut-Landmann *et al.*, 2007; Robinson *et al.*, 2009), as well as a potential role in the process of wound healing (Pickert *et al.*, 2009).

The discovery of T_H17 sparked interest in potential additional T_H cell subsets, but since a unique transcriptional regulator has not been identified, these will not be discussed in this chapter. These briefly consist of follicular T helper (T_{FH}) cells, predating the discovery of T_H17 cells and in which the expression of Bcl-6 seems required, which direct humoral immune response via the organization of germinal centers (Breitfeld *et al.*, 2000; de Vinuesa *et al.*, 2000; Yu *et al.*, 2009); T_H9 cells, which produce IL-9 and seem to be involved in airway hypersensitivity reactions and immunity against helminthes (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008b); T_H22 cells, which produce IL-22 but not IL-17 and take part in immune response at mucosal surfaces such as the skin in humans (de Jong *et al.*, 2010; Eyerich *et al.*, 2009; Trifari *et al.*, 2009); and last, regulatory type 1 (T_R1) cells, which share the transcriptional regulator with T_H1 cells but no longer produce IFNγ while they maintain the expression of IL-10 associated with an immunomodulatory function (Gabrysova *et al.*, 2009; Saraiva and O'Garra, 2010).

3. THE ROLE OF CYTOKINES IN T HELPER CELL DIFFERENTIATION

The initiating events resulting in T_H cell activation and differentiation take place in highly organized lymphoid tissues, such as lymph nodes (LNs) and the spleen. These structures are necessary to increase the potential for an encounter between an antigen and a rare antigen-specific lymphoid cell. In addition, they serve to create a microenvironment permissive of the development of an appropriate immune response. T cell activation does not take place immediately upon microbial invasion. It is the epithelial cells of the skin, lung, and intestine and cells of the

innate immune system that detect pathogens or their products. Pathogen-associated molecular patterns (PAMPs) activate host cells via conserved pattern recognition receptors (PRRs), thereby initiating the recruitment of phagocytotic innate immune cells (Trinchieri and Sher, 2007). It is worth to note that at least several subsets of phagocytotic cells are known, reflecting different cell lineages as well as tissue-specific cell types, each with their own characteristic cytokine and surface molecule profiles. The uptake of microbes or their products allows further processing and the transport of antigen, primarily with the help of dendritic cells (DCs), which migrate via the afferent lymph to the secondary lymphoid organs (von Andrian and Mempel, 2003). Upon arrival at the secondary lymphoid organs, circulating naive T_H cells scan multiple DCs to identify a peptide–major histocompatibility complex (MHC) for which their T cell receptor (TCR) has sufficient affinity. Importantly, the antigen is presented in the context of several additional factors each influencing the processes that ultimately determine lineage differentiation of T_H cells.

3.1. Setting the cytokine microenvironment

One of the most important components of the microenvironment which has a profound impact on the activation and differentiation of naive T_H cells is the cytokine milieu (for overview, see Hirota *et al.*, 2010). Its composition is primarily determined by innate immune cells, which produce factors according to the PAMPs they have encountered. It is thought that each species or class of microorganism may be distinguished by a particular combination of PAMPs. This allows a level of microorganism identification by cells of the innate immune system, facilitating a more tailored response. Cytokines enhance innate immune cell activation, but if the invading pathogen fails to be cleared, they will also set the stage for the subsequent adaptive immune response.

An additional factor of importance is the cellular composition at the site of infection. It has become increasingly clear that epithelial cells, those forming the first barrier protecting the sterile tissues, play an important role in initiating immune responses. Epithelial cells have a large arsenal of PRRs and are able to produce a variety of immune factors, including cytokines and chemokines (Swamy *et al.*, 2010). It stands to reason that not all epithelial cells are similarly equipped with PPRs and effector molecules, for example, intestinal epithelial cells encounter the largest burden of commensal and pathogenic microorganisms, while the epithelial cells within the deeper regions of the lung would encounter largely sterile conditions. Integrated within the tissues are multiple subsets of DCs which can differ in the expression of PRRs and their cytokine arsenal. The skin has a specialized subset of DC, the Langerhans cells, while the intestine is home to several identified subsets (Iwasaki, 2007; Merad *et al.*,

2008). These many site-specific elements emphasize the tight management of immune responses that need to be tailored to the threat of the invading microbial species as well as the prevention of immune pathology to often fragile tissues.

3.2. T helper cell differentiation

T_H cells can be identified via their characteristic production of cytokines. Importantly, selective cytokines also play a major role in T_H commitment, which stands at the basis of most *in vitro* T_H cell differentiation protocols aimed at obtaining highly polarized subsets. How cytokines control differentiation of T_H cells *in vivo* is, however, still divisive. The central question is if a particular T_H subset development is mainly instructed by cytokines, or if cytokines primarily act to reenforce a predetermined fate via the provision of growth and survival signals (Coffman and Reiner, 1999).

T_H1 are generated with the help of IFNγ or IL-12 (Hsieh *et al.*, 1993; Manetti *et al.*, 1993), T_H2 with IL-4 (Kopf *et al.*, 1993), iT_{REG} with TGFβ (Chen *et al.*, 2003), and last, T_H17 with the concerted actions of TGFβ and IL-6 (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006a). *In vitro* these cytokines are both required and sufficient to allow the differentiation of the distinct T_H lineages; however, *in vivo* additional layers of complexity are encountered. Although further mediators may not be directly required for T_H cell lineage initiation, there are many factors that can alter T_H cell functionality. Some cytokines are T_H-derived, and are involved in strengthening the emerging T_H subset. In this way, IFNγ promotes T_H1 development, IL-4 supports T_H2 cells, and TGFβ initiates the iT_{REG} differentiation program, while IL-21 may succor the T_H17 lineage (Chen *et al.*, 2003; Korn *et al.*, 2007; Le Gros *et al.*, 1990; Lighvani *et al.*, 2001; Noben-Trauth *et al.*, 2002; Swain *et al.*, 1990). Importantly, these autologous produced cytokines serve an additional role by suppressing the emergence of alternative fates, for example, IFNγ suppressed T_H2 and T_H17 development, IL-4 inhibits the T_H1 and T_H17 lineages (Harrington *et al.*, 2005; Park *et al.*, 2005; Yamane *et al.*, 2005), while T_{REG} can inhibit both T_H1 and T_H2 induction (Gorelik *et al.*, 2000, 2002). However, the new T_H17 subset appears to have upset this direct balance between the subsets. Although a dichotomous relationship with iT_{REG}, but not nT_{REG}, has been proposed (Bettelli *et al.*, 2006), this depends on the production of IL-6 by a third cell type and not on T_H subset autologous secretion. Further, the prototypic T_H17 cytokines, IL-17A and IL-17F, are currently not known to affect the generation of other T_H cell subsets.

Many additional facets of T_H cell function can be altered by the local cytokine environment. IL-1 family members seem to be of particular importance, IL-1β enhancing T_H17 cell development, IL-18 empowering

T_H1 cells, while IL-33 can alter T_H2 cell cytokine expression (Schmitz *et al.*, 2005; Sutton *et al.*, 2006; Takeda *et al.*, 1998; Yoshimoto *et al.*, 1998). It is important to note that the expression of the required receptors making T_H cells susceptible to these modulating cytokines is only initiated after activation and possibly some lineage commitment.

3.3. Lineage transcription factors

Cytokines signaling is controlled via the actions of the transcription factors transducer and activator of transcription (Stat). These connect the cytokine receptors via members of the Janus kinase (Jak) family with specific gene activation in the nucleus. In parallel with specific cytokines driving distinct T_H lineages, members of the Stat-family are of critical importance for particular T_H cell subset development (Table 5.1). In addition to Stat proteins, transcription factors act as master regulators of effector differentiation. These are Tbet (Tbx21) for T_H1 (Szabo *et al.*, 2000), Gata3 for T_H2 (Zheng and Flavell, 1997), Foxp3 for T_{REG} (Hori *et al.*, 2003), and Rorγt for T_H17 (Ivanov *et al.*, 2006). Importantly, these factors are both required and sufficient for the basal development of one of the T_H cell subsets. When ectopically expressed under neutral culture conditions, Tbet, Gata3, and Rorγt are sufficient to induce IFNγ, IL-4, and IL-17 synthesis, respectively, while Foxp3 is sufficient to induce a phenotype similar to T_{REG} (Hori *et al.*, 2003; Ivanov *et al.*, 2006; Ouyang *et al.*, 2000; Szabo *et al.*, 2000). Although most characteristic phenotypes of T_H cells are induced by the master transcription regulators, supplementary factors are required for additional regulation.

The lineage-determining factors can form self-reinforcing feedback circuits, whereby they induce their own expression as shown for Gata3, Tbet, as well as Foxp3 but not Rorγt (Afkarian *et al.*, 2002; Mullen *et al.*, 2001; Ouyang *et al.*, 2000; Zheng *et al.*, 2010). In addition, the lineage-determining factors are able to inhibit competing T_H cell fates via direct interactions. Tbet associates with Gata3, allowing direct inhibition, while Foxp3 inhibits Rorγt (Hwang *et al.*, 2005; Usui *et al.*, 2006; Zhou *et al.*, 2008). Lineage master switches are also implicated in the transactivation

TABLE 5.1 T helper cell lineage specific cytokines, Stat proteins, and master transcription factor (TF)

Subset	T_H1	T_H1	T_H2	T_H1/T_H2	T_{REG}	T_H17
Cytokine	IFNγ	IL-12	IL-4	IL-2	IL-2	IL-6, IL-21, IL-23
Stat member	Stat1	Stat4	Stat6	Stat5	Stat5	Stat3
TF	Tbet	Tbet	Gata3		Foxp3	Rorγt (Rorα)

of the prototypical cytokine genes, for example, at least three regions in the *Ifng* gene undergo T_H1-specific locus modifications both binding Tbet accompanied with a cofactor such as Stat5 (Bream *et al.*, 2004; Hatton *et al.*, 2006; Schoenborn *et al.*, 2007; Shnyreva *et al.*, 2004). However, Tbet may not be absolutely required for remodeling of the *Ifng* locus in the presence of Stat4 and exogenous IL-12 (Usui *et al.*, 2006). Further, Stat4 also appears to be critical in maintaining transcriptional accessibility and DNA modifications, indicating that Tbet requires Stat4 to achieve complete T_H1 fate determination (Fields *et al.*, 2002; Thieu *et al.*, 2008).

The intricate cross-regulation between the T_H1 and T_H2 subsets is also apparent at the transcriptional level. The *Ifng* locus contains regions that are involved in gene silencing, which depends on the two key transcriptional activators of T_H2 cell differentiation; Gata3 and Stat6 (Chang and Aune, 2007). Similarly to the *Ifng* locus, the T_H2 locus which encompasses the *Il4*, *Il13*, and *Il5* genes contains at least three conserved regions that are responsive to chromatin remodeling and enhancer activity from the master regulator Gata3 (Lee *et al.*, 2003; Mohrs *et al.*, 2001; Solymar *et al.*, 2002). This strongly suggests that T_H cells, with the possible exception of T_H17 cells, can become terminally differentiated, and remain largely fixed in their phenotype.

Cytokines appear to be the main determining factor in the initiation of T_H cell differentiation. However, in order to fulfill this function two essential components need to be in place, the cytokine receptors and the required signaling pathways. It is of interest to note that expression of IL-12Rβ2 and IFNγR1 as well as IL-4Rα is low in naive T cells and the expression level is not increased upon *in vitro* stimulation under neutral (T_H0) conditions for IL-12Rβ2 or IL-4Rα (Fig. 5.1). However, these cytokine receptors are differentially expressed at high levels after T_H cell lineage commitment. The expression of IFNγR1 is increased upon activation under all, including neutral, conditions, which may explain the relative bias toward outgrowth of the T_H1 subset. This is often observed during *in vitro* T cell culture whereby the presence of IFNγ will result in the induction or outgrowth of the T_H1 cell subset.

Stat proteins do not appear to be upstream of the lineage master regulators. Induction of IFNγ and IL-4 can take place in the absence of Stat4 or Stat6 (Farrar *et al.*, 2001; Finkelman *et al.*, 2000; Grogan *et al.*, 2001; Kaplan *et al.*, 1998; Kurata *et al.*, 1999; Mullen *et al.*, 2001; Ouyang *et al.*, 2000). However, although the expression of prototypical cytokines can be induced, the absence of Stat activation results in defective T_H cell responses (Kaplan *et al.*, 1996a,b; Mullen *et al.*, 2001). This would suggest that cytokines may not necessarily be the prime mediators of initial T_H cell fate determination, but an essential secondary stimulus mediating outgrowth and stabilization of a particular lineage.

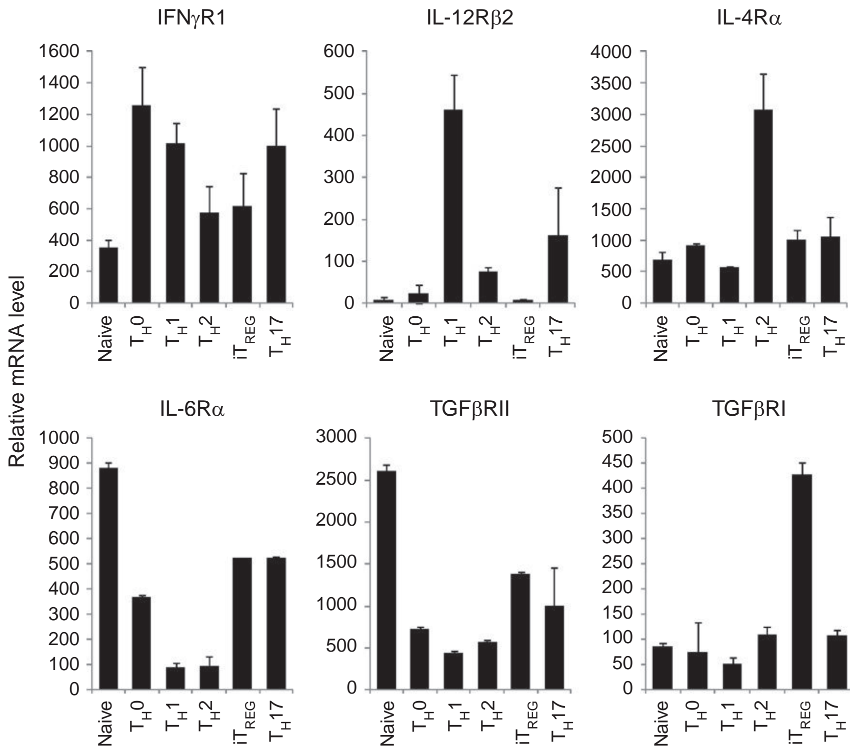

FIGURE 5.1 Cytokine receptor expression profile of T_H cell subsets. Gene transcript analysis determined for indicated cytokine receptors on FACS-sorted $CD4^+CD25^-CD44^{int}$ naive T cells after 3 days of indicated polarization conditions.

3.4. The unstable T_H17 subset

It is of interest to note that receptors required for T_H17 differentiation are already highly expressed on naive T cells, both IL-6Rα and TGFβR type II (Fig. 5.1). A self-reinforcing feedback loop, such as is present in T_H1, T_H2, and iT_{REG} cells, has neither been reported for Rorγt nor is there any functional evidence on the direct or indirect influence of Rorγt on Tbet, Gata3, or Foxp3. This suggests that T_H17 may not be able to achieve a state of terminal differentiation, but may instead be under the control of the local environment allowing a high degree of immunoadaptation. Furthermore, this raises the question if T_H17 cells exist as a fully differentiated subset or if they mainly serve a short-term goal as an extension of the innate immune system.

There are currently no experimental data that suggest the initiation of T_H17 cell differentiation without required cytokine stimulation. Cells from mice expressing a dominant negative form of the TGFβRII contain

severely diminished numbers of T_H17 in the lymphoid tissues (Veldhoen *et al.*, 2006b), but some ''leakage'' of the transgene which is coexpressed with the endogenous receptor cannot be excluded. A recent report suggests that TGFβ-independent T_H17 development may take place in the intestine, although with reduced efficiency when compared to its development in the presence of TGFβ (Ghoreschi *et al.*, 2010). In the absence of TGFβ, IL-23 appears to be the essential factor to generate T_H17 cells. Since the IL-23-receptor is absent on naive T cells, IL-6 was shown to be required and sufficient for its expression. However, the combination of IL-6 and IL-23 was previously assessed and shown not to be able to induce IL-17 (Bettelli *et al.*, 2006), while IL-23 was shown not to be essential for *in vivo* and *in vitro* T_H17 generation (Bettelli *et al.*, 2006; Khader *et al.*, 2007; Mangan *et al.*, 2006; Martin *et al.*, 2009; McGeachy *et al.*, 2009; Veldhoen *et al.*, 2006a).

IL-6-deficient mice have reduced, but not absent, numbers of T_H17 cells (Korn *et al.*, 2007; Martin *et al.*, 2009). Korn *et al.* could attribute this to the contribution of IL-21, which is produced by many cell types including T_H17. In contrast to IL-17, it appears that IL-21 has the capacity to promote T_H17 development in an autologous manner, while inhibiting the production of IFNγ (Korn *et al.*, 2007; Nurieva *et al.*, 2007; Wei *et al.*, 2007; Zhou *et al.*, 2007). This would indicate that in parallel to T_H1 and T_H2 cross-regulation, IL-21 is the T_H17 cytokine equivalent of IFNγ and IL-4, an autologous product that enhances differentiation of the subset that produces it while directly inhibiting the fates of others.

Both IL-6 and IL-21 preferentially activate Stat3, a crucial pathway for T_H17 development, as its absence results in greatly impaired T_H17 differentiation *in vivo* and *in vitro* (Chen *et al.*, 2006; Durant *et al.*, 2010; Mathur *et al.*, 2007; Yang *et al.*, 2007). However, it is currently not clear if T_H17 lineage initiation is Stat3-dependent or only the outgrowth and survival of this subset. Stat3 has indeed been implicated in the regulation of genes involved in cell survival and proliferation (Bourillot *et al.*, 2009; Durant *et al.*, 2010; Hirano *et al.*, 2000). Durant *et al.* revealed a nonredundant and lymphocyte intrinsic role for Stat3 in T cell proliferation under inflammatory conditions. This is in agreement with previous reports suggesting an important contribution for IL-6 in T cell survival in an inflammatory context (Atreya *et al.*, 2000). It remains to be established if Stat3 can directly bind the *Rorc* gene. However, in stark contrast to T_H1 and T_H2 development, the expression of the T_H17 lineage-determining factor, Rorγt, may depend on Stat3 (Mathur *et al.*, 2007). Forced expression of active Stat3 in *Rorc*-deficient cells induces some IL-17 production, suggesting that Stat3 itself is not sufficient for IL-17 expression (Zhou *et al.*, 2007). It is possible that Stat3 binding to intergenic sites contributes to the regulation of T_H17 gene expression, most likely via control of the accessibility of the *Il17a*, *Il17f*, *Il21*, and *IL-23R* genes (Durant *et al.*, 2010; Wei *et al.*, 2007).

If the role of Stat3 is crucial for the initiation of the T_H17 program, this would suggest that the role of the cytokines IL-6 and TGFβ for the initiation of T_H17 development is more important than those involved in the initiation of T_H1/T_H2 development. This is in agreement with the expression of the respective receptors on naive precursors (Fig. 5.1). It is further important to note that the decreased expression of IL-6Rα upon T cell activation does not render T cells less susceptible to the actions of IL-6. Soluble IL-6Rα can be secreted in combination with IL-6 by several innate cell types; the binding to the constitutively expressed gp130 on T cells allows continued IL-6 stimulation (Jones *et al.*, 2010). This is one layer of *in vivo* complexity which is highly undervalued based on *in vitro* data alone. The survival and proliferation of T_H17 cells seems under the control of yet another cytokine that requires Stat3-dependent signaling, IL-23. The role of IL-23 in T_H17 biology was known prior to the discovery of the *de novo* development of this subset (Cua *et al.*, 2003; Langrish *et al.*, 2004; Murphy *et al.*, 2003). IL-23 deficiency allows for the initiation of Th17 cells, both *in vitro* and *in vivo*, but severely impacts the survival and functioning of these cells (McGeachy *et al.*, 2009). Interestingly, the induced expression of the IL-23 receptor is Stat3-dependent and can be regulated by both IL-6 and IL-21(Ghoreschi *et al.*, 2010; Nurieva *et al.*, 2007; Zhou *et al.*, 2007).

From an early stage, it was recognized that T_H17 cells can produce the prototypical T_H1 cytokine IFNγ both *in vitro* and *in vivo* (Acosta-Rodriguez *et al.*, 2007; Mangan *et al.*, 2006). This may have important implications for lineage commitment. T_H17 cells may not be terminally differentiated but an intermediate stage on its way to be fully committed. In agreement with this notion, it was recently shown that in contrast to T_H1 cells, T_H17 cells are short lived and do not give rise to a population of memory cells (Pepper *et al.*, 2010). Our recent generation of a mouse expressing Cre recombinase under the control of the *Il17a* gene (IL-17A^{Cre}) allowed the fate mapping of those cells that actively transcribe the *Il17a* gene (Hirota *et al.*, 2011). Interestingly, we could show that under chronic stimulatory conditions, which promote the production of IL-23 (Veldhoen *et al.*, 2006b), T_H17 cells can switch off the expression of IL-17A and continue their existence as T_H1 cells in a IL-23-dependent manner (Hirota *et al.*, 2011; Lee *et al.*, 2009). Importantly, these ex-T_H17–T_H1 cells have a distinctly different phenotype compared with T_H1 cells which did not undergo the T_H17 cell developmental program (Hirota *et al.*, 2011). However, not all inflammatory conditions resulted in T_H17 to T_H1 conversion, but the T_H17 program was in all cases terminated. The intriguing implication would be that some immune activation events, or more precisely immune responses at certain anatomical sites like the mucosae of the nasal tract and skin (Hirota *et al.*, 2011; Pepper *et al.*, 2010), can result in a powerful T_H17 response including the recruitment of neutrophils but

this does not give rise to long-term immunological memory. Further, an earlier finding showed that, in the absence of IL-23, the primary immune response against *Mycobacterium tuberculosis* is unaffected, but upon rechallenge, the memory response is severely impaired compared with a response in which IL-23 was present during the primary response (Khader *et al.*, 2007). This might provide important insights in the seemingly conflicting data in which T_H17 responses in the intestine are either protective or pathogenic (Kullberg *et al.*, 2006; Zenewicz *et al.*, 2008).

The restrictions of T_H17 cells to enter the memory pool could be an important mechanism of peripheral tolerance via deletion. This would be especially important at mucosal sites colonized with a large variety of commensal bacteria. The licensing of acute T_H17 inflammatory responses would prevent the microbial invasion of otherwise sterile sites and avoid the development of chronic infections. TGFβ, either derived from innate immune cells or autologously produced by T cells, appears important in maintaining the stability of the T_H17 subset during its first stages of development (Li *et al.*, 2007). Ghoreschi *et al.* highlight this importance, showing the inhibitory effect of TGFβ on the expression of the IL-23 receptor and the different gene expression profiles of T_H17 cells generated with or without IL-23. We would predict the T_H17 cells stimulated with IL-23 to be highly susceptible to T_H1 conversion and able to produce IFNγ, as indicated by the presence of the prototypical T_H1 transcripts for Tbet, Hlx, and the IL-18R1 in IL-23-stimulated T_H17 cells (Ghoreschi *et al.*, 2010). In addition, this would restore the classical role of TGFβ as an anti-inflammatory cytokine, despite its role in the initiation of T_H17 cells.

The role of IL-23 may be to instruct a signal of survival and lineage conversion, thereby altering the subset's functional properties while potentially preserving its TCR repertoire in the lymphocyte memory pool for future use against reinfection with the same antigen. Conversion of T_H17 may be required depending on the pathogen or inflammatory situation encountered. The initial recruitment of neutrophils may prove to be insufficient to clear or contain an invading pathogen. The subsets flexibility allows the switch to at least T_H1-like cells, changing the immune response toward enhancing macrophage activity and other IFNγ-associated effects. However, the initial influx of neutrophils, the most phagocytotic immune cell type, followed by additional activation of macrophages would increase the danger of substantial levels of immune pathology. Deregulated IL-23/IL-23R signaling, or persistent infections, could thus enhance chronic inflammation. This is in line with single nucleotide polymorphisms (SNPs) found in the IL-23R allele by genome wide association studies (GWAS) into mucosal disorders of the skin and intestine (Duerr *et al.*, 2006; Nair *et al.*, 2009).

Although human and mouse T_H1, T_H2, and T_{REG} cell development seem very similarly regulated, there is a still ongoing debate on the

discrepancies between mouse and human T_H17 cell differentiation (O'Garra *et al.*, 2008). The most striking difference, and the possible underlying reason behind these discussions, is the relative ease with which many mouse immunologists can generate highly polarized T_H17 cells from naive $CD4^+$ T cells precursors. Cultures with human $CD4^+$ T cells, including FACS-sorted cells from cord blood, can be differentiated into T_H17 cells only with great difficulty and with comparatively low purity. Although cell purities and activation status as well as serum and culture medium ingredients are easy to blame, this would suggest that we may not have understood the importance of a much more fundamental stimulus present in the *in vivo* microenvironment such as the composition of costimulatory molecules present during T_H17 cell initiation.

4. STRENGTH OF SIGNALING

Naive T cells encounter a complex environment containing a multitude of factors that can influence their ultimate fate. However, naive T cells often lack the equipment, that is, the receptors, to respond to these cues prior to their activation. It is debatable if lineage commitment is initiated instantly after TCR stimulation. Immediately after TCR activation, T cells have been reported to contain transcripts, not protein, for both IFNγ and IL-4, as well as low expression levels of several lineage-determining transcription factors (Grogan *et al.*, 2001). Importantly, the existence of these transcripts does not require the presence of Stat proteins, but these may be required at later time points to sustain cytokine expression correlating with the induction of Tbet or Gata3 (Thieu *et al.*, 2008; Yamane *et al.*, 2005). However, not all T_H cell responses require the characteristic lineage instructing cytokines, some T_H1 cell responses, especially those against viruses, are IL-12-independent (de Wit *et al.*, 2004; Oxenius *et al.*, 1999; Schijns *et al.*, 1998). In addition, some T_H2 cell responses, including those against parasites, are independent of IL-4 (Finkelman *et al.*, 2000; Jankovic *et al.*, 2000; King *et al.*, 2008; Voehringer *et al.*, 2006). These and other observations resulted in the thesis that other factors such as the duration and strength of signaling are important determinants in lineage fate decisions (Lanzavecchia and Sallusto, 2000).

4.1. The immunological synapse

When a TCR recognizes its cognate antigen presented in context of an MHC molecule, a specialized interface between T cell and APC is formed called the immunological synapse (IS) or supramolecular activation cluster (SMAC; Grakoui *et al.*, 1999; Monks *et al.*, 1998). This is a three dimensional structure via which immune cells interact and exchange

information bidirectionally. The SMAC was initially thought to be composed of concentric rings each made up with a specialized mix of molecules (Shaw and Dustin, 1997), with the central part (cSMAC) composed of the TCR and costimulatory molecules such as CD2, CD4 or CD8, and CD28, accompanied by important proximal signaling molecules such as the tyrosine kinases Lck and Fyn (Lee *et al.*, 2002b). Cytoskeletal reorganization resulting from the IS formation allows focused delivery of vesicles that release their content in the intercellular synaptic space via exocytosis.

The current IS concept predicts a highly dynamic IS which can continuously change during the T cell activation process (Dustin *et al.*, 2006). At the initiation event, some molecules of interest are concentrated at the contact site, but others can be brought in via rapid vesicle transport. Attempts to visualize these processes without the use of simplified lipid bilayers, but with *in vivo* live imaging showed minimal and only transient inclusion of TCRs into a central SMAC-like structure (Friedman *et al.*, 2010). Instead, the authors found rapid, antigen-dependent TCR internalization independent on T cell motility arrest of SMAC formation. The engaged, and even unengaged, TCRs seemed to be clustered in small islets or microclusters. Engagement of the TCR with peptide–MHC results in a stimulus of which the time and duration are determined by several factors such as the on–off rate of the TCR, the antigen concentration, and the composition of costimulatory receptors present on the APC (Aleksic *et al.*, 2010; Cemerski *et al.*, 2007).

Importantly, cytokine receptors can also cluster in the synapse where they can be exposed to the concentrated cytokines secreted into the synaptic microenvironment, thereby potentially profoundly influencing T_H differentiation, such as recently shown for IL-12 (Pulecio *et al.*, 2010). However, the majority of studies of the IS have made use of previously activated, often $CD8^+$, T cells. Technically, live imaging of subcellular signaling complexes expressed at physiological densities in intact tissues has been enormously challenging. It is therefore, as yet, largely unknown how and which cytokines may play a role in the initial contact between T cell and APC ultimately resulting in T_H lineage commitment.

4.2. Strength of signaling

In contrast to effector and memory cells, naive T cells require at least three signals for their activation and differentiation. Importantly, all these signals are provided through the IS, emphasizing its pivotal role in the exchange of information. The first signal is the engagement of the TCR with the peptide–MHC complex. The second is dispensable for previously activated cells and is provided by the collectively called costimulatory molecules. The third comes from the afore mentioned cytokines, secreted via focused delivery or generally secreted into the local milieu.

The accumulated signals derived from the APC side of the IS initiate specific but integrated signaling cascades of particular strength and duration. It is worth to note that T cells respond to the initial stimuli provided by the APCs with their own feedback signals which can modify the APCs performance. This results in enhanced signaling processes, thereby strengthening the T_H commitment process. However, when multiple T cells are engaged by the same APC, this could allow cross-regulation of T cells with different TCR specificities and allow committed effector or memory T_H cells, or other cell types such as NK-, NKT-, B-cells, or T_{REG}, to influence the differentiation of new T_H cells.

The first molecular event occurring after TCR engagement is thought to be the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) elements, each containing two phosphorylation sites, found in the four polypeptides of the TCR complex (CD3γ, CD3δ, CD3ε, and TCRζ). Ligands that induce stronger functional responses result in increased phosphorylation of TCR complex ITAMs and, as a result, increased TCR internalization (Hemmer *et al.*, 1998; Itoh *et al.*, 1999; Liu *et al.*, 2000). This process is mediated by the Src tyrosine kinases family members Lck and Fyn but does neither involve the phosphorylation of all possible ten ITAMs nor necessarily result in dual phosphorylation of these motifs (Samelson, 2002). These protein modifications result in the formation of protein docking sites, thereby assembling components that initiate a chain of signaling events in the cell. The phosphorylation status of the combined ITAMs thus allows a large degree of signaling fine tuning. It has been shown that the strength of antigen stimulation, in terms of quality as well as quantity, can influence the signaling cascades downstream of the TCR, thereby impacting the development of T_H lineages (Constant and Bottomly, 1997).

4.2.1. TCR affinity

$CD4^+$ T cells recognize antigenic peptide presented on MHC class II molecules positioned in a peptide-binding cleft, held in place by anchor residues. Antigenic specificity of the TCR depends only on a few residues of its amino acid chains which ''recognize,'' through hydrogen bonds, three to five residues in the antigenic peptide and residues in the MHC molecule (Sette *et al.*, 1987). The limited interactions that take part in the recognition process suggest that small changes in the peptide composition may have significant effects on the ability to trigger the TCR. This is especially the case when direct or primary contact residues are altered. Interestingly, changes in secondary residues do activate the TCR specific for the original peptide but alter the signaling strength (Sloan-Lancaster and Allen, 1996). As such, signals resulting in the secretion of cytokines and T_H cell proliferation, as well as changes in T_H cell phenotype, can be

altered with one amino acid substitution (Evavold and Allen, 1991; Sloan-Lancaster and Allen, 1996).

T_H cells expressing a transgenic TCR where shown to produce IFNγ upon stimulation with its native peptide, whereas a low-affinity altered peptide ligand (APL) resulted in IL-4 production (Pfeiffer *et al.*, 1995; Tao *et al.*, 1997b). Concordantly, similar results were obtained when a change was made in a single residue in the TCR, responsible for peptide recognition (Blander *et al.*, 2000). Further, the outcome of T_H differentiation is also influenced by the haplotype of the MHC II molecules (Murray *et al.*, 1992). Since the studies on APLs were performed using transgenic TCR models, the significance of the observed changes in T_H cell differentiation in physiological conditions remains unclear. After all, a highly varied TCR repertoire is generated under stringent selection criteria, and the encounter with antigen is known to preferentially select medium and high affinity TCRs (Fasso *et al.*, 2000; Malherbe *et al.*, 2004; Savage *et al.*, 1999). Only when affinity competition for peptide ligands in polyclonal $CD4^+$ responses is absent can T_H2 cells with a low-affinity TCRs develop (Milner *et al.*, 2010).

The concept of peptide affinity is common to most APL-studies, whereby high affinity ligands correlate with T_H1 cell responses and weak ligands with T_H2 type responses (Chaturvedi *et al.*, 1996; Kumar *et al.*, 1995). Although the physiological relevance of the observations made with APLs has remained elusive, within the constraints of a bimodal T_H1–T_H2 response, they were once considered a tool for immunotherapy. In a mouse autoimmune model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), pre- or coadministration of a low-affinity APL was reported to inhibit the development of a, at the time considered, predominant T_H1-mediated disease via a switch to a T_H2 type response (Nicholson *et al.*, 1995). There are currently no data on the alterations in TCR-agonist peptides and the development of T_H17 responses.

4.2.2. Antigen dose

In 1995, two seminal studies by the O'Garra and Bottomly laboratories established through *in vitro* studies the extent by which TCR ligation strength can determine the functional differentiation of naive $CD4^+$ T cells (Constant *et al.*, 1995; Hosken *et al.*, 1995). Both groups associated the induction of T_H2 cells with a relatively low antigen dose and T_H1 cells with a higher dose. The induction of IFNγ by exposure to high antigen dose is in agreement with observations that strong TCR engagement results in increased and sustained Erk activation, which is reported to inhibit additional Gata3 expression and may therefore decrease the initiation of T_H2 cell development (Jorritsma *et al.*, 2003).The level of expression of Gata3 depends on the strength of TCR signaling received during

T cell development in the thymus (Hernandez-Hoyos *et al.*, 2003) and may thus be different between naive T cells expressing a different TCR. Interestingly, the Hosken *et al.* study revealed that in cultures in the presence of DC, the highest antigen concentrations, similarly to those with the lowest, also induced T_H2 cell development. This is in line with Stat6-independent T_H2 differentiation which does not depend on initial increases of Gata3 expression. Instead, it relies on the presence of high amounts of IL-2, as encountered during strong antigen stimulation, and Stat5 signals (Yamane *et al.*, 2005).

There is limited *in vivo* data regarding the influence of antigen dose on T_H differentiation. Studies using immunogenic peptides are in line with the *in vitro* observations where low dose of antigen leads to T_H2 and high dose to T_H1 differentiation (Chaturvedi *et al.*, 1996; Pfeiffer *et al.*, 1995). Additional studies titrated whole microorganisms, such as viruses and helminthes, thereby titrating TCR-agonists as well as the PAMPs and thus changing expression levels of costimulatory molecules and cytokines (Bancroft *et al.*, 1994; Bretscher *et al.*, 1992; Darrah *et al.*, 2007; Parish and Liew, 1972). There is very little data on the role of concentrations of individual PAMPs and their influence in shaping T_H immune response (Eisenbarth *et al.*, 2002), but we can assume that the effect of low or high dosage of various PAMPs on APCs results in the differential expression of surface molecules and levels of cytokines.

In stark contrast to thymic nT_{REG} development which depends on a relative strong TCR ligation (Hsieh *et al.*, 2004; Liston and Rudensky, 2007), the induction of iT_{REG} was shown to be dependent on weak TCR stimulus (Turner *et al.*, 2009). Strong antigenic stimulation inhibits its development due to the activation of the PI3K/Akt pathway (Haxhinasto *et al.*, 2008; Sauer *et al.*, 2008). Since PI3K positively regulates Erk signaling, this suggests that the inhibition of T_H2 and iT_{REG} development is linked. Besides the local cytokine environment, T cell production levels of IL-2 might also play a decisive role, with T_H2 development requiring more Stat5 activity (Zhu *et al.*, 2003). However, there is currently no mechanistic insight on how the signals resulting in T_H2 and iT_{REG} development are quantitatively different.

T_H17 cells are reported to develop, like T_H1, after relatively strong antigenic stimulation (Gomez-Rodriguez *et al.*, 2009; Iezzi *et al.*, 2009). Since different transgenic models were used in these studies, this does not allow for direct comparison of the obtained results with other reports. Therefore, the position of T_H17 cells with respect to antigen dose and T_H1 and T_H2 cell development is currently not clear. High antigen dose has also been shown to play a role in the differentiation of T_{FH} cells (Fazilleau *et al.*, 2009), which suggests that strength of TCR signaling is only one component of a mixture of factors ultimately determining the T_H fate. It is also worth noting that there are currently no reports of T_H17 cell

generation in the absence of at least one of the initiating cytokines. In the absence of IL-6, IL-21 is required (Korn *et al.*, 2007), while a report claiming T_H17 development in the absence of TGFβ needs to include IL-6 and IL-23 (Ghoreschi *et al.*, 2010).

4.2.3. Costimulation

The multimolecular IS complex is built around the TCR–peptide–MHC interaction. This crucial contact is accompanied by many other proteins important for cell adhesion and activation such as members of the CD28-, integrin- (Melton *et al.*, 2010), and Notch-families, the latter of which is discussed elsewhere (Amsen *et al.*, 2009).

4.2.3.1. A role for CD28 and CTLA-4? CD28, which is constitutively expressed, provides an essential costimulatory signal to naive T lymphocytes upon recognition of their cognate antigen. Ligation of CD28 is a necessary early step in T cell activation, required for cell proliferation, survival, and cytokine production (Gmunder and Lesslauer, 1984; Lindstein *et al.*, 1989; Parry *et al.*, 1997). The CD28 binding partners, CD80 and CD86 (B7.1 and B7.2) are expressed on APCs (Azuma *et al.*, 1993; Freedman *et al.*, 1987; Linsley *et al.*, 1990). Their expression is substantially increased upon PRR engagement, thereby licensing the activation of naive T cells (Acuto and Michel, 2003). However, T cell activation events are tightly regulated, and 24–48 h after activation, they express a receptor that shares homology with CD28 but shows higher affinity for CD80 and CD86; CTL-associated molecule (CTLA)-4, providing an inhibitory signal (Brunet *et al.*, 1987; Linsley *et al.*, 1991; Walunas *et al.*, 1994). The importance of this feedback mechanism is highlighted in CTLA4-deficient mice which manifest a large and lethal lymphoproliferative disease (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995).

The effect of CD28 costimulation is closely linked with the TCR signaling-strength. It supports IL-4 production in combination with weak TCR signals, however, it does not promote T_H2 differentiation under high antigen dose (Tao *et al.*, 1997a), which results in IFNγ production instead (Rogers and Croft, 2000). These data are consistent with reports emphasizing the importance of Erk in T_H differentiation. Despite the increase in CD28-mediated IL-2 production (Fraser *et al.*, 1991), supportive of T_H2 development (Cote-Sierra *et al.*, 2004), sustained Erk phosphorylation inhibits Gata3 expression and thereby T_H2 cell development (Jorritsma *et al.*, 2003). However, prolonged engagement of CD28 was shown to be important for T_H2 differentiation (Jorritsma *et al.*, 2003). Although CD28 signaling is important for T_H activation and T_H1 and T_H2 development, examination of CD28-deficient mice in the C57Bl/6 and BALB/c background demonstrated surprisingly normal T_H1 and T_H2 responses (Brown *et al.*, 1996). Thus, the precise role that CD28 plays in T_H cell

differentiation remains to be defined. It is reported that CD28 costimulation is also important for T_H17 differentiation; however, this is in agreement with its general requirement in T_H activation (Park *et al.*, 2005). More interestingly, prolonged CD28 signaling can inhibit IL-17 production (Bouguermouh *et al.*, 2009), in line with the inhibitory effect of IL-2 and Stat5 activation of T_H17 development (Laurence *et al.*, 2007).

Cross-linking of CTLA-4 during activation of T cells reduces their production of IL-2 (Krummel and Allison, 1995). In opposition to the supportive role of CD28 engagement in cytokine mRNA stabilization, such as IL-2, CTLA-4 inhibits T_H2 cell (Bour-Jordan *et al.*, 2003; Oosterwegel *et al.*, 1999), as well as T_H1 cell differentiation and cytokine production (Alegre *et al.*, 1998). In paradox, while partly dependent on IL-2 for their induction and maintenance, CTLA-4 seems to be involved in iT_{REG} induction (Perez *et al.*, 1997; Samoilova *et al.*, 1998; Zheng *et al.*, 2006). The role of CTLA-4 could similarly be predicted to enhance T_H17 initiation, however, results have been ambiguous (Babu *et al.*, 2009; Bouguermouh *et al.*, 2009).

4.2.3.2. A role for ICOS–ICOSL? CD28 is considered to be the primary cosignaling molecule on $CD4^+$ T cells because of its constitutive expression, and it is routinely used in the *in vitro* generation of all T_H cell subsets. However, new members of the B7/CD28 family have been identified, this includes B7-H2 or ICOS-ligand (Swallow *et al.*, 1999). This protein shares sequence homology with both B7-1 and B7-2 but it does not bind to either CD28 or CTLA-4. Instead, it binds to a CD28 homolog, the inducible costimulator (ICOS), which is expressed on activated T cells (Hutloff *et al.*, 1999). ICOS was shown to be important for T_H2 differentiation, but its requirement is dependent on the experimental system used, and ICOS deficiency can also result in reduced T_H1 cell development (Kopf *et al.*, 2000; Rulifson *et al.*, 1997; Tsuyuki *et al.*, 1997; Zheng *et al.*, 2006).

More recent studies implicate ICOS in development of T_H17 and T_{FH} cells (Bauquet *et al.*, 2009; Paulos *et al.*, 2010). Interestingly, ICOS-ligand is known to be expressed on many epithelial cells (Kim *et al.*, 2005). Mucosal immunization protocols, including the intranasal route, are strongly associated with T_H17 immune responses (Pepper *et al.*, 2010; Zygmunt *et al.*, 2009). In addition, at the two largest epithelial sites, the skin and the intestine, prominent T_H17 cell populations can be found, suggesting that ICOS–ICOSL interactions may have an important role in the initiation of T_H17 differentiation (Furio *et al.*, 2010). Signaling via ICOS was shown to be able to induce optimal IL-17A secretion by T_H17 cells (Park *et al.*, 2005). Further, it can amplify T_H17 responses via c-Maf induction and subsequent transactivation of its autologous stimulatory cytokine IL-21 (Bauquet *et al.*, 2009).

A recent study used ICOS-ligand for *in vitro* T_H17 generation from human cord-blood and achieved an impressive polarization of IL-17 producing T_H cells (Paulos *et al.*, 2010). In agreement with previous reports, CD28 stimulation, but not ICOS, was shown to enhance T_H2 polarization via increased production of IL-2, which was not achieved after ICOS ligation. This is in line with Laurence *et al.*, who showed the inhibitory effect of IL-2 on T_H17 development via Stat5 activity. Indeed, costimulation via ICOS, but not CD28, resulted in high T_H17 polarization. Besides the reduction in IL-2 production, ICOS ligation enhanced c-Maf transcripts and the production of IL-21, all important in T_H17 initiation. CD28 stimulation of cord-blood cells in the presence of IL-2 neutralizing antibodies and exogenous IL-21 could enhance the production of IL-17 to a level comparable with ICOS stimulation alone. Interestingly, ICOS stimulation could maintain the T_H17 cells found in peripheral blood, in stark contrast to CD28, but it did not preferentially enhance IL-17 single- or IL-17/IFNγ double-producers.

ICOS-ligand is expressed in many tissues, such as B cells, macrophages, DC, and other cell types including endothelial cells and epithelial cells (Aicher *et al.*, 2000; Gonzalo *et al.*, 2001; Yoshinaga *et al.*, 1999), and ICOS-ligand overexpression can result in autoimmunity (Tafuri *et al.*, 2001; Yu *et al.*, 2007). ICOS is not constitutively expressed on naive T cells but is induced following T cell activation (Coyle *et al.*, 2000; Hutloff *et al.*, 1999; Yoshinaga *et al.*, 1999). Its expression can be detected as early as 1 h after activation and it is clear that ICOS ligation can enhance T cell proliferation and influence T cell effector functions (Dong *et al.*, 2000; McAdam *et al.*, 2001). However, ICOS-deficient mice are susceptible to T_H17-dependent myelin oligodendrocyte glycoprotein (MOG)-induced EAE (Dong *et al.*, 2000; Galicia *et al.*, 2009). This suggests that ICOS may not be directly involved in the initiation of T_H17, but may play a role in its subsequent function. In line with this notion, both CD28 and ICOS stimulation resulted in equal induction of Tbet and RORγt, with differential expression only apparent after several days (Paulos *et al.*, 2010). Further, *in vivo* blocking of ICOS does not prevent but exacerbates the induction of EAE, while its neutralization during the onset of symptoms does abrogate the disease (Rottman *et al.*, 2001).

4.2.3.3. A role for CD40–CD40L? ICOS cross-linking results in the expression of another important costimulatory molecule; CD40-ligand (CD40L; Grewal and Flavell, 1996; Watts and DeBenedette, 1999). CD40L ligation has been shown to be important for T_H17 induction with high antigen dose (Iezzi *et al.*, 2009), and is required in EAE (Grewal *et al.*, 1996). A recent study indicated that CD40L-CD40-dependent feedback from T cell to DC enhances IL-6 production, essential for T_H17 differentiation (Perona-Wright *et al.*, 2009). This suggests that T_H17 initiation may

depend sequentially on TCR, ICOS, and CD40L interactions. However, many studies have highlighted the role of CD40L cross-linking in T_H1 differentiation (Blazar *et al.*, 1997; Campbell *et al.*, 1996; Cella *et al.*, 1996; Koch *et al.*, 1996; Stuber *et al.*, 1996). *In vitro* activation of T cells with high antigen dose induces CD40L expression, which upon ligation with CD40 on DC stimulates the production of IL-12. Importantly, T cell stimulation with low dose of antigen, resulting in T_H2 development, fails to induce CD40L (Ruedl *et al.*, 2000). Although this is in agreement with observations that expression of CD40L is upregulated by IL-2 (Skov *et al.*, 2000) and inhibited by IL-4 (Lee *et al.*, 2002a), there is no data on the expression of CD40L during high IL-2-dependent T_H2 development. In addition, there is currently no additional insight for the seemingly contradictory role of CD40L in both T_H1 and T_H17 differentiation. However, the intricate relationship between these two subsets may not rule out the importance of CD40L for both.

Importantly, the costimulatory molecules should not be seen in isolation but as a carefully orchestrated response by APCs, timely organized in the IS within the first hours of stable T cell–APC interaction, to optimize an appropriate immune response to encountered pathogens or their products. The combinational presence, their concentration, and the kinetics of their expression will have a substantial influence on the initiation of T_H subsets. Future advances in high-resolution live imaging focusing on the sequential fast acting processes in the IS can provide important answers, linking the APCs encounter with a pathogens' molecular pattern composition with the events occurring at the interface between APC and T cells. This could elucidate the role of individual costimulatory molecules and their (sequential) engagement pattern required for distinctive signal to the antigen-specific T cells. This is than subsequently reinforced by the T cell feedback signals, enhancing the development of a particular T_H cell subset. It remains to be resolved when and how cytokine and cytokine receptor signals become important in driving the process of T_H cell subset initiation, stabilization, and expansion.

5. ENVIRONMENTAL FACTORS

In addition to PAMPs, environmental factors have frequently been associated with providing the trigger enabling or enhancing the development of autoimmune or allergic responses in genetically predisposed individuals. Many years of research have highlighted the influence of chemicals that can often indirectly influence T_H cell differentiation and function. However, a more direct effect on T_H development was recently reported with the discovery of the role of *all*-trans retinoic acid (ATRA) and aryl hydrocarbon ligands.

5.1. Retinoic acid

ATRA is a dietary metabolite which can be generated by DC from vitamin A. It was initially shown to play a role in T_H2 differentiation and suppress T_H1 development (Stephensen *et al.*, 2002). TGFβ signaling acts via intracellular proteins called Smads, including Smad3, that transduce signals from TGFβ to the nucleus. Smad3 has also been shown to be induced by ATRA (Osanai *et al.*, 2007). In T_H2 cells, Smad3 expression may regulate the cytokine secretion profile via a direct interaction with Gata3 (Blokzijl *et al.*, 2002). This suggests that ATRA may also have an effect on the differentiation of T_H9 cells which require the concerted activity of IL-4 and TGFβ for their development (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008b).

More recently, ATRA has been associated with the enhanced induction of iT_{REG} and reduced T_H17 development (Coombes *et al.*, 2007; Osanai *et al.*, 2007; Sun *et al.*, 2007). Interestingly, the presence of ATRA can enhance iT_{REG} differentiation even in the presence of exogenous IL-6 or IL-21 and strong TCR stimulation (Benson *et al.*, 2007; Mucida *et al.*, 2007). The effects of ATRA are likely to be at least partially mediated by the nuclear retinoic acid receptor (RAR)α and involve increased Smad3 signaling activity (Pendaries *et al.*, 2003; Schambach *et al.*, 2007; Xiao *et al.*, 2008). However, RARα is not expressed in naive T_H cells and its induction seems dependent on TGFβ signaling (Schambach *et al.*, 2007), indicating that ATRA does not directly affect naive T cell polarization. Interestingly, ATRA stimulation is reported to result in reduced expression of the IL-6 and IL-23 receptor, thereby making the T cells refractory to subsequent regulation by these cytokines *in vitro* (Xiao *et al.*, 2008). Although EAE disease-severity was reduced in the presence of ATRA, no increases in T_{REG} populations were observed *in vivo*. This suggests that ATRA is not able to prevent T_H17 development, in line with its induced expression and trans-IL-6 signaling (Jones *et al.*, 2010), but could reduce the influence of IL-21 and IL-23 on T_H17 cells as highlighted by the reduced numbers of T_H17 found, as well as the reduced production of both IL-17 and IFNγ in the presence of ATRA (Xiao *et al.*, 2008).

5.2. The aryl hydrocarbon receptor

Interestingly, another nuclear ligand dependent transcription factor was found to be differentially expressed in T_H17 cells with some expression in T_{REG}, the aryl hydrocarbon receptor (AhR) (Quintana *et al.*, 2008; Veldhoen *et al.*, 2008a). Its activation in T_H17 results in high expression of the immunomodulatory cytokine IL-22 (Veldhoen *et al.*, 2008a, 2009). Man-made ligands for AhR can be found in cigarette smoke, charcoal-

grilled food, and industrial contaminants, which appears to make the level of AhR-activity highly dependent on lifestyle and the environment (Stockinger *et al.*, 2009). As such, AhR expression in T_H17 cells could provide an important mechanistic insight in the relation between industrial hydrocarbon and the rise in autoimmune disorders.

However, it seems unlikely that this evolutionary highly conserved system is preserved in mammals to respond to such contaminants alone. The levels of AhR-activity change during the seasons, with highest activity during the summer months (Paigen *et al.*, 1981). Interestingly, this correlates with the occurrence of new lesions and immune activity in multiple sclerosis patients, with a two to three times higher likelihood in March–August than during the rest of the year, correlating strongly with regional climate data and solar radiation in particular (Meier *et al.*, 2010). In line with this observation is the close relationship of AhR with proteins determining the circadian rhythm. Interestingly, proteins with similar ligand-binding domains as AhR primarily detect changes in energy status (Gu *et al.*, 2000; Veldhoen and Duarte, 2010). This suggests that AhR responds to endogenous ligands generated after environmental cues such as light. In agreement, photoproducts of the amino acid tryptophan have been shown to be high affinity ligands and to be present *in vivo* (Oberg *et al.*, 2005).

AhR is widely expressed in many cell types present throughout the body. As such, the precise role of AhR in the immune system, and its induced expression in T_H17 cells, in particular, should probably be seen in the context of the surrounding tissues. The expression of AhR within the T_H17 subset may itself be under tight regulation. T_H17 cells generated *in vitro* with TGFβ and IL-6 show high levels of AhR expression (Ghoreschi *et al.*, 2010; Veldhoen *et al.*, 2008a), however, those stimulated with IL-23 seem to reduce its expression level (Ghoreschi *et al.*, 2010). We previously reported the presence of high levels of AhR in T_H17 freshly isolated from draining LNs after immunization (Martin *et al.*, 2009; Veldhoen *et al.*, 2008a). Our recent studies with an IL-17 fate reporter mouse confirmed these results. In addition, we can now show that T_H17 cells that have switched their T_H17 cell phenotype toward a T_H1 cell phenotype retain the expression of AhR as well as the IL-1R1 (Hirota *et al.*, 2011). This indicates that those T_H1 cells that are T_H17 cell-derived are still susceptible to AhR-ligands as well as IL-1. In agreement, natural killer (NK) cells that can selectively produce IL-22 express IL-1R1 and AhR (Hughes *et al.*, 2010). The role of IL-1 in autoimmune disorders is well documented (Sutton *et al.*, 2006), but it remains to be seen if it is required in maintaining and activating both T_H17 and T_H1, or in the transition phase between T_H17 to T_H1. The role of AhR is also of great interest, as the ability to interfere with its activity may hold potential therapeutic benefits.

6. CONCLUSION

The discovery of a third major subset of T_H cells seemed to initially resolve some contradictory experimental observations. However, there now seem to be even more questions to be resolved than before. The relative contributions that cytokines and the initial stimulatory signals (TCR signaling-strength, costimulatory molecules, and cell type composition) encountered by T cells upon activation make toward the initiation of a particular T_H cell subset have as yet to be resolved. In addition, we are now faced with a high degree of plasticity, which seems particularly high in the new T_H17 cell subset. The flexibility of this subset could have intriguing implications for the maintenance of peripheral tolerance, immunity at mucosal barrier sites, and the development of autoimmune disorders. A detailed understanding of the elements that determine the initiation, maintenance, quiescence, or the conversion toward another T_H subset, while retaining characteristics of its previous T_H17 cell existence, would no doubt proof extremely useful in therapeutic immune interventions.

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