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CHAPTER 1

Learning from Leprosy: Insight into the Human Innate Immune Response

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Contents

Abstract Investigation into the innate immune response in leprosy has provided insight into host defense and immunopathology in human infectious disease. A key advance has been the delineation

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of pattern recognition receptors that detect pathogen-associated molecular patterns of the bacterium that causes leprosy, *Mycobacterium leprae*. From this knowledge, it has been possible to determine the cytokine responses as well as macrophage and dendritic cell differentiation programs that contribute to host defense and tissue injury in leprosy. These insights provide targets for therapeutic intervention to modulate innate immune responses against microbial infection in humans.

In 1884, when Metchnikoff discovered phagocytes (Metchnikoff, 1884) and the process of phagocytosis, he created what is now the modern concept of innate immunity, indicating that the host response against foreign pathogens involves the (1) rapid recognition of pathogens, (2) uptake, and (3) subsequent killing of the invaders, ''Whenever the organism enjoys immunity, the introduction of infectious microbes is followed by the accumulation of mobile cells, of white corpuscles of the blood in particular which absorb the microbes and destroy them'' (Mechnikov, 1908). Immunologists have since linked phagocytosis to an array of microbicidal and digestive mechanisms. The internalization and subsequent killing of pathogens are not only key to the innate immune response but also in promoting antigen presentation and initiation of the acquired immune response. However, mycobacteria have evolved many mechanisms to circumvent the innate immune response of host cells, in fact surviving and proliferating within the macrophage (MΦ), the primary phagocyte of the innate immune response. Through study of mycobacterial disease, many details of the innate immune response have been elucidated, this review will focus on the study of human leprosy to contrast host defense versus pathogenesis at each stage of the innate immune response.

1. LEPROSY AS A MODEL

Leprosy, caused by the intracellular pathogen *Mycobacterium leprae*, offers an attractive model for investigating the regulation of human immune responses to infection. First, the disease itself still poses a significant health and economic burden within developing countries and shows clustering to limited geographical regions or ethnic groups within a country (Bakker *et al.*, 2002; Baumgart *et al.*, 1993; Bloom, 1986; Britton and Lockwood, 2004; Durrheim *et al.*, 2002). Although treatment of leprosy patients with multidrug regimens has reduced the number of active cases to approximately 0.5 million, the number of new cases per year is not declining (Britton and Lockwood, 2004). Furthermore, patients ''cured'' of the infection have permanent neurologic deficits, can relapse,

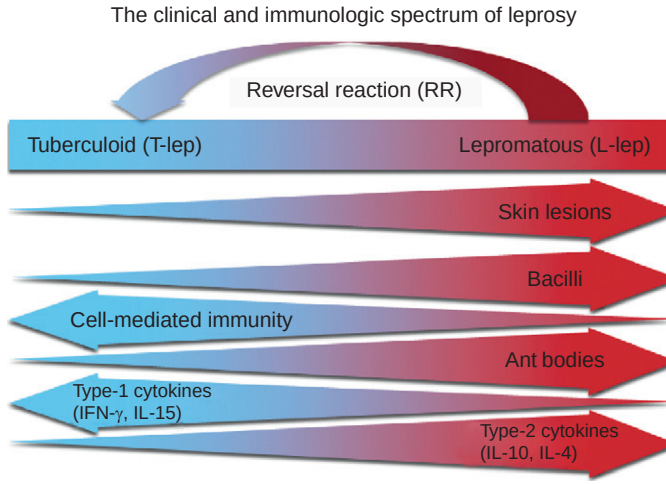

FIGURE 1.1 Clinical and immunologic spectrum of leprosy. Clinical manifestations of leprosy correlate with the immunologic response from leprosy patients. Lepromatous leprosy patients have a humoral (Th2) response ineffective at containing the intracelluar pathogen resulting in disseminated infection. In contrast, tuberculoid leprosy patients have a robust cell-mediated (Th1) immune response effective at localizing the infection.

and/or subsequently undergo tissue-damaging reactions, all leading to functional impairment and disability. Secondly, leprosy is a disease that presents as a spectrum (Fig. 1.1) in which the clinical manifestations correlate with the nature of immune response to the pathogen (Ridley and Jopling, 1966), providing an extraordinary opportunity to investigate resistance versus susceptibility to a microbial pathogen in a human disease. Here, we provide a comprehensive assessment of how investigation of leprosy histology, gene expression patterns, genetic polymorphisms, and cellular immune response have been utilized to investigate mechanisms by which the innate immune system determines outcome in human infectious disease. Although the focus of the present discussion relates to the innate immune response in leprosy, in some instances, we have included information from studies of the related mycobacterial species *M. tuberculosis*.

Patients with tuberculoid leprosy (T-lep) are relatively resistant to the pathogen as the infection is localized. The number of lesions is few and bacilli are infrequently detected, although tissue and nerve damage are both frequent. At the opposite end of this spectrum, patients with lepromatous leprosy (L-lep) are relatively susceptible to the pathogen as the infection is disseminated. Skin lesions are numerous and growth of the pathogen is unabated, with numerous bacilli detected in MΦ. These diverse

clinical presentations correlate with the level of cell-mediated immunity (CMI) against *M. leprae*. The standard measure of CMI to the pathogen is the Mitsuda reaction or lepromin skin test, a 3-week response to intradermal challenge with *M. leprae*. The test is positive in T-lep patients but negative in L-lep patients. Interestingly, serum antibody levels against *M. leprae* are greater in L-lep patients, indicating that humoral responses do not contribute to host defense against mycobacteria.

The mechanisms that contribute to the inverse correlation between CMI and humoral immunity were initially investigated according to the distribution and function of T cells at the site of disease. In leprosy skin lesions, CD4+ T cells predominate in the T-lep form; whereas, CD8+ T cells predominate in the L-lep form (Modlin *et al.*, 1983). Furthermore, the cytokine patterns of these T cell subsets show a striking correlation with the disease type (Cooper *et al.*, 1989; Yamamura *et al.*, 1991, 1992). CD4+ T cells that produce the type-1 or Th1 cytokine pattern including IFN-γ predominate in T-lep lesions; whereas, CD8+ T cells that produce the type-2 or Th2 cytokine pattern including IL-4 predominate in L-lep lesions. Subsequent investigation of cytokine patterns in leprosy lesions revealed that the local expression of the innate type-1 cytokines IL-12, IL-18, and GM-CSF predominate in T-lep lesions, whereas the type-2 cytokines IL-10 and IL-5 characterize L-lep lesions (Garcia *et al.*, 1999; Salgame *et al.*, 1991; Sieling *et al.*, 1994; Yamamura *et al.*, 1991). The biological relevance of the Th1 versus Th2 cytokines has been investigated at each step of the innate immune response (Fig. 1.2).

2. RECOGNITION OF *M. LEPRAE* BY THE INNATE IMMUNE SYSTEM

Cells of the innate immune system express germ-line encoded pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs) that are shared among groups of pathogens. Several PRR–PAMP pairs have been shown to be involved in the innate immune response to *M. leprae* and related mycobacteria.

2.1. Toll-like receptor 2/1 (TLR2/1)

From the study of mycobacteria, significant progress has been made toward understanding the innate immune receptors that recognize and mediate host responses to mycobacteria. Several Toll-like receptors (TLRs) mediate innate immune recognition of *M. tuberculosis* and related species. Experiments performed in the Modlin lab (Brightbill *et al.*, 1999) and others (Aliprantis *et al.*, 1999) led to the exciting finding that microbial lipoproteins trigger host responses via TLR2, requiring the acyl functions

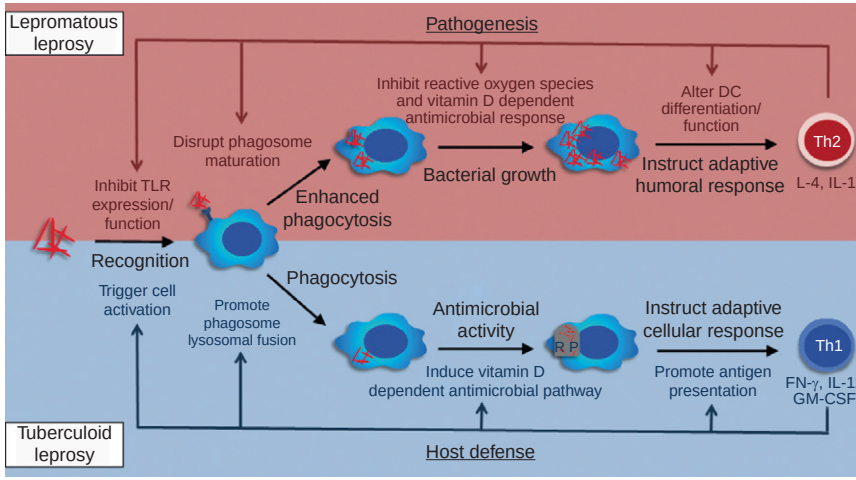

FIGURE 1.2 Leprosy as a model for host defense versus pathogenesis for the innate immune response to mycobacterial infection. The spectrum of leprosy provides an ideal human model to investigate each step of the innate immune response: recognition, phagocytosis, killing, and instruction of Th2 versus Th1 adaptive immune response. Furthermore, the Th1 versus Th2 cytokines affect each step of the innate immune response to further cause pathogenesis versus host defense, respectively.

for activity. Subsequently, triacylated lipoproteins were found to activate TLR2/1 heterodimers (Takeuchi *et al.*, 2001), whereas diacylated lipoproteins were found to activate TLR2/6 heterodimers (Takeuchi *et al.*, 2002) and activate an inflammatory cytokine response (Brightbill *et al.*, 1999; Gehring *et al.*, 2004; Pecora *et al.*, 2006; Sieling *et al.*, 2008). TLR2 also mediates the response to the lipoarabinomannan (LAM) from rapidly growing mycobacteria (Means *et al.*, 1999). TLR2 has also been shown to interact with a number of accessory molecules and receptors to modulate their recognition of mycobacterial PAMPs. For instance, TLR2 physically interacts with RP105, a PRR phylogenetically related to TLR4, in the recognition of 19-kDa lipoprotein from *M. tuberculosis* (Blumenthal *et al.*, 2009). Furthermore, the C-type lectin receptor, Dectin-1, has been reported to partner with TLR2 for recognition of avirulent mycobacterial species, but not pathogenic species. The class A scavenger receptor, macrophage receptor with collagenous structure (MARCO), is utilized preferentially to ''tether'' the predominant cell wall glycolipid, trehalose 6,6′-dimycolate (TDM) to the MΦ and activate the TLR2 in combination with CD14. However, MΦs that lack MARCO expression rely on scavenger receptor A (SR-A), which is less effective at binding TDM, resulting in markedly lower levels of pro-inflammatory cytokines (Bowdish *et al.*, 2009). These data help to explain the differential response to

mycobacterial infection of various MΦ populations which differ in their expression of scavenger receptors.

The expression and activation of TLRs was investigated in leprosy (Krutzik *et al.*, 2003). Initial studies indicated that activation of monocytes/MΦ by killed *M. leprae* required TLR2–TLR1 heterodimers for induction of cytokine responses, suggesting the presence of triacylated lipoproteins in the pathogen. A genome-wide scan of *M. leprae* detected 31 putative lipoproteins. Synthetic lipopeptides representing the 19- and 33-kD lipoproteins activated both monocytes and dendritic cells (DC) to release pro-inflammatory cytokines. Additional studies demonstrated that the *M. leprae* 33-kD lipoprotein (Yamashita *et al.*, 2004) and the *M. leprae* major membrane protein-II (Maeda *et al.*, 2005), also a lipoprotein, triggered TLR2 responses, requiring the acyl functions and the polypeptide region for optimal activity. The activation of the TLR2/1 heterodimer by *M. leprae* lipopeptides triggered the production of TNF-α, as part of the acute inflammatory response and IL-12, which instructs the adaptive type-1 or Th1 cytokine response (Krutzik *et al.*, 2003).

Immunohistochemical analysis of leprosy skin lesions revealed TLR2 and TLR1 were more strongly expressed on MΦ in lesions from the localized T-lep form as compared with the disseminated L-lep form of the disease. The type-1 and type-2 cytokine patterns differentially affected TLR2/1 activation by an *M. leprae* lipopeptide. The type-1 cytokines IFN-γ, GM-CSF, IL-12, and IL-18 enhanced TLR2/1 activation, whereas the type-2 cytokines IL-4 and IL-10 inhibited activation. Furthermore, although IL-10 inhibited TLR2 and TLR1 activation it did not downregulate TLR2 or TLR1 expression as compared with IL-4, which inhibited both TLR2 expression and TLR2/1 activation. In contrast, the type-1 cytokines IFN-γ, IL-12, and IL-18 upregulated TLR2/1 heterodimer responses and IFN-γ and GM-CSF increased TLR1 expression in monocytes. Thus, the local cytokine pattern triggered during microbial infection regulates the innate immune system through two different and independent mechanisms, one directly affecting TLR activation and the other modulating TLR expression. These findings indicate that in humans, the regulated expression and activation of TLR2 and TLR1 contributes to the outcome between the host response and the microbial invader.

A number of additional mechanisms have been identified, which regulate TLR function in leprosy. Activation of LILRA2, which is more highly expressed in L-lep versus T-lep lesions, inhibited TLR2/1-induced IL-12 release but preserved IL-10 release (Bleharski *et al.*, 2003). Similarly, oxidized phospholipids inhibited TLR2/1-induced IL-12 release but preserved IL-10 release (Cruz *et al.*, 2008). The ability of *M. leprae* to upregulate CORO1A, also known as tryptophan aspartate-containing coat protein (TACO), known to be expressed in MΦ containing *M. leprae in vitro* and in L-lep but not T-lep disease lesions (Suzuki

et al., 2006), was shown to downregulate TLR2-mediated signaling (Tanigawa *et al.*, 2009).

2.2. TLR2 and TLR1 SNPs

Several investigators have examined whether polymorphisms in the *TLR2* and *TLR1* genes contribute to the pathogenesis of leprosy. Initially, a TLR2 SNP was found to be associated with susceptibility to the L-lep form of leprosy (Kang and Chae, 2001). Investigation of this TLR2 SNP by transfection of the mutant TLR2 into cells lacking the wild-type gene revealed diminished innate responses (Bochud *et al.*, 2003; Kang *et al.*, 2002, 2004; Schroder *et al.*, 2003). However, this TLR2 SNP was subsequently identified in a pseudogene and found not to encode a TLR2 transcript (Malhotra *et al.*, 2005b).

In contrast, there is compelling data to suggest that polymorphisms in the *TLR1* gene may contribute to diminished TLR2/1 responses to lipopeptides, and to the pathogenesis of leprosy. As reviewed previously (Modlin, 2010), the TLR1 T1805G SNP (I602S), rs5743618 affects TLR1 surface expression and subsequent activation by mycobacterial lipopeptides (Johnson *et al.*, 2007; Misch *et al.*, 2008). The TLR1 I602S SNP was associated with a decreased incidence of leprosy (Johnson *et al.*, 2007) and protection against reversal reaction (Misch *et al.*, 2008). The TLR1 A743G SNP (N248S) rs5433095 is in linkage disequilibrium with TLR1 1805, but had little effect on function in transfection studies when separated from the TLR1 1805 allele (Omueti *et al.*, 2007). TLR1 248SS is associated with protection against leprosy (Schuring *et al.*, 2009). It would seem puzzling that a polymorphism that would reduce TLR2/1 signaling would protect against leprosy and reversal reaction. However, Johnson *et al.* reason that the TLR response may be critical during the acute infection, but a moderation of the innate response may be beneficial in chronic infectious diseases such as leprosy (Johnson *et al.*, 2007). Consistent with this hypothesis is the finding that TLR2/1 activation can lead to tissue injury, including nerve damage in leprosy (Oliveira *et al.*, 2003).

2.3. Other TLRs and PRRs

As reviewed previously (Modlin, 2010), TLR4 has been reported to be required for the host defense against *M. tuberculosis* (Means *et al.*, 1999), and shown to mediate the response to mycobacterial heat shock proteins (Bulut *et al.*, 2005), although these studies are frequently criticized because the recombinant proteins used may contain amounts of endotoxin sufficient to activate TLR4 (Gao and Tsan, 2003; Marincek *et al.*, 2008). Two TLR4 SNPs, TLR4 D299G and T399I were found to be associated with a protective effect against leprosy (Bochud *et al.*, 2009). Finally, TLR9 has a

role in recognition of bacterial CpG DNA, participating in the response to mycobacteria (Bafica *et al.*, 2005). A polymorphism in TIRAP, a signaling molecule downstream of the TLRs, TIRAP S180L has been associated with protection against leprosy infection (Hamann *et al.*, 2009).

A comprehensive study of TLR pathway gene polymorphisms in susceptibility to tuberculosis analyzed 149 SNPs in 18 genes involved in the TLR pathway (Davila *et al.*, 2008). Four polymorphisms, all in the *TLR8* gene, showed statistical evidence of association with resistance to tuberculosis and were prevalent in various populations. It is reasonable to consider whether TLR8 polymorphisms play a role in the innate immune response in leprosy infection.

Nucleotide-binding oligomerization domain 2 (NOD2) is a cytoplasmic receptor belonging to the NOD-like receptor family, that recognizes peptidoglycan, including that derived from mycobacteria, by sensing muramyl dipeptide (MDP) (Girardin *et al.*, 2003; Yang *et al.*, 2007). Triggering of NOD2 by MDP activates NF-κB through the adaptor molecule, receptor-interacting protein kinase RIP2. In addition, MDP can also activate the inflammasome, by recruitment of caspase-1, leading to the proteolytic cleavage and activation of IL-1β (Delbridge and O'Riordan, 2007). NOD2 deficiency in a mouse model of tuberculosis resulted in increased susceptibility (Divangahi *et al.*, 2008; Gandotra *et al.*, 2007). NOD2 polymorphisms have been associated with susceptibility to tuberculosis in humans (Austin *et al.*, 2008). NOD2 was found to mediate the response to mycobacteria in human monocytes and synergize with lipoprotein in activating monocyte cytokine responses (Ferwerda *et al.*, 2005), but also synergized with transfected DNA in activating the IFN-β pathway that may be detrimental to host defense against mycobacteria (Leber *et al.*, 2008). Relevance of NOD2 in the pathogenesis of leprosy was demonstrated in a recent genome-wide association study on Han Chinese which identified SNP variants of NOD2 and RIP2 that confer susceptibility to leprosy, regardless of disease classification. Furthermore, tests of heterogeneity of association between clinical forms indicated that the SNPs rs9302752 of NOD2 and rs42490 of RIP2 were more strongly associated with L-lep versus T-lep patients; however, further genetic polymorphisms may be found with a direct genome-wide association study of the clinical forms of disease (Zhang *et al.*, 2009).

3. PHAGOCYTOSIS OF MYCOBACTERIA

3.1. Evasion of phagosome–lysosomal fusion by mycobacteria

In Metchnikoff's model of innate immunity, recognition of the microbial pathogen was followed by phagocytosis (Metchnikoff, 1884). A key cell of the mammalian innate immune system that mediates phagocytosis of

microbial pathogens is the MΦ. Phagocytosis is a receptor-mediated process that involves the ingestion of particulate material and microorganisms into phagosomes. In general, phagosomes mature and subsequently fuse with lysosomes for destruction of the pathogen. However, some pathogens, such as mycobacteria, have adapted to block this fusion.

A number of studies addressing the MΦ surface receptors involved in *M. leprae* or *M. tuberculosis* uptake have implicated the C-type lectin receptors (Maeda *et al.*, 2003) or the complement receptors (Kang and Schlesinger, 1998). The C-type lectin receptors recognize specific carbohydrate structures found in the components of the cell wall of pathogens including those found in the mycobacterial cell envelope. CD209, also known as DC-SIGN, and the mannose receptor bind the mannose-capped lipoarabinomannan (ManLAM) on the cell wall of mycobacteria (Maeda *et al.*, 2003).

Another C-type lectin, the mannose receptor, in conjunction with the complement receptors, CR1, CR3, and CR4 also promote uptake of *M. leprae* by MΦ (Schlesinger, 1993). CR3 receptor can facilitate phagocytosis of mycobacteria through complement opsonins or lectin-based phagocytosis which requires cholesterol (Cywes *et al.*, 1996; Peyron *et al.*, 2000). In fact many lipids are key to normal phagocytosis. Phosphatidylinositol (PI) constitutes approximately 10% of the total lipid on the inner leaflet of the plasma membrane (Yeung and Grinstein, 2007) and is a major component of the forming phagosome. Phosphorylation of PI-3-phosphate [PI(3)P] by the class III PI3K, hVps34 (Fratti *et al.*, 2003) is required for proper phagosome maturation. As a survival strategy, *M. tuberculosis* secretes the glycosylated LAM to inhibit the production of PI(3)P. Together, these effectors decrease vacuolar PI(3)P to arrest phagosome maturation and effectively prevent fusion with the lysosome (Vergne *et al.*, 2003, 2005).

In leprosy infection, TACO, which is highly expressed in L-lep skin lesions, is recruited, from the plasma membrane to the phagosomal membrane to play an essential role in inhibiting the phagosome–lysosome fusion, as well as in the survival of bacilli within MΦ (Suzuki *et al.*, 2006). The phagosomal localization is transient in MΦ exposed to dead mycobacteria, whereas localization is quite stable when live bacilli are used. TLR2 activation decreased TACO expression, which may affect phagosome–lysosomal fusion; however, *M. leprae* infection inhibited TLR-mediated TACO suppression (Tanigawa *et al.*, 2009).

3.2. Pathogenic foam cell formation in mycobacterial infection

In addition to phagocytosis of microbial pathogens, MΦs also have a scavenger function to remove extracellular material, in particular, MΦ phagocytosis of oxidized lipoproteins, such as oxidized low-density

lipoprotein (oxLDL), maintains proper lipid homeostasis within tissues (Greaves and Gordon, 2009; Mosser and Edwards, 2008), but can lead to foam cell formation in a variety of chronic infectious and noninfectious inflammatory disorders. In the different forms of leprosy, conventional histology indicates a distinct difference in the lipid content of the MΦ. In T-lep lesions, the MΦs are thought to be activated, resembling epithelial cells, and are therefore called epithelioid cells. In L-lep lesions, the MΦs, besides containing numerous bacilli, have a characteristic foamy appearance, first described by Rudolf Virchow in 1863. ''In the fresh state they have one characteristic that is especially noteworthy, i.e., their tendency to form a sort of vacuole, apparently from taking up water, so that under the circumstances they acquire a wholly physaliferous appearance'' (Virchow, 1863). It has generally been thought that such cells, termed Virchow cells, lepra cells, or foam cells, are MΦs containing large amounts of lipids including phospholipids and fatty acids, presumed to be of mycobacterial origin (Sakurai and Skinsnes, 1970).

By examining the gene expression profiles of T-lep and L-lep lesions (Bleharski *et al.*, 2003), host lipid metabolism genes were revealed to be differentially upregulated in the L-lep versus T-lep lesions (Cruz *et al.*, 2008). Moreover, the lipid within the foam cells in the L-lep lesions was found to include host-derived oxidized phospholipids. *In vitro*, mycobacteria induced in MΦ the intracellular accumulation of a specific host-derived oxidized phospholipid, 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine (PEIPC), providing a mechanistic link to the foam cells found in atherosclerosis lesions. In atherosclerosis, foam cells are derived from monocytes that enter the subendothelial space, differentiate into MΦs, and endocytose modified forms of LDL (Navab *et al.*, 2004). The foam cells in atherosclerosis are defined by the intracellular presence of distinct components of oxLDL: oxidized phospholipids, esterified cholesterol, and apolipoprotein B (ApoB); the latter is also expressed in L-lep lesions (Ridley *et al.*, 1984). Foamy, lipid-laden MΦs are also a frequent pathological observation in tuberculosis (Cardona *et al.*, 2000; Florey, 1958; Gunn, 1961; Hernandez-Pando *et al.*, 1997; Osler, 1892; Ridley and Ridley, 1987), with mycobacterial components such as mycolic acid able to promote MΦ accumulation of cholesterol ester and lipid body formation (D'Avila *et al.*, 2006; Kondo and Kanai, 1974, 1976; Korf *et al.*, 2005). The ability of mycobacteria to promote lipid body formation was also shown to involve TLR2 signaling (D'Avila *et al.*, 2006) and PPAR-γ activation (Almeida *et al.*, 2009). The accumulation of lipids in phagosomes within MΦ in L-lep lesions involves lipid-droplet associated proteins adipose differentiation-related protein (ADRP) and perilipin (Tanigawa *et al.*, 2008), further suggesting that the foam cells in L-lep lesions represent a dysregulation of host lipid metabolism.

Complementary to the correlation of mycobacterial infection and an increased amount of lipids, mycobacteria are critically dependent on the use of host-derived lipids and enzymes. For instance, mycobacteria require the isocitrate lyase genes as part of the glyoxylate shunt to metabolize fatty acids for generation of ATP during *in vivo* growth (Munoz-Elias and McKinney, 2005), and metabolism of host-derived fatty acids is required for the synthesis of mycobacterial lipids and virulence factors including phthiocerol dimycocerosate, sulfolipid-1, and polyketidesynthase-derived phenolic glycolipid (Jain *et al.*, 2007; Reed *et al.*, 2004). Additionally, among the mycobacteria, *M. leprae* is the most dependent on the host metabolic pathways due to genomic decay. Sequencing of the *M. leprae* genome showed massive gene decay as compared with *M. tuberculosis*; entire metabolic pathways are purged from the *M. leprae* genome, and it has been proposed that *M. leprae* has retained only essential pathways in its adaptation to intracellular survival (Cole *et al.*, 2001). In summary, the accumulation of host-derived oxidized phospholipids, increased host lipid metabolic pathways in L-lep lesions, and dependence of *M. leprae* on host lipid metabolism suggests a link between host lipid metabolism and innate immunity, contributing to the pathogenesis of mycobacterial infection.

4. ANTIMICROBIAL ACTIVITY

4.1. Vitamin D and innate immunity

The ability of TLRs to trigger a direct antimicrobial activity is central to their role in innate immunity. In mouse monocytes, TLR2-induced antimicrobial activity is dependent on the generation of NO, but in human monocytes, TLR activation did not induce NO, nor was TLR-induced antimicrobial activity NO dependent (Thoma-Uszynski *et al.*, 2001). By investigating the gene expression profile of TLR-activated human monocytes and MΦs, a key antimicrobial mechanism was found to involve induction of the 25-hydroxyvitamin D3-1α-hydroxylase (CYP27b1), which converts the 25D into the active 1,25D form, upregulation and activation of the vitamin D receptor (VDR), and downstream induction of the antimicrobial peptide cathelicidin (Krutzik *et al.*, 2008; Liu *et al.*, 2006, 2007; Martineau *et al.*, 2007; Wang *et al.*, 2004). The mechanism by which TLR2/1 activation upregulated expression of CYP27b1 and the VDR was found to involve TLR induction of IL-15 and its receptor components (Krutzik *et al.*, 2005). TLR also induced the antimicrobial peptide DEFB4, by simultaneous triggering of IL-1β activity and activation of the VDR (Liu *et al.*, 2009). Induction of antimicrobial peptides required the presence of 25D-sufficient human serum and was not observed with 25D-insufficient human serum or fetal calf serum, but could be restored by the addition of exogenous 25D3.

Low serum vitamin D levels are associated to both tuberculosis disease progression and susceptibility (Grange *et al.*, 1985; Wilkinson *et al.*, 2000), and there is some evidence that indicates that the vitamin D antimicrobial pathway may contribute to disease outcome in leprosy. Several studies suggest that polymorphisms in the VDR are associated with the type of disease, specifically the Taq I polymorphism (Fitness *et al.*, 2004; Goulart *et al.*, 2006; Roy *et al.*, 1999). Analysis of gene expression profiles in leprosy lesions indicated that the vitamin D antimicrobial pathway gene signature was differentially expressed in T-lep versus L-lep lesions (Montoya *et al.*, 2009). The finding that a polymorphism in the NF-κB1 putative binding site of DEFB1 (human β-defensin 1), DEFB1 G668C is associated with L-lep (Prado-Montes de *et al.*, 2009) further indicates a role for antimicrobial peptides in host defense in leprosy.

A previously unappreciated innate immune defense mechanism, autophagy is a fundamental biological process in which cytoplasmic material is enclosed in a double-membrane vacuole called an autophagosome, which promotes fusion with lysosomes for degradation. Autophagy induced via serum starvation or IFN-γ promoted phagosome–lysosomal fusion and autophagy has been demonstrated to contribute to intracellular killing of mycobacteria (Alonso *et al.*, 2007; Gutierrez *et al.*, 2004). Interestingly, the autophagic pathway also converges with the VDR-cathelicidin program. Autophagy induced by 1,25D was dependent on cathelicidin expression in the antimicrobial response to *M. tuberculosis* (Yuk *et al.*, 2009). Cathelicidin promoted the colocalization of mycobacterial phagosomes with autophagosomes to promote antimicrobial activity.

4.2. Divergence of macrophage phagocytic and antimicrobial programs in leprosy

In addition to their phagocytic function, MΦs also mediate an antimicrobial activity against infectious agents. The mechanisms which regulate these MΦ antimicrobial and phagocytic functions are central to our understanding of innate immune responses against microbial pathogens. Although MΦ infiltration is prominent in all lesions, MΦs in the self-healing T-lep form are well differentiated and rarely contain bacteria, whereas, MΦs in the disseminated L-lep form are characterized by abundant intracellular bacilli and foam cell formation as the result of the accumulation of host- and pathogen-derived lipids (Cruz *et al.*, 2008). Despite the histologic differences of MΦs infiltrating T-lep versus L-lep lesions, the frequency of CD209 expressing MΦ is similar across the spectrum of disease (Krutzik *et al.*, 2005).

Even though MΦ in both T-lep and L-lep lesions expressed CD209 (Krutzik *et al.*, 2005), we thought it possible that these MΦs were distinct in their functional programs, and consequently differentially contributed

to the pathogenesis of leprosy. The induction of these MΦs functional programs are known in part to be influenced by the distinct T cell cytokine patterns at the site of infection (Jullien *et al.*, 1997; Yamamura *et al.*, 1991), however, we reasoned that the cytokines produced by the innate immune response have direct effects on the MΦ functional programs.

Of the innate immune cytokines known to regulate MΦ function, T-lep lesions express IL-15 (Jullien *et al.*, 1997; Yamamura *et al.*, 1991), whereas, L-lep lesions are characterized by the expression of IL-10 (Jullien *et al.*, 1997; Yamamura *et al.*, 1991), prompting the comparison of the IL-15 and IL-10 induced MΦ differentiation (Montoya *et al.*, 2009). Both IL-10 and IL-15 upregulated CD209 expression on monocytes, however, they induced MΦ functional programs. IL-10 induced the phagocytic pathway, including a scavenger receptor program, resulting in the phagocytosis of mycobacteria and oxLDL. IL-10 derived MΦ co-expressed CD163 and the scavenger receptors SRA-I, CD36, and MARCO. In contrast, IL-15 induced the vitamin D-dependent antimicrobial pathway, yet the cells were less phagocytic. IL-15 derived MΦs were CD163 negative and lacked scavenger receptor expression (Fig. 1.3). The differential regulation of MΦ

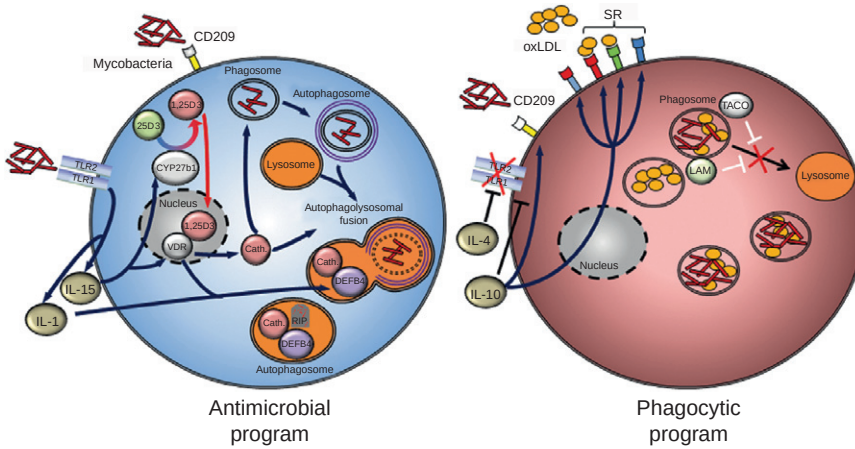

FIGURE 1.3 Divergent macrophage programs for antimicrobial activity versus phagocytosis. Exogenous or TLR-induced IL-15 triggers upregulation of the vitamin D receptor (VDR) and CYP27b1. CYP27b1 converts the inactive 25D3 to the active 1,25D3 form of vitamin D which can then bind the VDR and initiate transcription of cathelicidin and, in conjunction with IL-1, results in DEFB4 production. Cathelicidin initiates autophagy and autophagolysosomal fusion resulting in killing of mycobacteria by the antimicrobial peptides in the autophagosome. Alternatively, IL-10 induces a scavenger receptor program resulting in enhanced phagocytosis of mycobacteria and oxidized lipid resulting in foam cell formation and microbial persistence. Additionally, IL-10 and IL-4 can suppress TLR expression and/or signaling, while LAM and TACO have distinct mechanisms for suppressing phagolysosomal fusion.

functional programs was confirmed by immunohistochemical and expression array analysis of leprosy lesions: the MΦ phagocytic gene expression signature was prominent in the clinically progressive L-lep form, whereas the vitamin D-dependent antimicrobial pathway predominated in the self-limited T-lep form of the disease and in patients undergoing reversal reactions, in which there is clinical conversion from the multibacillary to the self-limited form. Furthermore, the *in vitro* phenotype of the MΦ programs correlated with the *in vivo* phenotype of leprosy skin lesions. L-lep skin MΦs were CD209+CD163+ and contained *M. leprae*, lipoproteins, and host-derived phospholipids, while T-lep MΦs were CD209+CD163− (Cruz *et al.*, 2008; Montoya *et al.*, 2009).

Polymorphisms in the IL-10 locus have been investigated with conflicting results (Fitness *et al.*, 2004; Franceschi *et al.*, 2009; Malhotra *et al.*, 2005a; Moraes *et al.*, 2004; Pereira *et al.*, 2009; Santos *et al.*, 2002). Paradoxically, one IL-10 promoter polymorphism, 819C/T, was associated with susceptibility to leprosy, and could lead to decreased IL-10 production (Malhotra *et al.*, 2005a; Pereira *et al.*, 2009; Santos *et al.*, 2002). In summary, these data indicate that MΦ programs for phagocytosis and antimicrobial responses are distinct and differentially regulated in innate immunity in bacterial infections.

5. DENDRITIC CELL FUNCTION IN LEPROSY

The ability of the innate immune system to instruct the adaptive T cell response is a part of an effective host defense against intracellular pathogens. This instructive role of the innate immune system is primarily mediated by DCs, professional antigen-presenting cells (Martin-Fontecha *et al.*, 2003) that are highly efficient in activation of T cell responses that provide CMI against the pathogen (Banchereau and Steinman, 1998). DCs process antigens and present them to CD8+ T cells via class I MHC molecules and to CD4+ T cells via class II MHC molecules. In addition to the polymorphic MHC molecules, the CD1 family of nonpolymorphic antigen-presenting molecules presents non-peptide lipid and glycolipid antigens to T cells (Moody and Porcelli, 2003). Like T cells reactive against peptide antigens, CD1-restricted T cells are highly specific in their recognition characteristics, as they are restricted by a single form of CD1. Three different classes of CD1b-presented mycobacterial lipid antigens have been structurally defined, and several lines of evidence support an important role for CD1 in microbial defense. First, mycobacteria-specific group I CD1-restricted T cells release high levels of IFN-γ (Sieling *et al.*, 1995), which is required for effective CMI against such organisms. Second, mycobacteria-reactive CD1-restricted T cells show a high degree of cytolytic activity *in vitro*

against antigen-pulsed CD1+ mononuclear phagocytes (Beckman *et al.*, 1994; Sieling *et al.*, 1995), and also recognize and lyse CD1+ targets infected with live virulent *M. tuberculosis* bacilli (Stenger *et al.*, 1997). Therefore, the presentation of mycobacterial lipid antigens through CD1+ DCs represents a major pathway effective in controlling mycobacterial infection. L-lep lesions are characterized by a marked deficit in CD1b+ DCs, both in the dermis and epidermis (Miranda *et al.*, 2007; Sieling *et al.*, 1999; Simoes Quaresma *et al.*, 2009), a potential mechanism for the reduced cell-mediated immune responses in these lesions (Ridley and Jopling, 1966).

Through use of genetic profiling in leprosy lesions (Bleharski *et al.*, 2003; Lee *et al.*, 2007), members of the LILR family were discovered to be increased in the L-lep skin lesions; inhibitory receptors subsequently found to interrupt DC differentiation from myeloid precursors. LILRA2 protein expression was increased in the L-lep versus T-lep skin myeloid cells. Activation of LILRA2 on peripheral blood monocytes impaired GM-CSF induced differentiation into immature DC, as evidenced by reduced expression of DC markers (MHC class II, CD1b, CD40, and CD206), but not MΦ markers (CD209 and CD14). Furthermore, LILRA2 activation abrogated antigen presentation to both CD1b- and MHC class II-restricted, *M. leprae*-reactive T cells derived from leprosy patients. Impaired DC differentiation and function was also evident through expression of oxidized phospholipids induced by mycobacterial infection. Oxidized phospholipids suppressed both lipid and protein antigen presentation by CD1b and MHC class II, respectively. High-density lipoprotein (HDL), through associated enzymes and reverse cholesterol transport, is a physiological antagonist to oxidized phospholipids and was able to rescue DC differentiation and antigen presentation normally suppressed during mycobacterial infection. Interestingly, HDL from the serum of L-lep patients is impaired and is not able to rescue DC differentiation (Cruz *et al.*, 2008). These data point to striking similarities in the pathogenesis of foam cell formation in mycobacterial infection to atherosclerosis, implying a common metabolic and inflammatory process.

Establishing specific markers to distinguish DCs from MΦ has remained controversial. Previously, CD209 was thought to be specifically expressed by DC, based upon its induction by GM-CSF and IL-4 *in vitro* (Sallusto and Lanzavecchia, 1994), expressing classical DC markers such as CD1 molecules but also co-expressing CD209 (Krutzik *et al.*, 2005). However, cells co-expressing CD1 and CD209 could not be readily detected in normal human tonsil, skin, and in leprosy lesions. Instead, CD1 and CD209 were expressed on distinct nonoverlapping cell populations (Krutzik *et al.*, 2005; Ochoa *et al.*, 2008). CD209+ cells clearly co-expressed MΦ markers including CD16, CD64, and CD68 but not the DC markers CD1a, CD1b, DC-LAMP, CD11c, CD63, and CD83.

Furthermore, a variety of studies demonstrate CD209 expression on tissue MΦ, including skin (Ochoa *et al.*, 2008; Zaba *et al.*, 2007), lung (Soilleux *et al.*, 2002; Van den Heuvel *et al.*, 1999), CNS (Fabriek *et al.*, 2005), placenta (Bockle *et al.*, 2008), and adipose tissue (Zeyda and Stulnig, 2007). The phenotypes of MΦ and DC found *in situ* can best be reproduced *in vitro* by culturing peripheral blood monocytes with IL-15 which results in a CD209+CD1− MΦ phenotype or GM-CSF alone, resulting in an immature DC phenotype, CD1+CD209− (Krutzik *et al.*, 2005). Also, TLR activation via GM-CSF can induce a DC differentiation pathway, however, peripheral monocytes from L-lep patients do not differentiate into CD1+ DC following TLR activation (Krutzik *et al.*, 2005). Furthermore, expression of the costimulatory protein, B7.1, is also decreased in L-lep lesions (Santos *et al.*, 2007), further suggesting impaired CMI. The activation and maturation of DC can be directly inhibited by the pathogen itself, in contrast to other mycobacteria (Murray *et al.*, 2007), suggesting that the pathogen specifically subverts the generation of functional antigen-presenting cells. Together these studies suggest that *M. leprae* contributes to reduced cell-mediated immune responses in leprosy by disrupting the differentiation of DC and their antigen-presenting capacity.

In conclusion, leprosy provides an exciting model to investigate the mechanisms by which the human innate immune system contributes to host defense versus susceptibility to microbial infection. These insights not only provide insights into the human innate immune response to microbial pathogens, but identify potential therapeutic targets for intervention in leprosy and other infectious diseases worldwide.

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CHAPTER 2

The Immunological Functions of Saposins

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Abstract Saposins or sphingolipid activator proteins (SAPs) are small, nonenzymatic glycoproteins that are ubiquitously present in lysosomes. SAPs comprise the five molecules saposins A–D and the GM2 activator protein. Saposins are essential for sphingolipid degradation and membrane digestion. On the one hand, they bind the respective hydrolases required to catabolize sphingolipid molecules; on the other hand, saposins can interact with intralysosomal membrane structures to render lipids accessible to their degrading enzymes. Thus, saposins bridge the physicochemical gap between lipid substrate and hydrophilic hydrolases. Accordingly, defects in saposin function can lead to lysosomal lipid accumulation. In addition to their specific functions in sphingolipid metabolism, saposins have membrane-perturbing properties. At the low pH of lysosomes, saposins get protonated and exhibit a high binding affinity for anionic phospholipids. Based on their universal principle to interact with membrane bilayers, we present the immunological functions of saposins with regard to lipid antigen presentation to CD1-restricted T cells, processing of apoptotic bodies for antigen delivery and cross-priming, as well as their potential antimicrobial impact.

1. INTRODUCTION

Cells perform endocytosis to capture extracellular material and to remove used plasma membrane components. From endosomes, macromolecules can be either recycled to the plasma membrane or delivered to lysosomes for subsequent destruction. Lysosomes are acidic membrane-enclosed organelles representing the terminal degradative compartment of the endocytic pathway. They contain more than 60 different soluble hydrolytic enzymes specialized in the degradation of macromolecules. In addition, lysosomes are equipped with sphingolipid activator proteins (SAPs) that belong to the large and divergent family of saposin-like proteins (SAPLIPs). SAPLIP domains have been identified in relatively small proteins of about 80 amino acids in length, including the lung surfactant-associated protein B (SP-B), the tumorolytic proteins NK-lysin and granulysin, cytolytic proteins from amoeba, and several plant aspartic proteases. SAPs comprise the five molecules saposins A–D and the GM2 activator protein. Saposins A–D are produced in acidic endosomal compartments upon sequential proteolytic

cleavage of their single polypeptide precursor termed prosaposin (pSAP). Saposins are nonenzymatic, acidic, heat-stable, and protease-resistant molecules of about 8–11 kDa with essential functions in sphingolipid degradation and membrane digestion. The modes of action of SAPs include binding and stimulation of glycosidases required for sphingolipid degradation, as well as interaction with intralysosomal membranes to render lipids accessible to their respective degrading enzymes. Thus, saposins bridge the physicochemical gap between membrane lipid substrates and water-soluble hydrolases. Moreover, the multimolecular association of saposins, lipid bilayers, and CD1 glycoproteins facilitates the loading of CD1 molecules with lipid antigens for subsequent activation of lipid-reactive T cells. In this chapter, we present the current knowledge pertaining to the biology of saposins in lysosomal sphingolipid degradation and membrane digestion and highlight its implications for immunological processes, such as lipid antigen presentation, processing of apoptotic bodies for cross-priming, and direct antibiotic function.

2. SAPOSINS IN LYSOSOMAL GLYCOSPHINGOLIPID DEGRADATION AND MEMBRANE DIGESTION

Glycosphingolipids (GSLs) are a class of lipids present in the plasma membrane of eukaryotic cells (Kolter and Sandhoff, 2005). Structurally, GSLs share a common hydrophobic ceramide moiety that acts as a membrane anchor, which is coupled to a hydrophilic oligosaccharide chain. GSLs play important roles in the structural organization of membranes and cellular interactions with microbes or toxins (Hakomori, 1981; Karlsson, 1989). GSLs are generated along the secretory pathway by the sequential, combinatorial addition of monosaccharide units, starting with the initial elongation of ceramide by glycosidic binding of either D-glucose or D-galactose in β-configuration. In particular, β-D-glucosylceramide undergoes elongation upon the stepwise action of specific glycosyltransferases to produce GSLs with complex oligosaccharide chains, such as gangliosides, globosides, and cerebrosides (Ichikawa and Hirabayashi, 1998). Alternatively, the addition of a phosphorylcholine moiety to ceramide produces sphingomyelin, a major constituent of the membrane of nerve cells and a dominant species among sphingolipids (Hooghe-Peters *et al.*, 1979). Eventually, sphingolipids reaching the plasma membrane become a part of structural microdomains enriched in cholesterol (Simons and Ikonen, 1997). Degradation of sphingolipids is initiated upon internalization of membrane patches through diverse mechanisms including endocytosis, phagocytosis, or autophagy, and commences in acidified compartments of the endosomal route for terminal degradation

in lysosomes (Luzio *et al.*, 2007). In contrast to soluble molecules, degradation of membranes is a more delicate process since the limiting membrane of lysosomes must remain intact to avoid the leakage of potentially hazardous enzymes into the cytosol. Thus, prior to reaching lysosomes, sphingolipids are sorted to intraendosomal membranes and degraded on the surface of intralysosomal vesicles (ILVs) upon exposure to soluble glycosidases (Sandhoff and Kolter, 1996). Sphingolipid degradation proceeds in a sequential pathway that assures the stepwise removal of monosaccharide units from the nonreducing end of the oligosaccharide chain (Fig. 2.1). Ultimately, ceramide is disassembled to sphingosine and fatty acid for subsequent reuse in metabolic pathways. As sphingolipid degradation proceeds, the length of the sugar headgroup inevitably shrinks in size, thereby becoming less accessible to water-soluble glycosidases. To overcome this physicochemical obstacle and to bring sphingolipids and their respective enzymes in close proximity, mammalians possess five saposins encoded by two genes (Rorman *et al.*, 1992). The first gene encodes pSAP, the common precursor to the four saposins A–D. The second gene encodes the GM2AP. The physiological significance of saposins in stimulating sphingolipid degradation is underscored by multiple reports of human sphingolipidoses with mutations in the *pSAP* gene leading to deficiencies of saposin function (O'Brien and Kishimoto, 1991). The five SAPs share a high degree of structural homology. However, SAPs show diverse ligand-binding properties and exist in multiple structural states that account for their distinct modes of action in sphingolipid degradation and membrane interaction.

2.1. Prosaposin

pSAP is a 524-amino acid glycoprotein that contains a 16-residue signal peptide sequence and five glycosylation sites. Importantly, pSAP is the common precursor to the four saposins A–D (Furst *et al.*, 1988; O'Brien *et al.*, 1988). In humans, pSAP exists as an intracellular molecule of 68 kDa and a major extracellular form of 73 kDa. Accordingly, pSAP is intracellularly targeted to lysosomes either via mannose-6-phosphate receptors or by sortilin (Lefrancois *et al.*, 2003). Alternatively, pSAP can be secreted and reendocytosed by mannose-6-phosphate receptors, low density lipoprotein receptor-related protein (LRP), or mannose receptors (Hiesberger *et al.*, 1998). Expression of pSAP and individual saposins is virtually ubiquitous and conserved among mammalian species (Kishimoto *et al.*, 1992). This is not surprising considering their important functions in sphingolipid degradation. Protein expression analyses revealed high concentrations of pSAP in the adult liver and body fluids, especially in the brain, semen, milk, serum, pancreatic juice, and bile (Kolter and Sandhoff, 2005). Further, pSAP and saposins are expressed in cells of hematopoietic

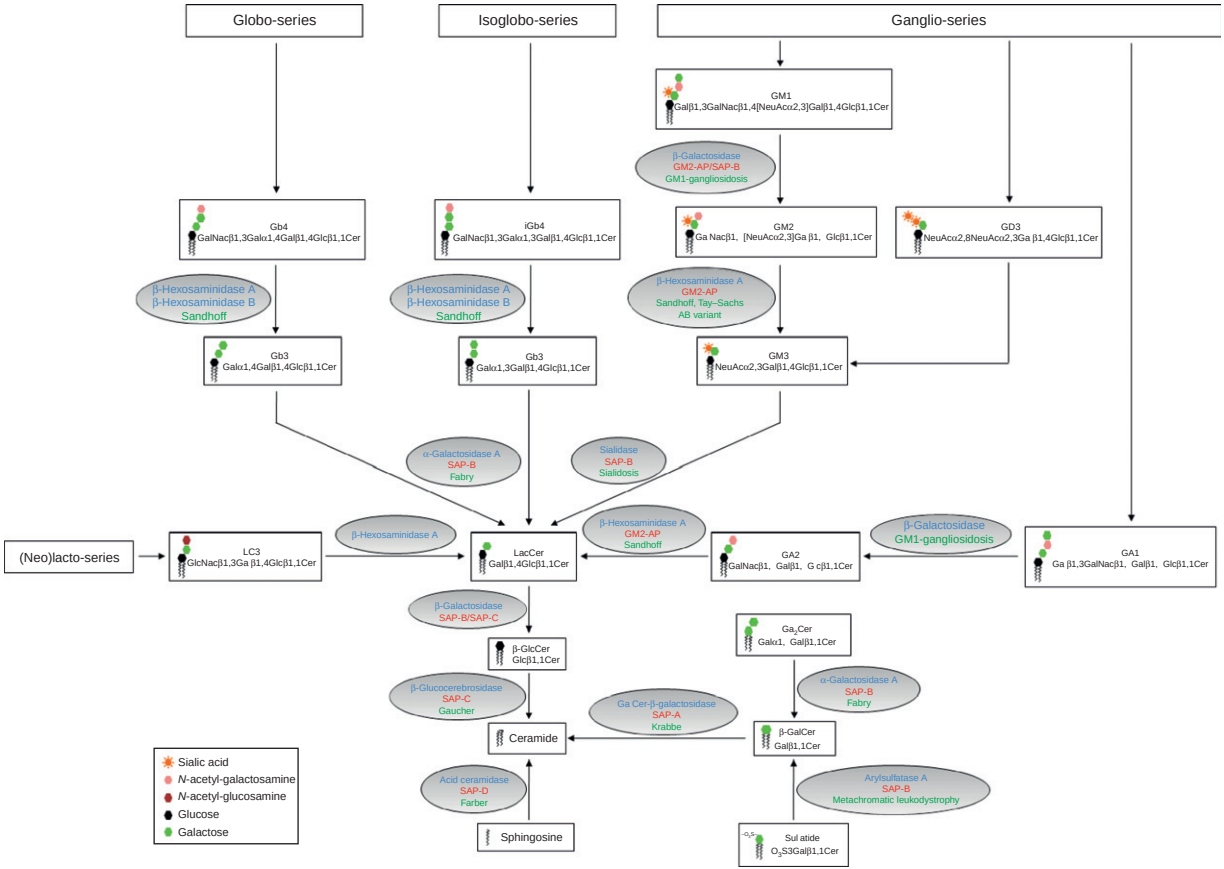

FIGURE 2.1 *Pathways of GSL degradation*. The graph depicts the degradative pathways of various GSL species, including the globo-, isoglobo-, ganglio-, and (neo)lacto-series. The names, formula, and structural icons of the respective GSLs are shown in the boxes. The ovals contain the enzymes (depicted in blue) and the saposins (shown in red) that are involved in the corresponding lipid degradation step. The associated lysosomal storage disease due to enzyme or saposin deficiency or both is indicated in green.

origin and nerve cells (Kondoh *et al.*, 1993; Sano *et al.*, 1989). pSAP is sequentially processed from its N-terminal end, starting with the cleavage of the SAP-A domain (Hiraiwa *et al.*, 1993). The mechanism through which pSAP is processed to the four saposins remains incompletely understood. Early studies suggested that pSAP proteolysis occurs at low pH and requires the action of proteases susceptible to inhibition by pepstatin A (Hiraiwa *et al.*, 1993). One of these candidate proteases was later shown to be cathepsin D (Hiraiwa *et al.*, 1997). *In vitro*, unprocessed pSAP can stimulate the degradation of sphingolipids to a similar extent like SAP-B, SAP-C, and SAP-D act on their respective enzymes (Kishimoto *et al.*, 1992). However, considering that pSAP proteolysis occurs in acidic compartments, its contribution to lysosomal sphingolipid degradation might be of limited physiological relevance. However, pSAP has been proposed to represent a neurotrophic factor and might also be involved in the transport of gangliosides (Hiraiwa *et al.*, 1992; O'Brien *et al.*, 1994). Point mutations in pSAP have been identified in patients lacking all four saposins, a disease referred to as combined SAP deficiency (Harzer *et al.*, 1989; Hulkova *et al.*, 2001). pSAP-deficient individuals and pSAP $^{-/-}$ mice show similar clinicopathological features and die during the neonatal period or at the age of 3–4 weeks, respectively, due to multiple organ failure (Fujita *et al.*, 1996). Analysis of pSAP-deficient cells reveals numerous electron-dense membrane inclusions, and pSAP $^{-/-}$ mice show massive accumulation of GSLs such as ceramide, β-glucosylceramide (β-GlcCer), β-galactosylceramide (β-GalCer), sulfatides, galabiaosylceramide (Ga_2Cer), lactosylceramide (LacCer), globotriaosylceramide (Gb3), and the ganglioside GM3 (Bradova *et al.*, 1993; Fujita *et al.*, 1996).

2.2. Saposin A

SAP-A activates the hydrolysis of β-GalCer by galactosylceramide β-galactosidase (Fig. 2.1) (Morimoto *et al.*, 1989). Accordingly, mice that carry a point mutation in the SAP-A domain of pSAP, and thus lack SAP-A expression, show tissue accumulation of β-GalCer (Matsuda *et al.*, 2001). SAP-A deficiency forms the basis of a chronic late-onset form of globoid cell leukodystrophy that resembles the disease of patients carrying genetic deficiency in galactosylceramide β-galactosidase (Krabbe disease) (Spiegel *et al.*, 2005). Under acidic conditions, SAP-A mobilizes lipids from liposomes in a reaction that is enhanced by bis(monoacylglycero)phosphate (BMP) or decreased by cholesterol (Locatelli-Hoops *et al.*, 2006). The crystal structure of human SAP-A has been resolved in its closed monomeric saposin-fold conformation and consists of four amphipathic α-helices folded in the shape of an oblate ellipsoid (Ahn *et al.*, 2006). Charged residues are located on the surface of the molecule, whereas

conserved hydrophobic residues are directed toward a small cavity. Elucidation of the SAP-A structure in its open, lipid-binding conformation remains to be determined.

2.3. Saposin B

SAP-B has been the first activator protein identified (Mehl and Jatzkewitz, 1964). SAP-B is required to stimulate the breakdown of sulfatide by arylsulfatase A, Gb3 and Ga_2Cer by α-galactosidase A, and LacCer by galactosylceramide β-galactosidase (Fig. 2.1). Accordingly, the latter GSLs are present in abnormally high concentrations in the urine of SAP-B-deficient patients and accumulate in the tissues of SAP-B $^{/}$ mice (Li *et al.*, 1985; Sun *et al.*, 2008). Further, SAP-B cooperates with GM2AP in the degradation of the ganglioside GM2 *in vitro* (Wilkening *et al.*, 2000). SAP-B can be considered as a nonspecific activator protein. Accordingly, SAP-B stimulates the hydrolysis of ceramide-free glycolipids by diverse glycosidases from animals, plants, and microorganisms (Li *et al.*, 1988). These special properties might explain as to why SAP-B stimulates the degradation of a broader spectrum of sphingolipids compared to other SAPs. Additionally, SAP-B binds and transfers the anionic phospholipid phosphatidylinositol (PI) between biological membranes (Ciaffoni *et al.*, 2006). Inherited defects of SAP-B lead to an atypical form of metachromatic leukodystrophy (MLD) with late infantile or juvenile onset (Kretz *et al.*, 1990; Schlote *et al.*, 1991; Wenger *et al.*, 1989). The crystal structure of human SAP-B displays a shell-like homodimer that consists of two V-shaped monomers (Ahn *et al.*, 2003). The concave inner surface of each monomer is lined with hydrophobic residues that create a large lipid-binding cavity when two monomers are associated. In its open conformation, the SAP-B dimer can directly interact with lipid membranes, promote the reorganization of lipid alkyl chains, and extract lipid substrates upon transition to the closed conformation.

2.4. Saposin C

SAP-C has been primarily described by O'Brien and colleagues as an activator of β-GlcCer degradation by glucosylceramide β-glucosidase (Fig. 2.1) (Ho and O'Brien, 1971). SAP-C might also stimulate the degradation of ceramide, β-GalCer, and galactosylsphingosine (Harzer *et al.*, 1997). Inherited deficiency of SAP-C causes a variant juvenile form of Gaucher disease (type III) with marked storage of β-GlcCer (Christomanou *et al.*, 1989; Matsuda *et al.*, 2004; Schnabel *et al.*, 1991). SAP-C exhibits a characteristic dual mode of action in sphingolipid degradation. Similar to SAP-A, SAP-C stimulates its respective enzyme partner in an allosteric manner (Berent and Radin, 1981). In parallel, SAP-C

can directly bind anionic phospholipids to destabilize membranes and to promote the association of β-glucosylceramidase with its lipid substrate for subsequent degradation (Vaccaro *et al.*, 1993). At low pH, SAP-C additionally triggers the fusion of vesicles containing anionic phospholipids *in vitro* (Vaccaro *et al.*, 1994, 1995; Wang *et al.*, 2003). Using SAP-C mutants, the fusogenic activity could be mapped to the lysine-rich amino-terminal portion of the molecule, indicating that electrostatic interactions between negatively charged lipids and positively charged saposin residues might be required (Qi and Chu, 2004). Finally, evidence suggests that SAP-C functions as a neurotrophic factor by stimulating neurite outgrowth and increasing choline acetyltransferase activity in neurons (O'Brien *et al.*, 1995). The structure of SAP-C in its closed conformation reveals a homodimer of boomerang-shaped intertwining monomers in an open, extended conformation, with solvent-exposed hydrophobic pockets (Hawkins *et al.*, 2005; Rossmann *et al.*, 2008). Accordingly, a ''clip-on'' model of vesicle fusion has been proposed. At lysosomal pH, SAP-C dimers unfold their hydrophobic pockets to interact with anionic phospholipids of membrane bilayers. Ultimately, SAP-C molecules, inserted into opposing lipid vesicles, clip one another through domain swapping, thus bringing the vesicles close enough for fusion.

2.5. Saposin D

SAP-D is the most abundant saposin in normal tissues with concentrations threefold higher than other SAPs (O'Brien and Kishimoto, 1991). SAP-D promotes the hydrolysis of ceramide by acid ceramidase *in vivo* (Fig. 2.1), as demonstrated by the accumulation of α-hydroxyl fatty acid ceramides in the kidneys and the cerebellum of SAP-D $^{-/-}$ mice (Matsuda *et al.*, 2004). Consequently, SAP-D-deficient animals show renal tubular degeneration and hydronephrosis, as well as progressive loss of Purkinje cells in the cerebellum, leading to ataxia. To date, inherited SAP-D deficiency has not been identified in humans. Of note, the phenotype of SAP-D $^{-/-}$ mice does not resemble human ceramidase deficiency (Farber disease) or its murine counterpart characterized by early embryonic lethality (Li *et al.*, 2002; Matsuda *et al.*, 2007). Similar to SAP-C, SAP-D poorly binds sphingolipids, but displays high affinity for anionic phospholipids at lysosomal pH (Tatti *et al.*, 1999). However, SAP-C and SAP-D are functionally different. SAP-D is a membrane disrupter, whereas SAP-C fuses lipid bilayers (Ciaffoni *et al.*, 2001). SAP-D spontaneously binds to membranes that contain anionic lipids, including BMP, PI, and phosphatidylserine (PS), in a reversible, pH-driven fashion (Ciaffoni *et al.*, 2003). By destabilizing membranes, SAP-D allows the formation of small vesicles derived from larger liposomes *in vitro* (Ciaffoni *et al.*, 2001). Multivesicular or multilamellar bodies found in the lumen of acidic cellular

compartments are specifically enriched for BMP (Kobayashi *et al.*, 1998, 2002). Therefore, it is tempting to speculate that SAP-D might regulate the homeostasis of internal endolysosomal membranes. SAP-D exists as a substrate-free closed helix bundle or in a V-shaped, open, and ligand-bound configuration in the presence of lipids. Prior to interaction with lysosomal membranes, SAP-D remains in a monomer–dimer equilibrium in the closed conformation. The acidic pH of lysosomes dramatically increases the surface hydrophobicity of SAP-D, thereby allowing the positively charged amino acids at the bottom of SAP-D to associate with the surface of intralysosomal membranes enriched in negatively charged lipids. The top of SAP-D subsequently rotates by 180° along its axis, thus positioning its hydrophobic residues into the membrane bilayer, and exposing positively charged residues to the solvent. Thereafter, SAP-D changes its configuration to the closed conformation, which forms the mechanistic basis for lipid extirpation from the bilayer. Ultimately, SAP-D leaves the membrane with bound lipid (Rossmann *et al.*, 2008).

2.6. The GM2 activator

The GM2AP is the fifth member of the SAP family. GM2AP is larger than saposins A–D, with a molecular weight of 20 kDa in its mature lysosomal form (Furst and Sandhoff, 1992). To reach lysosomes, newly synthesized GM2AP uses the major intracellular mannose-6-phosphate-mediated trafficking route (Rigat *et al.*, 1997). Mannose-6-phosphate receptors also allow the endosomal recapture of GM2AP from extracellular fluids upon endocytosis (Rigat *et al.*, 1997). The saposin function of GM2AP is required to stimulate the degradation of GM2 by β-hexosaminidase A (Hex-A) *in vivo* (Fig. 2.1) (Conzelmann and Sandhoff, 1979). Deficiency in GM2AP leads to the AB variant of GM2 gangliosidosis, an atypical form of Tay–Sachs disease with characteristic tissue accumulation of GM2 and related GSLs in neuronal lysosomes (Conzelmann and Sandhoff, 1978). GM2AP acts as a lipid transfer protein *in vitro* as indicated by its capacity to extract and carry GSLs from donor to acceptor liposomes (Conzelmann *et al.*, 1982). The structure of monomeric GM2AP consists of an eight strand, cup-shaped, antiparallel β sheet (Wright *et al.*, 2000). The monomer contains a hydrophobic cavity with dimensions that can accommodate the ceramide portion of GM2 and other lipids, lined by surface loops and a single short helix (Wright *et al.*, 2003). The most flexible of the loops contains the substrate-binding site and controls the entrance to the cavity to facilitate an open or a closed conformation. Accordingly, open, empty GM2AP binds to target membranes by using its hydrophobic loops and penetrates into the hydrophobic region of the bilayer. Subsequently, the lipid recognition site of the activator can interact with the substrate and insert its ceramide portion into the hydrophobic cavity. At this point, the

lipid-loaded activator may change to the closed conformation, allowing the complex to leave the membrane in a soluble state. Finally, GM2AP exposes GM2 to the water-soluble enzyme Hex-A for subsequent degradation in the lysosomal lumen (Kolter and Sandhoff, 2005).

2.7. Topology

The inner leaflet of the limiting lysosomal membrane is covered with a thick glycocalix composed of glycoproteins. This layer allows the lysosome to resist the low lumenal pH, and protects the inner limiting membrane from digestion by acid hydrolases or destabilization through lipid-binding molecules. Therefore, membrane constituents targeted for lysosomal degradation have to be sorted to small lumenal vesicles formed from endosomes by the inward budding of the limiting membrane into the lumen. Vesicle-rich endosomes are referred to as multivesicular endosomes or multivesicular bodies (MVBs) (Piper and Luzio, 2001). This topological transition exposes macromolecules originating from the outer leaflet of the plasma membrane to the endosomal lumen. Hence, the subsequent fusion of an MVB with a lysosome renders macromolecules accessible to hydrolases. Following this principle, intraendosomal vesicles serve as important devices in the delivery of used plasma membrane proteins and lipids to lysosomes (Futter *et al.*, 1996; Sandhoff and Kolter, 1996). Recently, the biogenesis and function of MVBs and ILVs has become a major focus for cell biologists, especially since the discovery of endosomal sorting complexes required for transport (ESCRT) (Luzio *et al.*, 2007). Yet, the sequence of events leading to their formation remains poorly understood. Three lipids play critical roles in the biogenesis and function of MVBs and ILVs. Firstly, phosphatidylinositol-3-phosphate is enriched on the cytosolic face of endosomes, and evidence suggests that it might be required for the formation of MVBs (Futter *et al.*, 2001; Odorizzi *et al.*, 1998). Secondly, cholesterol is highly enriched on intraendosomal vesicles, where it stabilizes the membrane (Hornick *et al.*, 1985). By contrast, cholesterol is almost absent from ILVs as it is removed from lysosomes by the Niemann–Pick disease protein C1 (NPC1) and NPC2 (Friedland *et al.*, 2003; Mobius *et al.*, 2003). Considering the negative impact of cholesterol on SAP-A- and SAP-B-mediated lipid mobilization from liposomes, physiological depletion of cholesterol from ILVs might favor lipid mobilization by SAPs (Locatelli-Hoops *et al.*, 2006; Remmel *et al.*, 2007). Thirdly, the anionic lipid BMP plays a crucial role in the topology of ILVs and is derived from mitochondrial cardiolipin during the process of autophagy, or BMP is produced from phosphatidylglycerol (PG) in the endoplasmic reticulum (ER) (Hullin-Matsuda *et al.*, 2009). In contrast to cholesterol, BMP is specifically enriched on ILVs, where it can account for up to 70% of the total membrane phospholipid content

(Kobayashi *et al.*, 1998, 2002). BMP functions in the formation of ILVs since BMP-containing liposomes can spontaneously form inward-budding profiles in a pH-inducible manner (Matsuo *et al.*, 2004). Remarkably, the presence of BMP in liposomes strongly enhances the stimulatory capacity of all five SAPs (Chu *et al.*, 2005; Ciaffoni *et al.*, 2003; Locatelli-Hoops *et al.*, 2006; Remmel *et al.*, 2007; Wilkening *et al.*, 2000). Taken together, these findings support a model in which the gradual depletion of cholesterol and subsequent integration of BMP into ILVs renders them more accessible for SAPs. Moreover, BMP enhances SAP-stimulated degradation of GSLs by specific glycosidases. Notably, the membrane-perturbing properties of SAP-C potentially mediate the fusion of multiple ILVs in order to promote their subsequent disruption by SAP-D.

3. SAPOSINS FACILITATE LIPID PRESENTATION TO CD1-RESTRICTED T LYMPHOCYTES

The majority of studies on antigen presentation have concentrated on molecules encoded by the major histocompatibility complex (MHC). However, over the past two decades, it has become evident that other molecules can also trigger T cell responses, including the family of lipid-presenting CD1 molecules. In addition to proteins, lipid antigens extend the spectrum of determinants that are potentially recognized by the immune system, and thus amplify the diversity of immune responses to fight intruders, for example, in the context of infection of the host with lipid-rich pathogens. In contrast to protein processing to peptides, cellular lipid acquisition challenges the host due to the physicochemical properties of fats. Accordingly, hydrophobic lipid antigens have to be extracted from aggregates or membranes and solubilized for subsequent transport. Subsequently, lipids are loaded onto specific antigen-presenting molecules such as CD1 proteins. For both steps, helper molecules like saposins are required (Fig. 2.2). On the other hand, some lipids have to be further subjected to structural editing, or processing, to reveal antigenic epitopes otherwise unavailable for recognition by T cells. In the following, we highlight the predominant roles played by saposins and lysosomal glycosidases in both processes.

3.1. Characteristics of antigen-presenting CD1 molecules

The organization of the CD1 complex is similar to MHC class I. Each *CD1* gene contains three exons encoding separate extracellular domains (α1, α2, α3) that noncovalently associate with β2-microglobulin (β2m) to form a stable transmembrane heterodimer of approximately 50 kDa (Brigl and Brenner, 2004). Five separate genes (*CD1A*, *CD1B*, *CD1C*, *CD1D*, and

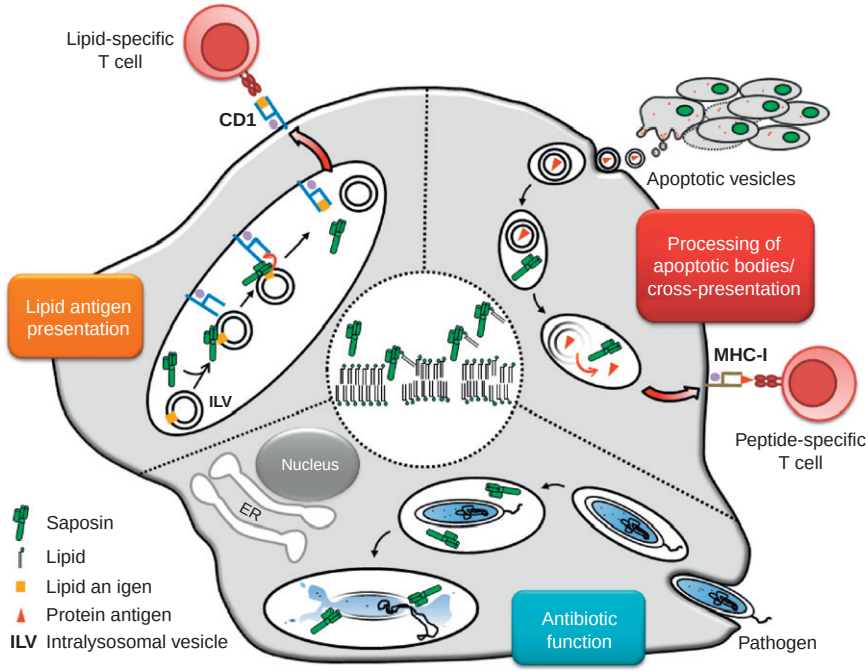

FIGURE 2.2 *Immunological functions of saposins*. The central circle demonstrates the universal mechanism of saposin action on lipid bilayers. Accordingly, saposin molecules insert into lysosomal membranes to induce bilayer disintegration and lipid extraction. Further, the graph represents the three immunological consequences of this mechanism. (1) Lipid antigen presentation: In lysosomes of APCs, saposins interact with intralysosomal vesicles (ILVs) that contain lipid antigens. Subsequently, SAPs facilitate the loading of CD1 molecules with lipid antigens to activate CD1-restricted lipid-reactive T cells. (2) Processing of apoptotic bodies / cross-presentation: Apoptotic bodies derived from tumor or infected cells are engulfed by phagocytes such as DCs or macrophages. Protein antigens contained in apoptotic vesicles are liberated in lysosomes upon membrane disintegration induced by saposins. This leads to antigen delivery from apoptotic bodies for subsequent cross-presentation of antigens to MHC-I-restricted $CD8^+$ T cells. (3) Antibiotic function: Pathogens like intracellular bacteria are phagocytosed by macrophages or DCs. At the acidic pH of lysosomes, saposins unfold their antimicrobial effects through direct attack on the membranes of pathogens.

CD1E) are mapping to chromosome 3 in humans. In mice, CD1 maps to chromosome 1 and consists of two genes (*CD1D1* and *CD1D2*). On the basis of sequence homology, CD1 proteins fall into one of two groups: group 1 CD1 molecules (CD1a, CD1b, and CD1c) and group 2 CD1 molecules (CD1d), with CD1e representing an intermediate. While CD1a-d proteins are mainly expressed on the surface of antigen-presenting cells (APCs), CD1e is exclusively found as a soluble molecule in lysosomes, and

therefore cannot present antigens to CD1-restricted T cells (Angenieux *et al.*, 2005). The subdivision of CD1 molecules correlates with functional differences. Accordingly, group 1 molecules present lipid antigens to T lymphocytes, while group 2 molecules display antigens to Natural Killer T (NKT) cells. Structurally, CD1 molecules contain a main hydrophobic groove that can be prolonged by further pockets (Moody *et al.*, 2005). Group 1 CD1 molecules are mainly expressed by cortical thymocytes and myeloid cells, such as dendritic cells (DCs) and Langerhans cells (Dougan *et al.*, 2007a). In addition, CD1c proteins are also expressed by marginal zone B cells and a fraction of peripheral blood B lymphocytes (Delia *et al.*, 1988). In humans, group 2 CD1d molecules are found on cells of the myeloid lineage as well as on few nonlymphoid cells. However, cortical thymocytes and mantle zone B cells in lymph nodes exhibit the highest levels of CD1d expression (Exley *et al.*, 2000). In mice, CD1d expression depends on the *CD1D1* gene. CD1d molecules are mainly expressed by APCs, including hepatic stellate cells (Winau *et al.*, 2007). In addition, cortical thymocytes as well as some activated T cells express CD1d proteins (Dougan *et al.*, 2007a). Expression of CD1d on the surface of APCs is enhanced by proinflammatory cytokines, such as interferon-β (IFN-β), IFN-γ, and TNF-α, as well as by toll-like receptor (TLR)-2 and TLR-4 ligands (Skold *et al.*, 2005). The peroxisome proliferator-activated receptor-γ (PPAR-γ) controls CD1d expression by triggering retinoic acid synthesis in human DCs (Szatmari *et al.*, 2006). Furthermore, a number of pathogens modulate the extent of CD1d expression. Viruses such as Kaposi's sarcoma-associated herpes virus (KSHV) and HIV downregulate the expression of CD1d on the plasma membrane (Andre *et al.*, 2005; Sanchez *et al.*, 2005). Finally, herpes simplex virus-1 (HSV-1) and vesicular stomatitis virus (VSV) can cause downmodulation of CD1d by suppressing CD1d recycling (Raftery *et al.*, 2006; Yuan *et al.*, 2006).

3.2. Intracellular trafficking of CD1 molecules

In analogy to MHC-I molecules, CD1 heavy chains are folded and associated with β2m within the lumen of the ER, in a process that involves calnexin and calreticulin, but excludes transporters associated with antigen processing (TAP) (Kang and Cresswell, 2002; Sugita *et al.*, 2007). At this stage, the pocket formed by the α1 and α2 helices of the heavy chain can be loaded with hydrophobic spacers such as neutral phospholipids to stabilize the nascent CD1 molecule (Gadola *et al.*, 2002). Accordingly, ER-resident lipid exchange proteins such as the microsomal triglyceride transfer protein (MTP) could fulfill this loading function (Dougan *et al.*, 2005, 2007b). From the ER, CD1 molecules transit along the secretory pathway until they reach the plasma membrane, where they follow a major pathway of internalization subdivided into distinct intracellular

trafficking routes. The type of endocytic compartment through which CD1 molecules transit largely depends on the presence of the amino acid sequence Y-X-X-Z (tyrosine-X-X-bulky hydrophobic residue) in the cytoplasmic moiety of the heavy chain (Chiu *et al.*, 2002). At the plasma membrane, this motif mediates recruitment of the adaptor molecule AP-2 by CD1b, CD1c, and CD1d molecules, and promotes their subsequent internalization through clathrin-coated pits (Sever, 2003). Following their traffic to endosomes, CD1b and CD1d molecules are further sorted to late acidic endocytic compartments by the recruitment of AP-3 through the same cytoplasmic motif (Sugita *et al.*, 2002). CD1b is almost exclusively located in lysosomes, whereas CD1c that does not bind AP-3 shows a broad localization throughout the endosomal pathway (Sugita *et al.*, 2007). Notably, there is no evidence for targeting motifs in the cytoplasmic tail of CD1a proteins. Nevertheless, they are found in recycling endosomes and traffic back to the plasma membrane in an ARF6-dependent manner (Sugita *et al.*, 1999). Finally, CD1e displays unique features in comparison to other CD1 molecules. Upon assembly, CD1e molecules are targeted to lysosomes without reaching the plasma membrane, where they exist in a cleaved soluble form and participate in the processing of microbial glycolipid antigens (de la Salle *et al.*, 2005). Ubiquitination of the cytoplasmic tail is a prerequisite for CD1e proteins to target lysosomes (Maitre *et al.*, 2008). In the endocytic system, CD1 molecules exchange lipids acquired in the secretory pathway with self or foreign lipids, and, except for CD1e, traffic to the plasma membrane where they display their lipid cargo to CD1-restricted T cells. Hence, the divergent trafficking routes of CD1 molecules may reflect an evolutionary adaptation to face the diversity of intracellular pathogen lifestyles through detection by the immune system.

3.3. CD1-restricted T cells

Michael Brenner and colleagues provided the first evidence that human CD1 molecules present lipid antigens to induce T cell responses (Beckman *et al.*, 1994). The cognate antigen presented in the context of CD1b proved to be mycolic acid, a lipid contained in the cell wall of *Mycobacterium tuberculosis*. Intriguingly, T lymphocytes restricted by group 1 CD1 molecules demonstrate favored reactivity with lipid antigens originating from the cell wall of mycobacteria. *M. tuberculosis* is the etiologic agent of tuberculosis, one of the most ancient and life-threatening infectious diseases worldwide (Kaufmann, 2006). Since humans represent the principal reservoir of *M. tuberculosis*, it is tempting to speculate that group 1 CD1-restricted T cells might have developed as a result of coevolution of host and pathogen to provide specific protection against *M. tuberculosis* infection. Both hydrophobic peptides and lipids or glycolipids from

mycobacteria can be presented by CD1 molecules. Accordingly, didehydroxymycobactin (DDM), a lipopeptide structurally related to siderophores, is presented to T cells in the context of CD1a (Moody *et al.*, 2004). Mannosylated phosphatidylinositides (PIMs), including lipoarabinomannan (LAM), and diacylated sulfoglycolipids (Ac_2SGLs) activate T cells when presented through CD1b (Gilleron *et al.*, 2004; Sieling *et al.*, 1995). Further, glucose monomycolate (GMM), produced upon interaction of biosynthetic pathways of host and pathogen, activates T cells restricted by CD1b (Moody *et al.*, 2000a). Finally, hexosyl-1-phosphoisoprenoids stimulate CD1c-dependent T cells (Beckman *et al.*, 1996; Moody *et al.*, 2000b). Lymphocytes restricted by group 1 CD1 molecules are found in all the major phenotypic subsets of T cells, including single-positive $CD4^+$ and $CD8^+$ T cells, as well as double-negative (DN) CD4 CD8 T lymphocytes (Porcelli *et al.*, 1992; Rosat *et al.*, 1999; Sieling *et al.*, 2000). Upon activation, CD1-restricted T cell clones develop T_H1 effector functions dominated by the production of IFN-γ and TNF-α (Rosat *et al.*, 1999; Sieling *et al.*, 1999). In addition, DN and $CD8^+$ CD1-restricted T cells exert potent cytotoxic functions toward *M. tuberculosis*-infected macrophages through Fas–Fas ligand interactions or the release of granulysin, respectively (Stenger *et al.*, 1997). Using these mechanisms, CD1b-restricted T cells effectively kill *M. tuberculosis*-infected macrophages in a CD1b-dependent manner (Stenger *et al.*, 1997). A feature shared by all CD1-restricted T cells is the basal recognition of CD1 molecules in the absence of foreign lipids (Porcelli *et al.*, 1989). Autoreactive responses by T cells can stimulate the maturation of DCs toward a proinflammatory phenotype, which may play a critical role with regard to the ensuing generation of adaptive immune responses (Spada *et al.*, 2000; Vincent *et al.*, 2002). Recognition of sulfatide or GM1 by CD1-restricted T cells could form the basis of this autoreactivity (Shamshiev *et al.*, 2000, 2002). In contrast to CD1b molecules, CD1a and CD1c are largely excluded from acidic subcellular compartments, and their antigen-presenting functions are not affected upon inhibition of endosomal acidification (Briken *et al.*, 2000; Porcelli *et al.*, 1992; Sieling *et al.*, 1995). Optimal binding of PIMs, GMM, and LAM require an acidic environment in which the α-helices of CD1b can partially unfold (Ernst *et al.*, 1998). Although these findings highlight an interesting mechanism that allows access of lipid antigens to the CD1b groove only upon their trafficking to the proper compartment, they cannot account for a model in which CD1b itself extracts lipids from membranes and thus, chaperoning helper molecules have to fill this gap. In this context, SAP-C has been identified as the critical saposin required for recognition of microbial lipid antigens by CD1b-restricted T cells (Winau *et al.*, 2004b). Accordingly, human pSAP-deficient fibroblasts expressing CD1b failed to present mycolic acid, GMM, and LAM for activation of antigen-specific CD1b-restricted T cell clones. Moreover,

T cell responses could be restored upon fibroblast reconstitution with SAP-C but not other SAPs (Winau *et al.*, 2004b). The underlying mechanism involved SAP-C-mediated extraction of LAM from membranes and subsequent transfer to CD1b (Fig. 2.2), as indicated by coprecipitation experiments identifying a direct interaction between SAP-C and CD1b (Winau *et al.*, 2004b). These findings demonstrated saposins as a missing link in antigen presentation of lipids to group 1 CD1-restricted T cells, and suggest that SAP-C dysfunctions potentially have adverse consequences concerning T cell immunity in infectious diseases like tuberculosis.

3.4. CD1d-restricted natural killer T cells

In contrast to humans, mice lack genes encoding group 1 CD1 molecules. Therefore, their repertoire of lipid-specific T cells is solely represented by lymphocytes restricted to CD1d molecules, namely NKT cells. Unique features of NKT cells include usage of an invariantly rearranged T cell receptor (TCR) α chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) paired with a limited set of TCR β chains, expression of diverse surface receptors characteristic for NK cells, and functional autoreactivity toward CD1d-expressing APCs *in vitro* (Bendelac *et al.*, 1995; Budd *et al.*, 1987; Dellabona *et al.*, 1994; Fowlkes *et al.*, 1987; Porcelli *et al.*, 1993). Further, NKT cells express intermediate levels of TCR at the cell surface and a phenotype of activated/memory T cells in naive and germ-free mice, as well as in human cord blood, which may reflect the consequence of continuous basal TCR stimulation with self antigens (D'Andrea *et al.*, 2000; Park *et al.*, 2000; van Der Vliet *et al.*, 2000). NKT cells are a heterogeneous population and dominated by a subset that reacts with the marine sponge-derived GSL antigen α-galactosylceramide (α-GalCer) presented by CD1d proteins (Chen *et al.*, 1997; Kawano *et al.*, 1997). By definition, NKT cells that respond to α-GalCer are referred to as invariant NKT (iNKT) cells. iNKT cells show exclusive usage of the TCR rearrangement Vα14-Jα18 coupled to Vβ8, Vβ7, or Vβ2 in mice, or the rearrangement Vα24-Jα18 associated with Vβ11 in humans, and promptly produce IFN-γ and interleukin-4 (IL-4) upon activation (Bendelac *et al.*, 2007). By contrast, NKT cells that fail to respond to α-GalCer stimulation are referred to as noninvariant NKT cells, or type II NKT cells. Notably, type II NKT cells use diverse TCRs, and owing to the lack of specific markers to track them, their biology remains poorly understood.

3.4.1. Invariant NKT cells

The relevance of iNKT cells to diseases such as cancer, infection, or autoimmunity, has been extensively reviewed elsewhere (Bendelac *et al.*, 2007; Godfrey *et al.*, 2004). One of the most exciting features in the biology of iNKT cells pertains to the modes of their activation. In the face

of infection, it has become evident that the host can use several pathways to activate iNKT cells. Firstly, activation may result from direct, TCR-mediated recognition of microbial lipid antigens presented in the context of CD1d-expressing APCs. Known antigens include α-glucuronosylceramides and α-galacturonosylceramides from *Sphingomonas* spp., diacylglycerols from *Borrelia burgdorferi,* the causative agent of Lyme disease, and phosphatidylinositol tetramannoside from *M. tuberculosis* (Fischer *et al.*, 2004; Kinjo *et al.*, 2005, 2006; Mattner *et al.*, 2005; Sriram *et al.*, 2005). Following a second pathway predominantly triggered by infection with Gram-negative lipopolysaccharide (LPS)-positive bacteria, such as *Salmonella typhimurium*, iNKT cells become activated upon recognition of self antigens presented by LPS-exposed DCs in an IL-12-dependent manner (Brigl *et al.*, 2003). In addition to TLR-4-mediated activation of iNKT cells triggered by LPS, several other axes of DC sensitization through TLR ligation have been identified (De Libero *et al.*, 2005). Accordingly, stimulation of DCs through the nucleic acid sensor TLR-9 results in the subsequent activation of iNKT cells (Paget *et al.*, 2007). In the latter case, neosynthesized β-linked self GSLs and type I interferons provided by DCs were strictly required. Accordingly, stimulation of DCs through TLR-4, TLR-7, or TLR-9, could influence the expression of various glycosyltransferases involved in the biogenesis of GSLs (Paget *et al.*, 2007; Salio *et al.*, 2007). Importantly, blocking the *de novo* generation of GSLs abrogated the responses by iNKT cells. Finally, increased expression of CD1d/GSL complexes, representing ligands for the iNKT cell invariant TCR, could be visualized at the surface of APCs stimulated with LPS or a TLR-8 agonist (Salio *et al.*, 2007). Thus, microbe-exposed APCs remodel the repertoire of self GSLs to produce dominant species that can be recognized by iNKT cells. How signals relayed through pattern recognition receptors can lead to selective induction of GSL antigens without compromising cellular lipid homeostasis remains to be clarified. Lastly, several recent studies indicate that iNKT cells could become activated in a pure cytokine-driven fashion without requirement for TCR tickling by CD1d–self-lipid complexes (Montoya *et al.*, 2006; Nagarajan and Kronenberg, 2007). Accordingly, MCMV-infected DCs could activate iNKT cells in an IL-12-dependent, but CD1d-independent manner (Tyznik *et al.*, 2008). In analogy to human CD1b, murine CD1d molecules primarily localize to lysosome-associated membrane protein 1 (LAMP-1)-positive organelles, indicating a trafficking route that includes late acidic compartments for acquisition of self lipids (Chiu *et al.*, 2002). Of note, deletion of the AP-3-binding motif in the cytoplasmic tail of CD1d (CD1-TD) depletes the molecule from late endosomal and lysosomal compartments, while surface expression of the mutant molecule is slightly increased (Chiu *et al.*, 1999). This tail modification leads to severe functional consequences specifically affecting iNKT cells. Accordingly, CD1-TD-expressing APCs fail to present self lipids and

exogenous α-GalCer to iNKT cell hybridoma, and CD1-TD knock-in mice show impaired production of iNKT cells in the thymus, which results in profound defects of iNKT cells in peripheral organs (Chiu *et al.*, 2002). Taken together, these findings identified late endocytic compartments as primary sites where CD1d molecules acquire self lipids or exchange self lipids with foreign antigens. Further, the endosomal route proved to be essential in the generation of CD1d–self-lipid complexes that are recognized by thymocytes, which ultimately facilitates iNKT cell development. Finally, these findings also suggested that lipid exchanges between CD1d and membranes might require helper molecules located in late endosomal and lysosomal compartments. First evidence supporting this hypothesis derived from studies performed with pSAP$^{-/-}$ mice, which are devoid of all four SAPs and selectively lack iNKT cells (Zhou *et al.*, 2004a). *In vitro*, DCs and thymocytes from pSAP$^{-/-}$ mice failed to stimulate iNKT cell hybridoma, but showed intact functions in the presentation of self lipids to noninvariant NKT cell hybridoma, as well as a normal capacity to process exogenous proteins for activation of various antigen-specific MHC-II-restricted T cells. In cell-free assays, recombinant SAP-A and SAP-C showed the highest efficiency in the exchange of trisialoganglioside GT1 loaded onto CD1d molecules with PS or sulfatide contained in liposomes, using an experimental pH that corresponded to lysosomes. However, SAPs alone could not extract GT1 bound to CD1d. By contrast, GM2AP extracted the ganglioside from CD1d but did not replace it (Zhou *et al.*, 2004a). Using murine pSAP-deficient cell lines transduced with human CD1d as APCs, another study concluded that recognition of α-GalCer by a human iNKT cell line could be enhanced by reintroduction of human pSAP. However, pSAP did not increase autoreactive responses by the iNKT cell line (Kang and Cresswell, 2004). Finally, reintroduction of mutant pSAP constructs, each lacking one of the four saposins, revealed that SAP-B-expressing APCs most efficiently enhanced α-GalCer presentation to NKT cells (Yuan *et al.*, 2007). However, no individual SAP proved to be absolutely essential in that process. In humans and mice, SAP-B seems to play a dominant role in the exchange of CD1d-bound self lipids acquired in the secretory pathway with self or foreign lipids present in lysosomes.

3.4.2. Noninvariant NKT cells

Similar to iNKT cells, type II NKT cells show reactivity to CD1d molecules expressed by APCs. In contrast to iNKT cells, type II NKT cells recognize CD1d-bound lipids that are loaded along the secretory pathway (Chiu *et al.*, 1999). The myelin-derived self GSL sulfatide, which previously has been identified as a self antigen comparably presented by CD1a, CD1b, and CD1c molecules, is specifically recognized by a subset of type II NKT

cells (Jahng *et al.*, 2004; Shamshiev *et al.*, 2002). Identification of this subpopulation using sulfatide-loaded CD1d tetramers revealed its specific enrichment in the central nervous system during experimental autoimmune encephalomyelitis (EAE). Interestingly, sulfatide treatment prevented antigen-induced EAE in wild type, but not in CD1d $^{-/-}$ mice. The underlying mechanism involved sulfatide-reactive type II NKT cells that prevented the production of IFN-γ and IL-4 by pathogenic myelin oligodendrocyte glycoprotein (MOG)-reactive T cells (Jahng *et al.*, 2004). More recent studies explored the requirements for type II NKT cell hybridoma recognition of sulfogalactosylsphingosine (lysosulfatide), a sulfatide derivative lacking the fatty acid constituent (Roy *et al.*, 2008). In this context and according to findings that type II NKT cells specialize in the recognition of antigens acquired along the secretory pathway, pSAP deficiency had no impact on the recognition of lysosulfatide by type II NKT cell hybridoma. However, at acidic pH, SAP-C enhanced the recognition of plate-bound CD1d molecules loaded with lysosulfatide by noninvariant NKT cells (Roy *et al.*, 2008).

4. SAPs STIMULATE THE PROCESSING OF LIPID ANTIGENS BY LYSOSOMAL GLYCOSIDASES

The lysosomal system is of considerable biomedical importance since its alterations are associated with numerous human diseases. To date, more than 50 monogenic human diseases that are primarily associated with lysosomal dysfunction have been identified, and the majority of these conditions are classified as lysosomal storage disorders (LSDs). LSDs are caused by deficiencies in membrane proteins that transport degradation products out of the lysosome, or they are due to defects in molecules involved in the processing or trafficking of lysosomal proteins and GSLs (Fig. 2.1). Pathways of GSL production and degradation have recently attracted the attention of lipid immunologists since analyses of mouse models of human LSDs have uncovered unexpected antigen-processing defects with major impact on CD1-restricted T cell responses.

4.1. Hexosaminidase B

The mouse model of Sandhoff disease that lacks the β-subunit of hexosaminidase A and hexosaminidase B (Hexb $^{-/-}$ mice) has been instrumental in the elucidation of an endogenous antigen recognized by iNKT cells, namely, the GSL isoglobotriaosylceramide (iGb3) (Zhou *et al.*, 2004b). *In vivo* analyses revealed a specific lack of iNKT cells in Hexb $^{-/-}$ mice, which suggested that functions of hexosaminidases in the catabolism of GSLs are required for the production of iNKT cell agonists in the thymus.

In line with this postulate, APCs from Hexb $^{-/-}$ mice expressing CD1d molecules failed to stimulate autoreactive responses by iNKT cell hybridoma, whereas responses to α-GalCer were preserved. By testing the potential antigenicity of several GSL species belonging to the globo-, isoglobo-, and neolacto-series, which are produced in lysosomes upon the action of hexosaminidases (Fig. 2.1), only iGb3 could stimulate murine and human iNKT cells (Zhou *et al.*, 2004b). Hence, by removing the terminal *N*-acetyl-β-D-galactosamine residue from iGb4, Hex-B produces iGb3 that is recognized by iNKT cells. Possibly due to missing self on CD1d molecules, either by lack of generation (Hexb $^{-/-}$) or by deficient CD1d loading (pSAP $^{-/-}$) of endogenous antigens, both knock-out strains fail to develop iNKT cells. Therefore, potential iGb3–SAP–CD1d interactions have been proposed. Accordingly, SAP-B could exchange CD1d-bound GT1 with free iGb3 or iGb4 (Zhou *et al.*, 2004b). However, while immunological and novel biochemical evidence points toward iGb3 as the natural self antigen, its physiological role remains vividly challenged by several studies in humans and mice (Christiansen *et al.*, 2008; Gadola *et al.*, 2006; Li *et al.*, 2009; Porubsky *et al.*, 2007; Speak *et al.*, 2007). Recently, SAP-B $^{-/-}$ mice were described to accumulate Gb3 in various tissues, which is in agreement with previous findings that SAP-B is required to activate the degradation of Gb3 by α-galactosidase A (Sun *et al.*, 2008). Since globosides and isoglobosides use the same degradation pathway, SAP-B $^{-/-}$ mice could provide an interesting model to test the possible accumulation of iGb3 or other potential endogenous antigens for iNKT cells.

4.2. α-Galactosidase A

First evidence uncovering the antigen-processing component of α-galactosidase A (α-Gal-A) derived from a study in which APCs from α-Gal-A $^{-/-}$ mice (Fabry disease) failed to present the synthetic disaccharide antigen Galα(1→2)α-GalCer to iNKT cell hybridoma (Prigozy *et al.*, 2001). Accordingly, removal of the terminal galactose residue by lysosomal α-Gal-A was required to expose the α-GalCer epitope to the invariant TCR. In the catabolism of globosides, α-Gal-A functions downstream of Hex-B to produce LacCer from Gb3 (Fig. 2.1). In addition, α-Gal-A degrades the galactolipid Ga_2Cer to β-GalCer (Ohshima *et al.*, 1997). Interestingly, α-Gal-A shows a broader substrate specificity than previously expected, since it can also remove terminal galactose residues bound in the α(1→3) configuration. Therefore, iGb3 that contains a terminal α(1→3)-branched galactose could represent a physiological substrate for α-Gal-A. Consequently, enzyme deficiency in α-Gal-A activity could lead to iGb3 accumulation. Unexpectedly, α-Gal-A $^{-/-}$ mice demonstrated a lack of iNKT cells (Prigozy *et al.*, 2001; Zhou *et al.*, 2004b). In contrast to Hexb $^{-/-}$ mice, this deficit was only partial and specific for iNKT cells located in

peripheral organs. Since Fabry and Sandhoff diseases have different etiologies and display diverse patterns of GSL storage, a generalized defect of iNKT cell selection has been proposed in LSD mice. Analyses of knock-out mouse models of Tay–Sachs disease in which hexosaminidase A is lacking (HexA $^{-/-}$ mice), Sandhoff disease (Hexb $^{-/-}$ mice), GM1 gangliosidosis (β-Gal $^{-/-}$ mice), and mice deficient for the endosomal transmembrane protein NPC1 involved in cholesterol homeostasis, revealed reduced frequencies as well as functional defects of iNKT cells (Gadola *et al.*, 2006; Schumann *et al.*, 2007). The hypothesis has been proposed that the degree of iNKT cell deficiency in each mouse model could be related to the extent of lipid stored, irrespective of specific lipid entities. Therefore, lipid storage itself could exert a nonspecific negative impact on the selection of thymic iNKT cell precursors. Upon immunological analysis of α-Gal-A $^{-/-}$ mice, we found specific loss of peripheral iNKT cells in accordance with previous reports (Zhou *et al.*, 2004b). These defects were the direct consequence of iNKT cells chronically exposed to self GSLs (unpublished observations). Moreover, DCs from α-Gal-A $^{-/-}$ mice induced CD1d-dependent production of IFN-γ and IL-4 by iNKT cells in the absence of exogenous antigen. Additionally, wild-type DCs treated with an inhibitor of α-Gal-A elicited NKT cell activation. Further, reconstitution of α-Gal-A-deficient DCs with recombinant enzyme, or iGb3 blocking in Fabry DCs, abrogated iNKT cell responses. In a more recent study analyzing iNKT cells in diverse animal models of LSDs, in which GSLs, glycosaminoglycans, or both accumulate, defective iNKT cell development could only be observed in mice affected by combined deficiency in sulfatase activity. However, these defects were generalized to other T cell subsets. By contrast, mice with single lysosomal enzyme deficiencies showed normal iNKT cell development (Plati *et al.*, 2009). In conclusion, constitutive or induced deficiency in α-Gal-A activity leads to accumulation of endogenous self antigens such as iGb3 for subsequent activation of iNKT cells.

4.3. α-Mannosidase

A decisive function of α-mannosidase in the processing of carbohydrate antigens has been clarified in the human system. Accordingly, DCs from a patient with congenital deficiency in α-mannosidase failed to present mycobacterial hexamannosylated phosphatidyl-myo-inositides (PIM_6) to a CD1b-restricted T cell line (de la Salle *et al.*, 2005). In detail, the enzyme is required for the stepwise degradation of PIM_6 to PIM species that contain fewer mannose residues, including the stimulating antigen PIM_2. Importantly, the generation of stimulating PIM_2 species required assistance by soluble CD1e molecules (de la Salle *et al.*, 2005). In contrast to SAPs, which are ubiquitously expressed and primarily required in the

catabolism of GSLs, CD1e is mainly expressed in immune cells, and could therefore specifically act as an immunological lipid transfer protein (Angenieux *et al.*, 2005). In analogy to saposins, whether CD1e uses a ''solubilizer'' or ''liftase'' mode of action remains to be clarified.

5. SAPOSINS DISRUPT VESICLES RELEASED BY APOPTOTIC CELLS

5.1. Implications for apoptosis

Upon hypoxia, trauma, or the effect of noxious substances, cells die by necrosis, which involves depletion of the intracellular ATP stores associated with cell swelling and rupture of cellular organelles (Winau *et al.*, 2005). Ultimately, necrotic cells burst and release their organellar and cytosolic content into the surrounding tissue, which subsequently causes inflammation. In sharp contrast to necrosis, apoptosis represents a regulated form of cell death that prevents inflammatory responses under physiological conditions (Winau *et al.*, 2005). Accordingly, apoptotic cells shrink, condense their DNA and organelles prior to fragmentation, release membrane blebs, and finally disintegrate into apoptotic bodies. Thus, apoptosis avoids cell leakage and secondary harmful inflammation, and represents a ''silent'' way of death that cells undergo during development and tissue homeostasis (Ravichandran and Lorenz, 2007). However, the silencing feature of apoptosis can be overridden in the context of infection or cancer, when apoptotic bodies become vehicles for antigens and tumor- or pathogen-associated molecular patterns that trigger immune responses (Winau *et al.*, 2004a). A hallmark of apoptosis is the exposure of PS, which is normally confined to the inner leaflet of the cytoplasmic membrane in living cells, on the surface of apoptotic cells (Savill *et al.*, 2002). Further, apoptotic cells release high amounts of chemoattractant nucleotides and lysophosphatidylcholine (LPC), which subsequently recruit phagocytes (Elliott *et al.*, 2009). Capture and subsequent internalization of apoptotic bodies involves specific recognition of externalized PS by diverse phagocytic receptors, including the scavenger receptor CD36, brain angiogenesis inhibitor 1 (BAI1), T cell immunoglobulin and mucin domain-containing molecule 4 (TIM-4), Mer tyrosine kinase, and stabilin-2 (Ravichandran and Lorenz, 2007). Finally, apoptotic bodies are incorporated into phagosomes, which subsequently mature, and eventually fuse with lysosomes for terminal degradation. Elucidation of these clearance pathways is of great interest since removal of apoptotic cells by DCs bears important implications for the establishment of immune tolerance (Albert *et al.*, 1998a, 2001; Kawane *et al.*, 2006). While rapid progress has been made toward the understanding of molecular

processes inherent to the delivery, recognition, and engulfment of apoptotic bodies, the mechanism of the critical final processing step, describing their lysosomal disintegration, remains largely unexplored. Ultrastructural examination of apoptotic vesicles by electron microscopy reveals similar features to ILVs. Therefore, we anticipated that the special mode of action of saposins on intralysosomal membranes could be used to disrupt apoptotic vesicles located in lysosomes, following phagocytosis by macrophages or DCs (Fig. 2.2). Of note, high content of anionic phospholipids in ILVs favors their solubilization upon functional interaction with specific SAPs in lysosomes (Ciaffoni *et al.*, 2001). We propose that PS on apoptotic bodies deploys its actual specific function inside the phagocytes, namely, as molecular target for saposins in lysosomes to facilitate disintegration of apoptotic vesicles (Fig. 2.2, unpublished observations).

5.2. Saposins facilitate antigen cross-presentation

Presentation of peptide antigens by MHC molecules to T lymphocytes classically comprises two major pathways. Following the MHC-I pathway, endogenous proteins that are synthesized inside the cells, such as antigens produced by viruses, are primarily located in the cytosol. Subsequently, the multienzyme complex of the proteasome degrades the proteins into peptide fragments, which are translocated into the ER through TAP (Goldberg and Rock, 1992; Shepherd *et al.*, 1993). After loading of MHC-I molecules in the ER assisted by the peptide-loading complex, consisting of tapasin, calreticulin, and Erp57, MHC-I-peptide complexes are transported to the cell surface of the APC for specific recognition by $CD8^+$ T cells (Degen *et al.*, 1992; York and Rock, 1996). By contrast, exogenous antigens derived from pathogenic bacteria, for example, are endocytosed by APCs for subsequent degradation by cathepsins in late endosomal / lysosomal compartments, prior to loading onto MHC-II molecules. Subsequently, $CD4^+$ T cells recognize complexes of MHC-II and peptide exposed on the APC surface. However, exogenous antigens can also be presented by MHC-I molecules in a process termed cross-presentation (Vyas *et al.*, 2008). Accordingly, the respective antigen crosses from the endosomal route to the MHC-I pathway. The activation of $CD8^+$ T cells by cross-presented antigens is referred to as cross-priming, and DCs are APCs uniquely equipped for cross-presentation (Bevan, 1976; Guermonprez *et al.*, 2002). To date, multiple mechanisms for the cellular pathway of cross-presentation have been proposed, which are likely not mutually exclusive (Vyas *et al.*, 2008). Moreover, several antigen vehicles have been described to have cross-priming abilities, including proteins, peptides, and heat-shock proteins (HSP) chaperoning peptides (Srivastava, 2002). In addition, apoptotic cells represent a potent device to deliver antigens to the cross-presentation pathway. Notably, apoptotic

bodies derived from tumors or host cells infected with viruses induce vigorous $CD8^+$ T cell responses (Albert *et al.*, 1998b). Further, macrophages infected with mycobacteria release apoptotic vesicles that are engulfed by uninfected bystander DCs for cross-priming of $CD8^+$ T cells (Schaible *et al.*, 2003; Winau *et al.*, 2006). Ultimately, immunization with apoptotic vesicles released by mycobacteria-infected macrophages elicits $CD8^+$ T cell responses and protects against tuberculosis (Winau *et al.*, 2006). Therefore, apoptotic bodies as mediators of antigen cross-presentation are part of a unique detour pathway that promotes T cell responses in tumor and infection immunity (Winau *et al.*, 2004a). However, it remains unclear as to how antigens enclosed in apoptotic bodies become accessible for cross-presentation to $CD8^+$ T cells. Our previous findings suggested that successful cross-priming requires pSAP-dependent processing of apoptotic vesicles in recipient DCs (Winau *et al.*, 2006). Thus, we propose that saposins unseal apoptotic bodies for antigen delivery in DCs and subsequent $CD8^+$ T cell responses (Fig. 2.2).

6. SAP-LIKE PROTEINS IN ANTIMICROBIAL DEFENSE

The family of SAPLIPs comprehends heterogeneous and functionally divergent proteins that share a conserved motif of six cysteine residues associated by three disulfide bonds (Munford *et al.*, 1995). This motif forms the characteristic ''saposin fold'' that allows SAPLIPs to interact with lipids (Bruhn, 2005). The SAPLIP domain can be regarded as an ancestral molecule since it is present both in humans and in one of the most primitive eukaryotes, namely amoebozoans. *Entamoeba histolytica* is a prototypical pathogenic amoebozoan that produces amoebapores. These molecules belong to the family of SAPLIPs and exert cytolytic activities against bacteria and human cells, by forming pores in the target cell membrane as a killing principle (Leippe *et al.*, 1994a,b). *E. histolytica* is the etiologic agent of human amoebiasis, and recent evidence suggests that amoebapores could be responsible for the tissue destruction upon infection (Bracha *et al.*, 2003). To date, 19 genes encoding SAPLIPs have been identified in *E. histolytica*. Considering that the primary function of amoebapores is the destruction of phagocytosed bacteria for nutritional purposes, a diversity of SAPLIPs might be required for subsequent digestive steps. Interestingly, SAPLIPs with similar lytic functions have been described in humans and other mammals. These include human granulysin and porcine NK-lysin that share the highest degree of homology among members of the SAPLIP family. Similar to amoebapores, granulysin and NK-lysin show a broad spectrum of antimicrobial activity, killing parasites, bacteria, and fungi (Ernst *et al.*, 2000; Leippe, 1995; Pena *et al.*, 1997; Stenger *et al.*, 1998). In combination with perforin, granulysin

released by T cells kills *M. tuberculosis* in macrophages by affecting the integrity of its cell wall, leading to subsequent osmotic bacterial lysis (Stenger *et al.*, 1998). NK-lysin is predominantly stored by T cells and NK cells in cytosolic granules, and is released upon activation (Andersson *et al.*, 1995). In addition, NK-lysin was found to be lytic against YAC-1 tumor targets, and especially potent at killing tumor cell lines that had increased surface levels of PS (Andersson *et al.*, 1995; Schroder-Borm *et al.*, 2005). By contrast, granulysin has been more extensively studied with regard to its tumorolytic functions against human T cell lymphoma of the Jurkat type (Kaspar *et al.*, 2001). Moreover, several studies could unequivocally demonstrate the importance of granulysin and antimicrobial proteins in various infectious diseases (Heusel *et al.*, 1994; Kagi *et al.*, 1994; Lowin *et al.*, 1994; Stenger *et al.*, 1998). To date, the structures of six SAPLIPs have been resolved, starting with the first crystal of NK-lysin (Liepinsh *et al.*, 1997). They all show the same α-helical fold of five helices connected by three disulfide bonds. Surprisingly, the predicted modes of action by lytic SAPLIPs suggest extremely diverse mechanisms used to achieve membrane permeabilization. In its active state, the pore-forming amoebapore A is a dimer stabilized by electrostatic interactions that involve a unique, centrally positioned histidine residue (Andra and Leippe, 1994). One side of the dimer is exclusively hydrophobic, which allows its insertion into membranes. Once docked, the protein oligomerizes to create ring-like pores that resemble channels (Gutsmann *et al.*, 2003). The histidine residue functions as a pH-dependent switch. Activation of this switch occurs at low pH and allows the dimerization that is crucial for the cytolytic function (Andra and Leippe, 1994). Considering its function, it is therefore not surprising that this residue is highly conserved among all amoebapore isoforms. By contrast, NK-lysin and granulysin permeabilize membranes in a monomeric state through an electrostatic process termed electroporation (Miteva *et al.*, 1999). Concerning both SAPLIPs, membrane recruitment occurs through interactions of positively charged residues in the SAPLIP and negatively charged phospholipids in the target membrane. Subsequent conformational changes allow the two halves of the molecules to slit the membrane in a scissor-like fashion, ultimately causing osmotic lysis (Anderson *et al.*, 2003). In *Naegleria fowleri*, the SAPLIP naegleriapores are encoded in larger multipeptide precursor structures, each potentially giving rise to multiple glycosylated naegleriapore-like molecules (Herbst *et al.*, 2004). Finally, functional characterization of a novel SAPLIP in *E. histolytica*, namely SAPLIP 3, revealed fusogenic activities similar to SAP-C in mammalians (Winkelmann *et al.*, 2006). Accordingly, we highlight the possibility that saposins might possess conserved evolutionary traits of ancient weapons which potentially mediate direct antimicrobial functions (Fig. 2.2).

7. CONCLUSIONS

Decades ago, saposins have been considered as negligible test tube activators of lysosomal glycosidases. Today, saposins emerge as critical components of membrane homeostasis and unexpected actors on the scene of immunology. In addition to their well-established capacities in sphingolipid degradation and membrane digestion, saposins also fulfill important immunological functions. Based on their universal principle to interact with membrane bilayers in lysosomes, SAPs exert versatile helper functions further defined by their respective interaction partners. In this context, saposins can mobilize lipids from membranes and associate with lipid-degrading enzymes, in order to degrade or generate antigenic epitopes. Further, saposins interact with antigen-presenting molecules to facilitate the loading of lipid antigens onto CD1 proteins for subsequent activation of lipid-reactive T lymphocytes. Additionally, the membrane-perturbing properties of SAPs can mediate the disintegration of apoptotic bodies for antigen delivery in APCs and subsequent cross-presentation. Finally, saposins could have antimicrobial functions through direct membrane attacks on pathogens.

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CHAPTER 3

OX40–OX40 Ligand Interaction in T-Cell-Mediated Immunity and Immunopathology

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Abstract T-cell activation is mediated not only by antigen stimulation through T-cell receptors but also by costimulatory signals through costimulatory molecules. Among several costimulatory molecules, the tumor necrosis factor (TNF) receptor family member OX40 plays a key role in the survival and homeostasis of effector and memory T cells. According to the conventional understanding of OX40 costimulation, an interaction between OX40 and OX40 ligand (OX40L) occurs when activated T cells bind to professional antigen-presenting cells (APCs). The T-cell functions, including cytokine production, expansion, and survival, are then enhanced by the OX40 costimulatory signals. Over the last half-decade, evidence has accumulated that OX40 signals are critical for controlling the function and differentiation of $Foxp3^+$ regulatory T cells, indicating a new aspect of OX40-mediated autoimmunity. Furthermore, the expression of OX40L by mast cells was shown to be important for controlling inflammation through regulatory T-cell function. Besides the essential role played by OX40 signaling in generating memory CD4 T cells, recent reports show that it also has a unique role in generating memory CD8 T cells. In addition, recent genome-wide association studies have identified single-nucleotide polymorphisms of the OX40L and OX40 genes that are related to cardiovascular diseases and SLE, providing direct evidence for the involvement of the OX40–OX40L interaction in human diseases. Here, we review recent progress on how the OX40–OX40L interaction regulates T-cell tolerance, peripheral T-cell homeostasis, and T-cell-mediated inflammatory diseases.

ABBREVIATIONS

Ab	antibody
Ag	antigen
APC	antigen-presenting cell
CTLA4	cytotoxic T-lymphcyte antigen-4

dsDNA	double stranded DNA
EAE	experimental autoimmune encephalomyelitis
GITR	glucocorticoid-induced TNF receptor-related protein
HTLV-I	human T-cell leukemia virus type I
IL-7R	IL-7 receptor α chain
iTreg	induced Treg
KLRG1	killer cell lectin-like receptor G1
MCMV	murine cytomegalovirus
MPEC	memory precursor effector T cell
nTreg	naturally occurring regulatory T cell
SLE	systemic lupus erythematosus
SLEC	short-lived effector T cell
Tcm	central memory T
TcR	T-cell receptor
Tem	effector memory T
Th1	T helper 1
Th2	T helper 2

1. INTRODUCTION

Optimal T-cell activation requires not only T-cell receptor (TcR) signals delivered by antigen (Ag) stimulation but also costimulatory signals provided by antigen-presenting cells (APCs) (Lenschow *et al.*, 1996; Mueller *et al.*, 1989). Although the interaction between CD28 expressed on T cells and CD80/CD86 on the surface of APCs is the best-known costimulatory signal, other costimulatory molecules, including tumor necrosis factor (TNF) receptor superfamily molecules, such as OX40 (CD134, TNFRSF4), CD27 (TNFRSF7), 4-1BB (CD137, TNFRSF9), and glucocorticoid-induced TNF receptor-related protein (GITR, TNFRSF18), can potently augment the activation of T cells to achieve a full-fledged response (Croft, 2003; Nolte *et al.*, 2009; Sugamura *et al.*, 2004; Wang *et al.*, 2009). In contrast to the other TNF receptor-type costimulatory receptors, which are expressed by naïve T cells to some extent before their activation, OX40 is not found on naïve or memory T cells, but is transiently induced upon Ag activation. Therefore, OX40 specifically provides a costimulatory signal to activated effector T cells (Croft *et al.*, 2009; Sugamura *et al.*, 2004).

The ligand for OX40 (gp34, OX40L, CD252, TNFSF4) was originally termed glycoprotein 34 (gp34) and was identified as a protein expressed on human T-cell leukemia virus type I (HTLV-I)-transformed T cells (Tanaka *et al.*, 1985). Cloning of the gp34 gene revealed that it belonged to the TNF superfamily (Miura *et al.*, 1991). Subsequently, gp34 was found to bind OX40 (Baum *et al.*, 1994; Godfrey *et al.*, 1994). Apart from

HTLV-I-infected T cells, OX40L expression was originally thought to be limited to professional APCs, such as activated B cells (Stuber *et al.*, 1995), dendritic cells (DCs) (Murata *et al.*, 2000; Ohshima *et al.*, 1997), macrophages (Weinberg *et al.*, 1999), and Langerhans cells (Sato *et al.*, 2002). Thus, the interaction between OX40 and OX40L provides an important costimulatory signal to activated T cells, mainly through a T-cell–APC interaction, that leads to the expansion and survival of specific Ag-activated T cells.

However, OX40L is now recognized to be expressed far more broadly than previously thought, including by vascular endothelial cells (Imura *et al.*, 1996; Matsumura *et al.*, 1999), mast cells (Kashiwakura *et al.*, 2004; Nakae *et al.*, 2006), activated NK cells (Zingoni *et al.*, 2004), and the responding CD4 T cells themselves (Soroosh *et al.*, 2006). Similarly, OX40 is now known to be expressed by $Foxp3^+$ regulatory T cells (Takeda *et al.*, 2004; Valzasina *et al.*, 2005), activated NKT cells (Marschner *et al.*, 2005; Zaini *et al.*, 2007), and activated T cells. Thus, new insight into the function of the OX40–OX40L interaction has been gained in recent years. In this review, among the many OX40-mediated physiological responses, we focus on the roles of the OX40–OX40L interaction in (1) the T-cell tolerance mediated by regulatory T cells, (2) the generation and homeostasis of memory T cells, and (3) inflammatory responses related to disease.

2. IMPACT OF THE OX40 COSTIMULATORY SIGNAL IN IMMUNE RESPONSES

2.1. The OX40–OX40L interaction in effector T-cell function

The expression of OX40 on CD4 and CD8 T cells is induced by TcR signaling, and peaks 48 h and 3–5 days after *in vitro* and *in vivo* Ag stimulation, respectively (Calderhead *et al.*, 1993; Gramaglia *et al.*, 1998; Mousavi *et al.*, 2008; Salek-Ardakani *et al.*, 2008). Unlike other costimulatory receptors, OX40 is not expressed by resting T cells, including naïve and memory T cells. Similarly, the expression of OX40L by APCs is induced following stimulation with CD40, lipopolysaccharide, or TSLP, and peaks 48–72 h after *in vitro* and *in vivo* stimulation (Ito *et al.*, 2005; Mousavi *et al.*, 2008; Murata *et al.*, 2000; Ohshima *et al.*, 1997). Therefore, significant interactions between OX40 and OX40L should occur 2 or 3 days after Ag recognition by T cells. This is later than the interactions between other costimulatory receptors and their ligands; for example, CD28 and its ligands interact earlier after Ag recognition, because CD28 is constitutively expressed by resting T cells (Coyle and Gutierrez-Ramos, 2001; Croft, 2003; Sharpe and Freeman, 2002). Consistent with the different expression kinetics for CD28 and OX40, CD28 signals are essential for

the activation of naïve T-cells, which is in turn critical for the generation of effector T cells. In contrast, OX40 mainly promotes the expansion (late proliferation) and survival of effector T cells. This was demonstrated by the observation that OX40-deficient T cells proliferate normally 2–3 days after TcR engagement, and differentiate into effector T cells at almost normal levels. However, by day 12–13, there is a marked reduction in their survival (Rogers *et al.*, 2001; Song *et al.*, 2004). These observations indicate that OX40 has a unique role as a late costimulator for the survival of effector T cells that have been recently activated.

In addition, several studies using various experimental settings have documented roles for the OX40–OX40 interaction in regulating T helper 1 (Th1) and T helper 2 (Th2) responses. There is much *in vitro* evidence for the preferential induction of Th2 cells by OX40 engagement (Flynn *et al.*, 1998; Ohshima *et al.*, 1998), and the first *in vivo* evidence for the critical involvement of OX40 in Th2 responses was demonstrated using an infection model with the parasitic protozoan, *Leishmania major*. Treatment of an *L. major*-susceptible mouse strain with a blocking monoclonal antibody (mAb) against OX40L to inhibit the OX40–OX40L interaction successfully controlled the infection by suppressing the Th2 responses (Akiba *et al.*, 2000). Consistent with this finding, an excessive interaction between OX40 and OX40L in OX40L-transgenic (Tg) mice in which OX40L is constitutively expressed by T cells, rendered C57BL/6 mice, which are normally resistant to *L. major* infection, susceptible to it by enhancing the Th2 responses (Ishii *et al.*, 2003). Recently, a functional association between thymic stromal lymphopoietin (TSLP), a unique cytokine that controls DC function, and OX40L has been extensively studied (Liu, 2009). Human myeloid DCs that were stimulated with TSLP effectively primed naïve T cells to produce the Th2 cytokines IL-4, IL-5, and IL-13 along with high levels of TNFα, leading to Th2-cell-mediated inflammation (Soumelis *et al.*, 2002). Later, the same group identified OX40L as a molecule that is specifically expressed on DCs stimulated with TSLP (Ito *et al.*, 2005; Wang *et al.*, 2006). Furthermore, blocking OX40L with a neutralizing Ab inhibited the production of Th2 cytokines and TNFα, and enhanced the production of IL-10 by CD4 T cells (Ito *et al.*, 2005). Similar to the TSLP-DC-derived OX40L, the Th2 responses induced by DCs stimulated with the helminth Ag, schistosome egg Ag (SEA) were suppressed by the absence of OX40L (Jenkins *et al.*, 2007).

However, this study also showed that although DC-derived OX40L critically enhanced and maintained Th2 responses, it simultaneously promoted IFNγ-producing Th1-cell priming. Indeed, in the presence of a certain Ag, complete Freund's adjuvant, or IL-12, OX40 strongly increases the Th1 responses by promoting the expansion and survival of Th1 effector cells under various conditions (De Smedt *et al.*, 2002; Gramaglia *et al.*, 2000; Ishii *et al.*, 2003; Ito *et al.*, 2005; Rogers and Croft,

2000). Since OX40 signaling enhances the survival of effector T cells whether the effector T cells are polarized or nonpolarized, the OX40-mediated survival of Th2 effector cells might explain the enhanced Th2 responses mentioned above. In the case of TSLP-mediated Th2 inflammation, TSLP might induce factors in DCs responsible for Th2 polarization apart from OX40L, which might then promote the survival and maintenance of already differentiated Th2 effector cells, and accordingly lead to the preferential induction of Th2 responses. In other words, a different factor from OX40L might be critical for the TSLP-associated Th2 polarization. Taken together, these findings suggest that the OX40–OX40L interaction plays important roles in both Th1 and Th2 responses by promoting the survival of effector T cells and the generation of memory T cells, rather than in the polarization of the Th cells.

2.2. OX40L expressed by cells other than professional APCs

Studies using intravital two-photon imaging of the lymph nodes demonstrated that by 24–48 h after Ag priming, interactions between naïve CD4 T-cell and DCs have ceased (Mempel *et al.*, 2004; Miller *et al.*, 2004), and that about half of the Ag-specific T cells leave the DCs and move into the T-cell area in the deep paracortex of the lymph nodes (Shakhar *et al.*, 2005). Since the OX40 expression on CD4 T cells continues for up to 10 days after Ag stimulation, the T cells that leave the DCs might encounter other OX40L-expressing cells, which might also provide OX40 signals to induce full-fledged CD4 T-cell responses. Supporting this notion, it was demonstrated *in vitro* that OX40L expressed by responder CD4 T cells themselves binds to OX40 on other responder CD4 T cells through T-cell–T-cell contact, to enhance T-cell survival (Soroosh *et al.*, 2006). Furthermore, in an adoptive transfer experiment in which TcR-Tg T cells were transferred into congenic mice, Ag immunization induced a lower survival of OX40L $^{-/-}$ TcR-Tg donor T cells than OX40L$^{+/+}$ TcR-Tg donor cells (Soroosh *et al.*, 2006). These results indicate an important role of OX40L expressed by the CD4 T cell itself. However, the same report also showed that OX40L expression on both the APCs and the responding T cells during T-cell activation was much more effective for promoting the survival of Ag-specific T cells than OX40L expression on either the APCs or the responding T cells alone. Therefore, these results do not exclude the importance of OX40L expressed on APCs, but rather support the idea that the OX40 signals to activated OX40$^+$ T cells are first provided by professional APCs during Ag recognition and then by other OX40L$^+$ cells during the effector phase.

Consistent with this two-step OX40L costimulation model (Fig. 3.1), several studies demonstrated that OX40L expressed by CD4$^+$ CD3 accessory cells (Kim *et al.*, 2003), B cells (Linton *et al.*, 2003), and mast cells

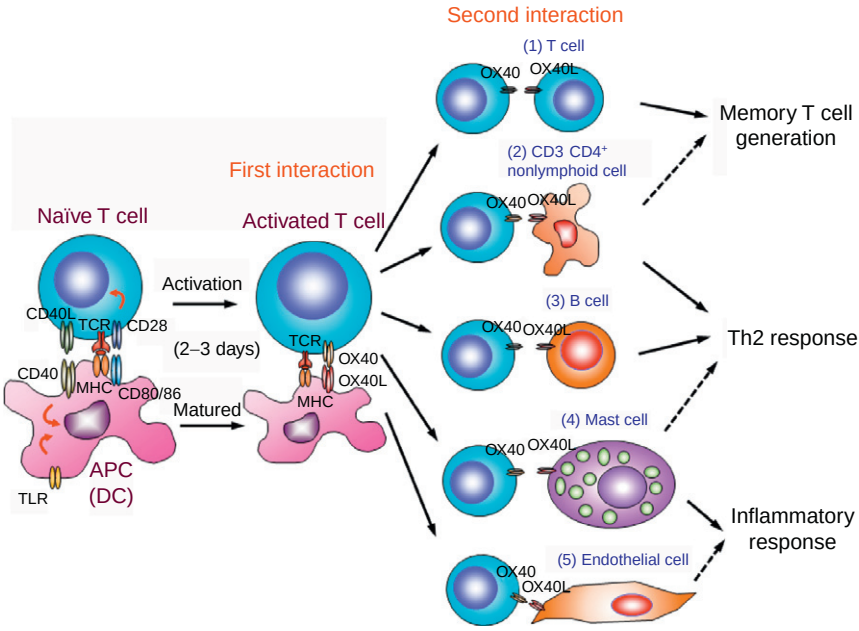

FIGURE 3.1 Two-step OX40L costimulation model. The interaction between OX40 and OX40L occurs during the T-cell–DC interaction 2 or 3 days after Ag recognition. After leaving DCs, the OX40-expressing T cell may interact with an OX40L-expressing cell other than DC, and receive an OX40 signal from the cell, which may provide essential signals for the generation of memory T cells, the enhancement of Th2 response, and the prolongation of inflammatory responses. Thus, the optimal interaction between OX40 and OX40L might be formed in two steps. After first interaction during naïve T-cell and DC interactions; (1) OX40L expressed on activated CD4 T cells interacts with OX40 expressed on other responder CD4 T cell, leading to the optimal generation of memory CD4 T cells (Soroosh *et al.*, 2006). (2) OX40L expressed on $CD3^{-}CD4^{+}$ accessory cells, which may be identical to the lymphoid tissue-inducer cells, promotes Th2 cell survival through the interaction with OX40 on Th2 cells (Kim *et al.*, 2003). (3) OX40L expression on B cells is required for *in vivo* Th2 development, but not Th1 development, under a certain condition (Linton *et al.*, 2003). (4) OX40L-expressing mast cells directly enhance effector T cell function through the interaction between OX40 on T cells and OX40L on mast cells (Kashiwakura *et al.*, 2004; Nakae *et al.*, 2006). (5) Endothelial cells also express OX40L (Imura *et al.*, 1996). OX40L expression on endothelial cells might be involved in vascular inflammation although any direct evidence has not yet been demonstrated. Solid arrows represent possible functions as demonstrated by experimental results, while dashed arrows represent hypothetic functions.

(Kashiwakura *et al.*, 2004; Nakae *et al.*, 2006) can also support T-cell function during the effector phase. Since the interaction between $OX40^{+}$ T cells and these $OX40L^{+}$ cells occurs after the T-cell–DC interaction, all of

these cells are potential candidates for critical components of this model. However, since the OX40L-expressing responding T cells do not express MHC class II (Ishii and Soroosh, unpublished result), whether the OX40 signals provided by the T cells themselves require Ag presentation is unclear. In spite of the importance of OX40L on these cells, further investigation will be necessary to verify the two-step OX40L costimulation model.

Mast cells play important roles in a variety of immune processes, such as pathogen clearance, inflammatory responses, and allergy. The interaction between mast cells and T cells has been shown to have a unique role in modulating T-cell function through the mast-cell expression of OX40L (Gri *et al.*, 2008; Kashiwakura *et al.*, 2004; Nakae *et al.*, 2006; Piconese *et al.*, 2009). Nakae *et al.* (2006) found that OX40L is constitutively expressed on mast cells in the BALB/c mouse strain, but not in the C57BL/6 strain. The coculture of T cells with OX40L-expressing mast cells that had been previously activated by stimulation with IgE and Ag enhanced the T-cell activation induced by anti-CD3 Ab. Importantly, blocking OX40L with a neutralizing mAb significantly suppressed the mast-cell-dependent T-cell activation responses, including their proliferation and production of IFNγ and IL-17.

More recently, the interaction between mast cells and $Foxp3^+$ regulatory T (Treg) cells was demonstrated *in vitro* and *in vivo* to modulate Treg-mediated immunosuppression and Th17-associated inflammatory responses (Gri *et al.*, 2008; Piconese *et al.*, 2009). Since mast cells exist in peripheral nonlymphoid tissues, these findings indicate an important role for OX40L that is different from T-cell priming or memory T-cell generation. If OX40-expressing inflammatory T cells interact with OX40L-expressing mast cells at the inflammatory site, the T-cell-mediated inflammation might be protracted by OX40 signaling. Therefore, the OX40L expressed on mast cells might be a therapeutic target for T-cell-mediated allergic diseases.

3. OX40 IN THE FUNCTION AND DEVELOPMENT OF REGULATORY T CELLS

The OX40–OX40L interaction is involved in autoimmune and inflammatory diseases in humans and mice. One of the mechanisms of the OX40-mediated autoimmunity is a disruption of T-cell tolerance. Mounting evidence suggests that immunological self-tolerance is maintained by several distinct mechanisms. The dominant tolerance, mediated by $Foxp3^+CD25^+CD4^+$ Treg cells, is particularly critical for keeping self-reactive T cells in check to avoid lethal autoimmunity in the periphery (Sakaguchi *et al.*, 2008). The first evidence that OX40 signals could

affect the Treg-mediated tolerance was demonstrated in 2004 (Takeda *et al.*, 2004). Since that report, *in vivo* and *in vitro* studies have demonstrated that the OX40–OX40L interaction can be an important regulator of the Treg-cell subset. OX40 is constitutively expressed on $Foxp3^+CD25^+CD4^+$ T cells and is an attractive candidate for modulating immunological self-tolerance. Below, we review the relationship between OX40 and $Foxp3^+CD25^+CD4^+$ T cells.

3.1. OX40 and the homeostasis of naturally occurring regulatory T cells

$Foxp3^+CD25^+CD4^+$ naturally occurring Treg (nTreg) cells are positively selected in the thymus as a unique population with a relatively high reactivity to self-antigens (Jordan *et al.*, 2001). They suppress the activation of self-reactive T cells through TGF-β, IL-10, and unknown molecular mechanisms (Shevach, 2009). One of the characteristics of Treg cells is the expression pattern of their surface molecules; their phenotype is similar to that of effector T cells, for example, $CD25^+$, cytotoxic T-lymphocyte antigen-4 $(CTLA4)^+$, $GITR^{hi}$, $CD44^{hi}$, etc. (Itoh *et al.*, 1999; Shimizu *et al.*, 2002; Takahashi *et al.*, 2000). Accordingly, a significant amount of OX40 is also expressed on their surface (Takeda *et al.*, 2004; Valzasina *et al.*, 2005).

The interaction between OX40 and OX40L, however, appears to be dispensable for the development of $Foxp3^+CD25^+CD4^+$ nTreg cells, because this population is still present in OX40- or OX40L-deficient knockout (KO) mice (Takeda *et al.*, 2004). Rather, the OX40 signaling influences the homeostasis of this nTreg population. One paper demonstrated that the number of $CD25^+CD4^+$ nTreg cells is significantly lower in OX40-KO mice than in their wild-type littermates (Takeda *et al.*, 2004). In contrast, the forced constitutive expression of OX40L in Tg strains increases the number of nTreg cells in both the spleen and thymus. Because $Foxp3^+CD25^+CD4^+$ nTreg cells proliferate constantly in an IL-2-dependent manner in normal mice (Setoguchi *et al.*, 2005), OX40 may serve as a costimulator for this turnover. This notion is supported by a series of adoptive transfer experiments (Takeda *et al.*, 2004). The transfer of nTreg cells along with nonregulatory CD4 T cells from wild-type mice into lymphopenic RAG2-KO mice induced the homeostatic proliferation of the nTreg cells, whereas the absence of OX40L in the recipient animals diminished their proliferation, and supplementation with excess amounts of OX40L dramatically enhanced it.

The regulation of Treg cell homeostasis by other TNFR superfamily members has also been reported. For example, a CD40 deficiency causes a significant decrease in Treg cells, and the transfer of $CD4^+$ T cells from CD40-KO mice into athymic nude mice induces organ-specific autoimmune diseases, probably due to the insufficient number of Treg cells

(Kumanogoh *et al.*, 2001). In addition, the administration of an mAb against GITR, which has high homology with OX40, into normal mice enhances the proliferation of Treg cells (Nishioka *et al.*, 2008). Therefore, costimulation through TNFR superfamily molecules including OX40 can regulate the size of the $Foxp3^{+}CD25^{+}CD4^{+}$ nTreg subset by controlling their homeostatic proliferation or the thymic production of these cells.

3.2. Role of OX40 in the function of nTreg cells

The effects of OX40 signals on the nTreg-cell-mediated immune regulation have been extensively examined *in vitro* and *in vivo* (Takeda *et al.*, 2004; Valzasina *et al.*, 2005; Vu *et al.*, 2007). In *in vitro* systems in which Treg cells, naïve T cells, and APCs are cocultured in the presence of TcR stimulation, Treg cells can suppress the activation and proliferation of naïve T cells (Fig. 3.2) (Takahashi *et al.*, 1998; Thornton and Shevach, 1998). The addition of an agonistic mAb against OX40 *in vitro* elicits the activation of naïve T cells, despite the presence of Treg cells (Takeda *et al.*, 2004; Valzasina *et al.*, 2005). Similarly, APCs from an OX40L-Tg mouse, in which OX40L is ubiquitously expressed, can stimulate naïve T-cell proliferation even in the presence of Treg cells (Takeda *et al.*, 2004).

To determine which cellular components were responsible for the abrogation of the Treg cells' suppressive effect, several groups have used Treg and naïve T cells from OX40-KO and wild-type mice or rats in various combinations (Takeda *et al.*, 2004; Valzasina *et al.*, 2005). However, the results of these experiments have not been consistent. One group reported that an enhanced OX40 signal caused by cross-linking the OX40 on naïve T cells is sufficient to abrogate of the Treg-mediated suppression (Takeda *et al.*, 2004), whereas other reports showed that the OX40 on both naïve T cells and Treg cells is responsible for abrogating the suppression (Piconese *et al.*, 2008; Valzasina *et al.*, 2005; Vu *et al.*, 2007). Differences in the experimental conditions could account for the inconsistency. A similar situation has arisen, regarding the effect of GITR on Treg-mediated suppression. While one group demonstrated that the GITR signals in native T cells are responsible for the GITR-mediated abrogation of Treg suppression (Stephens *et al.*, 2004), another reported that the GITR stimulation of Treg cells, but not of naïve T cells, is essential for the GITR-mediated abrogation (Shimizu *et al.*, 2002). Because OX40 and GITR share high homology at the amino acid level, these two molecules may use common mechanisms to break the Treg-mediated immune suppression *in vitro*.

The mechanisms by which OX40 signals can render naïve T cells resistant to Treg-mediated suppression are unresolved. For the OX40 expression on naïve T cells, OX40 signaling might induce robust IL-2 production by naïve T cells at the early phase of their activation, before

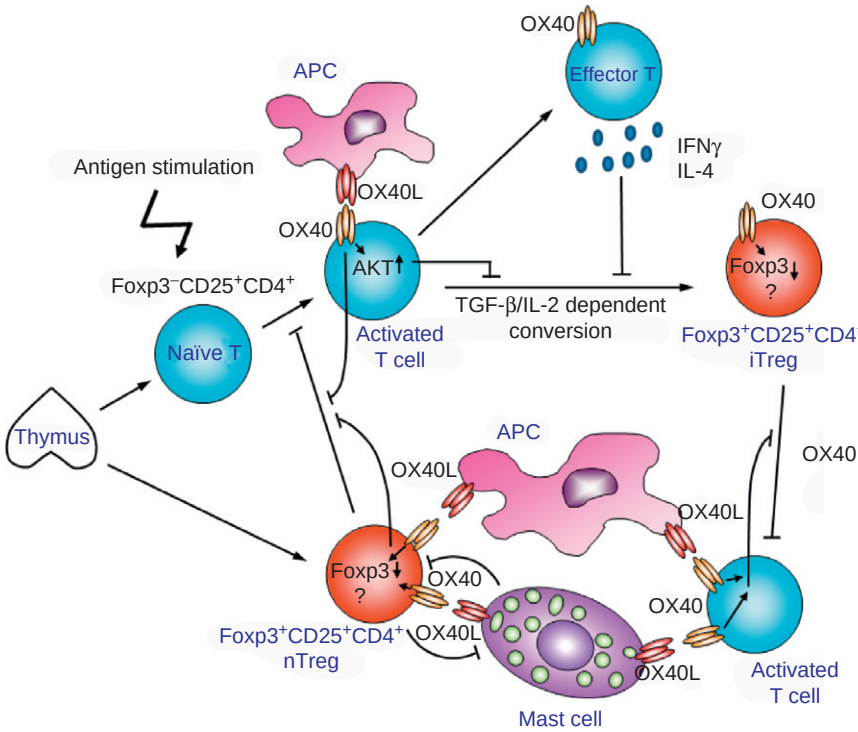

FIGURE 3.2 OX40–OX40L interaction in function and differentiation of Treg cells. Excess OX40 signals to both responder T cells and Treg cells suppress Treg-mediated immune suppression. OX40 signals into responder T cells render them resistant to Treg-mediated suppression. On the other hand, OX40 signals into Treg cells directly inhibit Treg-suppressive function although it is controversial whether OX40 signals might control the Foxp3 expression level in Treg cells. In addition, the deliberate OX40 stimulation inhibits the TGF-β-dependent differentiation of iTreg cells. The inhibition may be mediated in part by effector cytokines, such as IL-4 and IFNγ produced by effector T cells stimulated with OX40. Importantly, blocking OX40L markedly promotes iTreg differentiation and induces graft tolerance, which might be mediated by Treg cells. Therefore, OX40 is a possible molecular target for controlling T-cell-mediated autoimmunity. Furthermore, recent studies reported that the interaction between OX40L expressed by mast cells and OX40 expressed by Treg cells may mutually suppress mast-cell function and Treg cell-suppressive function (Gri *et al.*, 2008; Piconese *et al.*, 2009).

Treg cells start to exert their immune suppressive effects, thus abrogating the Treg cells' suppression. Alternatively, OX40 signals might provide survival signals to the naïve T cells that allow them to resist cytokine starvation (especially the lack of IL-2) (Scheffold *et al.*, 2005) and the consequent apoptosis, which could be caused by nTreg cells *in vitro* (Pandiyan *et al.*, 2007).

As regards signaling from OX40 on nTreg cells, one intriguing study demonstrated that the expression of Foxp3 was turned off in Treg cells when they were cultured with APCs from OX40L-Tg mice, although this strong OX40 stimulation did not influence the proliferation or survival of Treg cells (Vu *et al.*, 2007). The decrease in Foxp3 expression in the cultured Treg cells might account for the OX40-mediated abrogation of their suppressive function. A similar downregulation of Foxp3 was reported for TLR2 stimulation (Liu *et al.*, 2006). The downregulation of Foxp3 by OX40 signals, however, seems to contradict the higher frequency of Treg cells observed in OX40L-Tg mice (Takeda *et al.*, 2004). One explanation for his discrepancy is that it may arise from the artificial presence of OX40 and strong TcR stimulation *in vitro*, which does not occur normally *in vivo*, and might be necessary to elicit the disappearance of Foxp3 expression. Detailed studies exploring these molecular mechanisms are necessary. Recently, several groups reported the loss of Foxp3 expression *in vivo* using Foxp3 reporter mouse systems during inflammation in autoimmune conditions or during germinal center formation in the intestinal lymphoid tissues (Komatsu *et al.*, 2009; Tsuji *et al.*, 2009; Zhou *et al.*, 2009). According to these reports, $Foxp3^{+}CD4^{+}$ Treg cells can change their phenotype into that of pathogenic inflammatory T cells in inflamed tissues or into that of follicular helper T cells in Peyer's patches. It is possible that OX40 or TLR2 signals are involved in this process.

The role of OX40 *in vivo* has been examined in various experimental systems, including models of autoimmune disease, tumor immunity, and allergic inflammation. Inflammatory bowel disease (IBD) experimentally induced in lymphopenic mice, like RAG-2-KO mice, is frequently used as a simple model for Crohn's disease. The transfer of naïve T cells from wild-type mice into RAG-2 KO mice causes the disease in the recipient mice, and the coadministration of nTreg cells inhibits the development of the disease. One group demonstrated that strong OX40 signaling can disturb this Treg-mediated immune regulation (Takeda *et al.*, 2004). They showed that when OX40L-Tg mice on a RAG-2 KO background were used as recipients, the cotransfer of nTreg cells could not prevent the disease. This result suggests that OX40 signals can overcome the immune regulation by nTreg cells *in vivo* as well as *in vitro*.

As mentioned above, an interesting role of the OX40–OX40L interaction in the relationship between Treg cells and mast cells was reported. Gri *et al.* demonstrated that Treg cells suppress the degranulation of mast cells through the OX40–OX40L interaction *in vivo* and *in vitro*. Using an experimental system for inducing FcεRI-mediated acute-systemic-anaphylaxis in mice, which is dependent on mast cells, they compared histamine release among wild-type mice, Treg-depleted mice, and OX40-KO mice. Both Treg depletion and OX40 deficiency caused a similar, significant increase in circulating histamine, compared with its level in wild-type

mice, suggesting that Treg cells suppress the allergic reaction through an interaction between their OX40 and the OX40L expressed by mast cells (Gri *et al.*, 2008). The same group further demonstrated that, conversely, OX40L-expressing mast cells inhibited the Treg suppressive function, and that activated mast cells directly induced Treg proliferation and IL-17 production in an OX40–OX40L interaction-dependent manner (Piconese *et al.*, 2009). Thus, in addition to APCs, mast cells may play important roles in the OX40-mediated regulation of Treg cells.

3.3. Role of OX40 in tumor immunity controlled by Treg cells

This potent stimulatory effect by OX40 on not only naïve T cells but also Treg cells can enhance the immune response to tumors. In the microenvironment of tumors, the frequency of $Foxp3^{+}CD25^{+}CD4^{+}$ Treg cells is usually increased (Curiel *et al.*, 2004; Liyanage *et al.*, 2002; Wolf *et al.*, 2003; Woo *et al.*, 2001), and Treg cells often accumulate within the tumor mass. These Treg cells include nTreg cells and a group of cells called adaptive Treg or inducible Treg cells (iTreg cells). iTreg cells are induced from naïve T cells both *in vivo* and *in vitro* by exposure to TGF-β and relatively weak TcR stimulation (Apostolou and von Boehmer, 2004; Chen *et al.*, 2003; Cobbold *et al.*, 2004; Kretschmer *et al.*, 2005). Both Treg cell types hamper the development of effective tumor immunity (Ghiringhelli *et al.*, 2005; Liu *et al.*, 2007; Valzasina *et al.*, 2006; Zhou and Levitsky, 2007). Recent evidence suggests that OX40 may alter the Treg-cell-dominant environment to induce successful tumor immunity. One paper showed that the engagement of OX40 on both Treg cells and effector T cells was prerequisite for the successful eradication of a tumor, and that the generation of iTreg cells was blocked by the intratumoral injection of an agonistic anti-OX40 mAb (Piconese *et al.*, 2008). Another paper demonstrated that cyclophosphamide treatment along with the use of an agonistic OX40 Ab induced effective tumor immunity against B16 melanoma (Hirschhorn-Cymerman *et al.*, 2009). In this setting, the OX40 Ab might induce the expansion of Treg cells in the periphery, and the apoptosis of Treg cells in the tumor.

This pathway for controlling the conversion of naïve T cells into iTreg cells can explain the effective induction of robust immunity by OX40 stimulation and further indicates the usefulness of OX40 as a molecular target, particularly for tumor immune therapy. As mentioned above, the tumor environment effectively induces iTreg conversion. Piconese *et al.* (2008) demonstrated that the administration of an agonistic anti-OX40 Ab to tumor-bearing mice prevented the accumulation of iTreg cells in the tumor tissue in wild-type but not OX40-KO mice. Considering the results by Xiao *et al.*, it is possible that the OX40 signaling created a milieu abundant in IFN-γ in which iTreg cells were poorly induced, resulting in the activation of effector T cells and the subsequent eradication of the tumor.

3.4. Effects of OX40 on the development of adaptively induced Treg cells

During T-cell responses, naïve T cells can differentiate into several distinct effector lineages, such as Th1 and Th2 cells, by expressing the specific transcription factors, T-bet and GATA3, respectively, and they can become iTreg cells if Foxp3 expression is induced in them by TGF-β and weak TcR stimulation as described above. iTreg cells are generally similar to nTreg cells (Curotto de Lafaille and Lafaille, 2009). For example, they have common phenotypes with respect to their surface molecules, for example, $CD25^{+}$, CTLA-4^{+}, $GITR^{h}$, $OX40^{hi}$, etc. They cannot produce IL-2, and they proliferate poorly in response to Ag stimulation *in vitro* (Chen *et al.*, 2003). They also have immunosuppressive effects in both *in vitro* and *in vivo* experimental systems, including IBD mice (Fantini *et al.*, 2006), experimental allergy to house dust mite Ag (Chen *et al.*, 2003), and a type I diabetes model (Belghith *et al.*, 2003; Luo *et al.*, 2007).

Several groups have reported that the induction of iTreg cells is affected by costimulatory molecules, including OX40, *in vitro* (Benson *et al.*, 2007; So and Croft, 2007; Vu *et al.*, 2007; Wang *et al.*, 2008). The addition of an agonistic anti-OX40 Ab to an iTreg differentiation culture inhibits the expression of Foxp3 in the activated T cells, while the deliberate blockade of OX40 signals facilitates their differentiation (So and Croft, 2007; Vu *et al.*, 2007). Although the molecular mechanisms accounting for OX40's inhibitory effects remain to be clarified, the AKT-mTOR axis was reported to inhibit the production of iTreg cells from naïve T cells (Haxhinasto *et al.*, 2008), indicating that the activation of AKT by OX40 signaling might disturb the differentiation of iTreg cells (Song *et al.*, 2004). These costimulatory signals could eventually interfere with the transcriptional networks of NF-AT, Smad2/3, and STAT3, which are known to be essential for the *de novo* transcription of Foxp3 (Josefowicz and Rudensky, 2009).

The inhibitory effect of OX40 on the induction of iTreg cells was also confirmed in *in vivo* systems. Using a model of respiratory tolerance in which an Ag was given intranasally and the differentiation of iTreg cells was induced from naïve T cells, Duan *et al.* (2008) demonstrated that the coadministration of LPS hampered the induction of tolerance. Their detailed dissection of the mechanisms revealed that the expression of OX40L by DCs stimulated with LPS was required to suppress the development of the iTreg cells. In contrast, the administration of an anti-OX40L blocking Ab or the use of OX40-KO naïve T cells resulted in an enhanced number of iTreg cells after Ag administration (So and Croft, 2007; Vu *et al.*, 2007). An interesting indirect role of OX40 in inhibiting iTreg generation was also reported by Xiao *et al.* (2008). As mentioned above, OX40 regulates effector and memory T-cell formation. Xiao *et al.* demonstrated that IFN-γ derived from such effector or memory T cells could impede iTreg

differentiation. In contrast, Ruby *et al.* (2009) recently reported that OX40 stimulation could not suppress iTreg differentiation, but rather promoted iTreg expansion if IL-4 and IFN-γ were absent from the milieu.

3.5. Perspectives

As discussed above, substantial evidence suggests that OX40 can modulate various aspects of Treg cells: the homeostasis of nTreg cells, the effector function of nTreg cells, and the generation of iTreg cells (Fig. 3.2). In contrast to the apparent roles of OX40 in controlling nTreg function, its role in iTreg differentiation is still controversial, because of the complexity of the direct and indirect effects of OX40 on both iTreg and effector T cells. Nevertheless, by counteracting Treg-cell-mediated immune suppression, OX40 can provoke extremely efficient immune reactions, in addition to its well-known immunostimulatory effects on effector T cells. Therefore, OX40 is an attractive and promising molecular target for controlling immune responses. Further understanding of OX40's functions may lead to the development of effective clinical therapies.

4. OX40–OX40L INTERACTION IN THE GENERATION AND HOMEOSTASIS OF MEMORY T CELLS

The T-cell response to any acute antigenic stimulation has three distinct phases: expansion, contraction, and memory generation. Upon Ag stimulation, Ag-specific naïve T cells, which exist at a very low frequency, proliferate and differentiate into a large population of heterogeneous effector T cells (Blattman *et al.*, 2002; Moon *et al.*, 2007; Obar *et al.*, 2008; Obst *et al.*, 2005). After Ag removal, most of the Ag-specific T cells become senescent, terminally differentiated T cells that die by apoptosis, to prevent the potential risk for immunopathology, but a subset of effector T cells, which later differentiate into memory T cells, survives to confer protective immunity on the host (Ahmed and Gray, 1996; Dutton *et al.*, 1998). Although CD4 and CD8 T cells share common differentiation programs in response to acute Ag stimulation for their expansion, contraction, and differentiation into memory T cells, the efficiency and longevity of the memory T cells they generate are different (Seder and Ahmed, 2003). During acute viral infection, the magnitude of the expansion and consequent generation of memory T cells is much greater for the CD8 than the CD4 T cells (Homann *et al.*, 2001). Although it has been suggested that the initial CD8 T-cell responses and establishment of CD8 memory T cells are less dependent on costimulatory signals than the CD4 T-cell responses (Whitmire and Ahmed, 2001), recent studies clearly demonstrated that several members of the TNF receptor superfamily can costimulate CD8

T cells as well as CD4 T cells to generate memory T-cells. Among them, CD27, OX40 (CD134), and 4-1BB (CD137) can greatly impact the generation of memory CD4 and CD8 T cells by promoting the survival of effector T cells (Borst *et al.*, 2005; Croft, 2003; Sabbagh *et al.*, 2007; Salek-Ardakani and Croft, 2006). This part of the review focuses on the functional roles of the OX40 on T cells in the development, maintenance, and function of memory CD4 and CD8 T cells.

4.1. Role of OX40 in the generation and reactivation of memory CD4 T cells

OX40 promotes the expansion and survival of effector T cells. Since the surviving effector T cells are thought to become memory T cells, a larger pool of expanded effector T cells may directly lead to a larger pool of memory T cells. In fact, the $CD44^{hi}CD62L^{lo}$ effector memory population of polyclonal CD4 T cells in OX40L-KO mice is significantly smaller than that in wild-type mice (Murata *et al.*, 2002; Soroosh *et al.*, 2007). In contrast, OX40L-Tg mice in which constitutive OX40 signals are provided to $OX40^{+}$ effector T cells demonstrated a marked increase in effector memory CD4 T cells (Murata *et al.*, 2002). Similar to this result, the frequency of $CD44^{hi}CD62L^{lo}$ effector memory CD4 T cells was markedly increased when Ag-naïve mice were treated with an agonistic anti-OX40 mAb for long time (Soroosh and Ishii, unpublished observations). Interestingly, the $CD44^{hi}CD62L^{lo}$ effector memory population of polyclonal CD8 T cells in OX40L-Tg mice or mice treated with an agonistic anti-OX40 mAb is comparable to that in wild-type mice (Murata *et al.*, 2002; Ishii, unpublished observations). In a polyclonal T-cell system, OX40 promoted the generation of CD4 memory T cells preferentially over CD8 memory T cells, probably because the OX40 expression on CD8 T cells is much more transient than that on CD4 T cells (Croft *et al.*, 2009). Additional *in vitro* and *in vivo* studies using naïve TcR Tg CD4 T cells demonstrated that upon Ag stimulation, OX40-KO CD4 T cells divide normally and accumulate in the early priming phase. However, the OX40-deficient CD4 T cells are unable to maintain high levels of antiapoptotic molecules such as Bcl-2, Bcl-xL, and survivin (Rogers *et al.*, 2001; Song *et al.*, 2004, 2005), and consequently, the majority of them do not survive for long (Song *et al.*, 2004, 2005; Soroosh *et al.*, 2007). Collectively, these findings indicate that OX40 signals promote the survival of effector CD4 T cells, leading to the optimal generation of memory CD4 T cells.

Similar to the activation-induced OX40 expression on naïve T cells, OX40 is transiently expressed by memory T cells upon their restimulation with Ag, although the resting memory T cells do not express OX40 (Soroosh *et al.*, 2007). Therefore, the role of OX40 in the reactivation of memory T cells has also been examined. One group demonstrated in a

murine model of asthma that treatment with a blocking anti-OX40L mAb at the time of Ag rechallenge completely suppressed memory T-cell expansion and lung inflammation (Salek-Ardakani *et al.*, 2003). Since lung inflammation is considered to be mediated by the effector function of reactivated Ag-sensitized memory CD4 T cells, this result indicates that the blockade of OX40 signals at rechallenge is suppressive for the reactivation of memory CD4 T cells. However, the mechanism for the OX40-mediated reactivation of memory CD4 T cells, including whether or not it was directly induced by signaling through OX40 expressed on the reactivated memory CD4 T cells, was unclear.

In addressing this question, our recent study led us to propose a new scenario for the role of OX40 in the reactivation of memory T cells by Ag rechallenge. In an asthma model, the adoptive transfer of memory CD4 T cells from Ag-sensitized mice, but not naïve CD4 T cells, efficiently induced Ag-specific airway inflammation in the recipient mice upon Ag inhalation. Thus, this model is dependent on the Ag-specific reactivation of memory CD4 T cells. However, if the recipient mice lacked OX40 or NKT cells, Ag-inhalation failed to provoke the lung inflammation (Damayanti *et al.*, 2009). To investigate the effect of OX40 on NKT cells in the challenge phase, Ag-sensitized NKT-deficient mice that had received memory CD4 T cells were reconstituted with naive NKT cells before Ag inhalation. The reconstitution of the NKT-deficient mice with naïve NKT cells from wild-type mice completely restored the airway inflammation upon Ag challenge. By contrast, the transfer of NKT cells from OX40-KO mice did not restore the allergic airway response. Thus, OX40 expression by naïve NKT cells is required for the airway inflammation that is mediated by reactivated Ag-specific memory CD4 T cells. In addition, OX40 expression on NKT cells and OX40L expression on MHC class II^+ cells were found in the lung tissues when Ag was inhaled, suggesting a possible role of the OX40–OX40L system during NKT-cell–APCs interactions. Although this study did not address the role of OX40 on memory CD4 T cells in inducing lung inflammation, it showed that reactivation of the Ag-sensitized memory CD4 T cells requires the help of NKT cells that receive OX40 signals.

4.2. Differential requirement for OX40 in the generation of heterogeneous CD4 memory T cells

To understand how memory T cells develop, the lineage relationship among naïve T cells, effector T cells, and memory T cells still needs to be clarified. Although some studies support a linear model of memory T-cell differentiation, in which memory cells are derived directly from homogeneous effector cells, other data suggest that memory T cells are heterogeneous and generated by alternate pathways (Ahmed *et al.*, 2009;

Surh and Sprent, 2008). Recent studies have shown that, similar to $CD8^+$ memory T cells, $CD4^+$ memory T cells are heterogeneous and can be categorized into at least two subsets, effector memory (Tem) and central memory (Tcm) T cells, based on their phenotype, function, and anatomic distribution (Ahmadzadeh *et al.*, 2001; Masopust *et al.*, 2001; Reinhardt *et al.*, 2001; Sallusto *et al.*, 1999). Tem ($CD44^{hi}CD62L^{lo}CCR7^-$) cells reside in both lymphoid and nonlymphoid tissues, where they initiate protection immediately by producing high levels of effector cytokines at the site of the Ag encounter, although they have little proliferative activity. Therefore, Tem cells may also mediate organ-specific autoimmune and inflammatory responses. In contrast, Tcm cells ($CD44^{hi}CD62L^{hi}CCR7^+$), which lack full effector function, mainly localize to the secondary lymphoid tissues, where they mediate long-lasting protection through self-renewing cell expansion, and thus play important roles in protection from systemic infection (Ahmed *et al.*, 2009).

The development of TCR-specific MHC class I/peptide-tetramers has been a powerful tool for detecting and quantifying endogenous memory CD8 T cells, and their use has provided considerable information on the generation of the two subsets of memory CD8 T cells. However, because MHC class II/peptide-tetramer reagents are not widely available, it is still unclear how and when CD4 memory T cells are generated. In this context, understanding the mechanisms for the generation and maintenance of the two different subsets of memory CD4 T cells is critical, not only for our basic knowledge about memory T-cell biology but also for clinical applications, such as designing novel vaccines and new therapeutics against infections and autoimmune diseases.

Our recent studies using the adoptive transfer of OVA-specific TcR Tg OX40-KO T cells following *in vivo* stimulation with cognate Ag demonstrated that the absence of OX40 selectively reduces the generation of CD4 Tem cells but not Tcm cells in both lymphoid and nonlymphoid tissues. Furthermore, when Ag-primed TcR Tg cells that were generated during a 5-day *in vitro* culture in the presence of a blocking anti-OX40L mAb were transferred into untreated naive wild-type mice, the generation of the Tem-cell population was prevented, whereas the Tcm cells developed normally. Therefore, one of the major roles of OX40 signaling may be to imprint signals for persistent survival on the Tem precursors, for the optimal development of Tem cells (Soroosh *et al.*, 2007). This scenario of OX40-mediated imprinting is discussed in the section below on CD8 memory T cells.

The significant effect of OX40 signals during priming suggests that effective OX40 signals may be provided before or during the Tem/Tcm commitment, and that they preferentially induce the generation of Tem cells. This raises the question of whether OX40 might contribute to the Tem/Tcm commitment of effector CD4 T cells. To explain the process by

which the two subsets of memory T cells develop, at least three different models have been proposed (Ahmed *et al.*, 2009). In the linear differentiation model, naïve T cells first differentiate into Tem cells, which then give rise to Tcm cells. In the bifurcative model, following Ag recognition, one T cell can give rise to two daughter cells; the distal daughter cell, which leaves the APCs earlier, gives rise to Tcm cells, and the proximal daughter cell, which remains attached to the APCs longer, gives rise to Tem cells. In the third model, the self-renewing effector model, a naïve T cell can develop into a Tcm cell that can home to lymphoid tissues, where the Tcm cells give rise to Tem cells that can migrate to sites of inflammation (Ahmed *et al.*, 2009).

According to the first model (linear differentiation model), an increase in the Tem population should lead to an increase in the Tcm population; therefore, the OX40-mediated selective increase in the Tem population seems incompatible with this scenario. Among the three proposed models, the bifurcative differentiation model appears to best fit the preferential Tem generation by OX40. In this model, the asymmetrical division of a naïve T cell during T cell–APC contact yields Tem and Tcm progeny at the same time (Chang *et al.*, 2007). Thus, it is possible that the less-differentiated Tcm precursors that originate from the distal daughter cells never interact with the OX40L on the APCs, because OX40L is not expressed by APCs immediately after the T-cell–APC interaction starts. After Ag removal, the Tcm cells further undergo self-renewal proliferation; this process is highly dependent on homeostatic cytokines, which robustly promote the generation of Tcm cells, whether or not the Tcm precursor cells receive OX40 signals. In contrast, Tem precursor cells that were derived from the proximal daughter cell, which remained attached to the APC when the APC started to express OX40L, might proliferate in an OX40-dependent manner. If this scenario is true, the OX40–OX40L interaction might promote the survival of Tem precursor cells, but not contribute to the Tem/Tcm commitment.

Recently, the frequency of Tcm precursors generated during priming was shown to be closely correlated with the initial numbers of naïve T cells (Sarkar *et al.*, 2007). Therefore, when using the adoptive transfer of naïve T cells to examine the generation of memory T cells, the number of naïve CD4 T cells transferred might affect the result. Indeed, we found that if a larger number ($>10^7$) of naïve CD4 TcR Tg T cells were transferred, the immunization with a specific Ag induced a sizable Tcm cell pool (Ishii and Soroosh, unpublished observations). In contrast, when naïve TcR Tg CD4 T cells were transferred at physiologically appropriate numbers ($\simeq 10^4$), they mainly developed into $CD44^{hi}CD62L^{lo}$ Tem phenotypic cells, rather than $CD44^{hi}CD62L^{hi}$ Tcm cells, following Ag immunization, and they maintained the $CD44^{hi}CD62L^{lo}$ phenotype throughout the memory phase. Nevertheless, in both cases, the frequency

of Ag-specific $CD62L^{lo}$ cells (phenotypical Tem cells) was severely reduced by the absence of OX40 signals (Ishii and Soroosh, unpublished observation). In these contexts, in spite of the important effect of the initial number of naïve T cells on the differential generation of $CD4^+$ Tem and Tcm cells, OX40 is a strong modulator for increasing the frequency Tem cells, probably through the expansion and survival of Tem precursors beyond the contraction phase.

4.3. OX40 potentiates the generation and maintenance of memory CD8 T cells

Reports on murine models of viral infection have suggested that OX40 is dispensable for CD8 T-cell responses, in terms of the expansion of effector T cells, the generation of cytotoxic T cells, and viral clearance (Dawicki *et al.*, 2004; Hendriks *et al.*, 2005; Kopf *et al.*, 1999; Pippig *et al.*, 1999). However, in noninfectious models, such as contact hypersensitivity and allogeneic responses, the CD8 T-cell responses are reportedly impaired when the OX40 signals were absent (Chen *et al.*, 1999; Murata *et al.*, 2000; Sato *et al.*, 2002). OX40 was also shown to directly control primary and secondary CD8 T-cell responses to Ag when adjuvant was used, or when the Ag was expressed by tumor cells (Bansal-Pakala *et al.*, 2004; Song *et al.*, 2005). Under more physiological conditions using acute viral infection, several studies reported that a lack of OX40 signals diminishes the formation of viral Ag-specific memory CD8 T cells (Hendriks *et al.*, 2005; Humphreys *et al.*, 2007; Salek-Ardakani *et al.*, 2008). Hendriks *et al.* demonstrated, using an influenza virus infection model, that the absence of OX40 signals diminished the formation of viral Ag-specific memory CD8 T cells in both lymphoid and nonlymphoid tissues without affecting the primary expansion of CD8 T cells (Hendriks *et al.*, 2005). However, in a murine model of vaccinia virus infection, OX40 critically promoted the early expansion and subsequently impacted the generation of virus-specific memory CD8 T cells (Salek-Ardakani *et al.*, 2008). Thus, the mechanism by which OX40 contributes to the early expansion of CD8 T cells appears to be different depending on the experimental models used.

Interestingly, Humphreys *et al.* demonstrated that, among several epitopes of murine cytomegalovirus (MCMV), including M45, M57, m139, and M38, the expansion of CD8 T cells specific for M45, M57, or m139 was not affected by the absence of OX40, whereas the primary accumulation of M38-specific CD8 T cells was significantly impaired by the OX40 deficiency, in mice infected with MCMV (Humphreys *et al.*, 2007). Since these CD8 T-cell repertoires were activated to similar extents under the same conditions, the impact of OX40 might vary depending on each repertoire, which has an intrinsic affinity for the Ag/MHC complex. Thus, the strength of the TcR signals in each T cell might affect

its requirement for OX40 signals for its early proliferation. This notion may be supported by *in vitro* studies, in which T cells were activated with a different strength of Ag stimulation. For example, when T cells derived from TcR-Tg mice were stimulated with an Ag-peptide that had a high affinity for TcR (i.e., causing strong TcR signals), OX40 signals were not essential for the early proliferation and expansion of Ag-specific T cells *in vitro* (Rogers *et al.*, 2001; Song *et al.*, 2004, 2005; Soroosh *et al.*, 2006, 2007). In contrast, when the T cells were suboptimally activated with Ag (when polyclonal T cells were stimulated with anti-CD3 mAb or when TcR Tg T cells were stimulated with an Ag-peptide that had a low affinity for TcR), OX40 deficiency markedly suppressed the early proliferation and expansion of T cells (Murata *et al.*, 2000; Pippig *et al.*, 1999; Soroosh and Ishii, unpublished data). In this context, it is possible that strong TcR signals compensate for the lack of OX40 signals in the early proliferative response by T cells. Nevertheless, OX40 signals are essential for the survival of effector CD8 T cells, which leads to the optimal generation of CD8 memory T cells.

When using OX40 or OX40L-deficient mice are used to examine memory CD8 T cells, the CD4 T cells in these mice never receive OX40 signals either. Therefore, whether the effect of OX40 on memory generation and secondary responsiveness impacts CD8 T cells directly or affects them indirectly through CD4 T cells was an important question. To dissect the precise and exclusive role of OX40 in the development and maintenance of memory CD8 T cells, we performed several types of adoptive transfer experiments with OVA-specific TcR-Tg CD8 T cells following immunization with recombinant *Listeria monocytogenes* expressing OVA (r*LM*-OVA) as an acute bacterial infection.

First, to examine the direct effect of OX40 expressed on CD8 T cells, wild-type recipient mice transferred with OX40-deficient TcR Tg naive CD8 T cells were infected with r*LM*-OVA. The results showed that OX40 had only a minimal role in the early accumulation and effector function of Ag-specific CD8 T cells, confirming the redundant role of OX40 signaling during the effector phase, as previously shown. However, the absence of OX40 receptor on donor CD8 T cells in wild-type recipients, which expressed OX40 at normal levels on their other immune cells, severely reduced the number of memory CD8 T cells derived from the donor cells. This indicated that the OX40 expressed on CD8 T cells plays a critical role in the development of memory CD8 T cells (Mousavi *et al.*, 2008). This conclusion is supported by another study in a model of vaccinia virus infection (Salek-Ardakani *et al.*, 2008).

With regard to the precursor cells for memory CD8 T cells, Kaech's group demonstrated that the expression profiles of the IL-7 receptor α chain (IL-7R) and killer cell lectin-like receptor G1 (KLRG1) are useful for distinguishing between short-lived effector T cells (SLECs; IL-7R^{lo}

KLRG1^{hi}) and the memory precursor effector T cells (MPECs; IL-7R^{hi} KLRG1^{lo}) (Joshi *et al.*, 2007). Using these markers in the above bacterial infection model, we found that the targeted expression of OX40 in Ag-specific CD8 T cells significantly decreased the frequency of IL7R^{hi}KLRG1^{lo} MPECs during the effector phase, which was followed by a reduction in memory T cells. Furthermore, when MPECs derived from OX40-deficient effector cells generated in a wild-type recipient were transferred into a second host, the OX40-KO MPECs could neither survive nor differentiate into memory T cells, although MPECs from wild-type donor cells could (Mousavi *et al.*, 2008). These results, similar to the OX40-mediated survival of effector CD4 T cells, indicated that signals through the OX40 expressed by CD8 T cells play an important role in the survival of effector CD8 T cells including MPECs, leading to the optimal generation of memory CD8 T cells.

One of the most important characteristics of memory CD8 T cells is that they persist for a long time. Recent observations have demonstrated that an Ag-independent self-renewal process called basal homeostatic proliferation, which is considered to be mainly driven by IL-7 and IL-15, contributes to the longevity of memory T cells (Becker *et al.*, 2002; Goldrath *et al.*, 2002). In an adoptive transfer experiment, OVA-specific memory CD8 T cells that had been generated in the absence of OX40 signals were unable to self-renew in a second host in spite of the normal expression levels of the IL-7R, IL-2/15 receptor β chain, and γc chain on these memory T cells (Mousavi *et al.*, 2008). In other words, the blockade of the OX40 signals during priming only (by treatment with a blocking anti-OX40L mAb for 7 days after infection) was sufficient to suppress the future self-renewal ability of memory CD8 T cells (Mousavi *et al.*, 2008). Thus, the self-renewal program of memory CD8 T cells appears to be imprinted by OX40 signals onto the KLRG-1^{lo} memory precursors during priming. As mentioned above, the blockade of the OX40 signals in effector CD4 T cells during priming was also sufficient to suppress the generation of CD4 Tem cells (Soroosh *et al.*, 2007). Similarly, Borst's group observed that when Ag-specific memory CD8 T cells generated in the absence of OX40 signals during a primary infection with influenza virus were transferred into a wild-type host, the memory cells could not expand secondarily in response to infection in the host (Hendriks *et al.*, 2005). Thus, in a different infection model, the OX40–OX40L interaction may program CD8 T cells during priming for their future capacity to respond secondarily as memory CD8 T cells (Fig. 3.3). Since memory CD8 T cells that developed in the absence of OX40 signals exhibit cell-cycle arrest during basal homeostatic proliferation, which is mainly dependent on IL-7 and IL-15, it is possible that OX40 endows memory precursor cells with their constitutive responsiveness to these γc cytokines through an epigenetic change. Future analyses using genomic profiling in memory

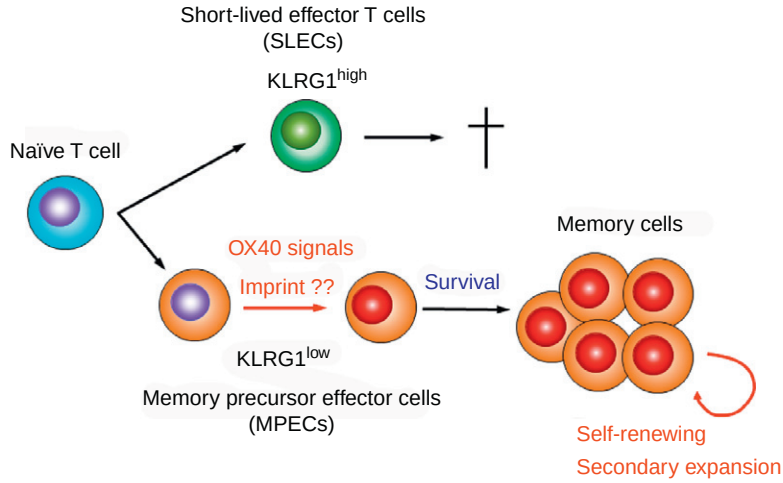

FIGURE 3.3 Hypothetic role of OX40 on the generation of memory CD8 T cells. When Ag-specific memory CD8 T cells generated in the absence of OX40 signals during a primary infection were transferred into wild-type host, the memory cells could not expand secondarily in response to infection in the host (Hendriks *et al.*, 2005) and failed to self-renew (Mousavi *et al.*, 2008). Therefore, the OX40–OX40L interaction may program activated CD8 T cells (probably KLRG1^{lo} MPECs) during priming for their future capacity as memory CD8 T cells. Since OX40-mediated immediate signals, such as transient NFκB activation, cannot explain these phenomena, OX40 stimulation during priming might induce an epigenetic change that destines memory T cells to functionally survive for a long time.

precursor cells generated in the presence and absence of OX40 signals should provide important insight, which could further our understanding of the molecular mechanisms of memory T-cell generation.

5. THE OX40 AND OX40L INTERACTION IN DISEASE

Associations between the OX40–OX40L interaction and disease have been intensely studied using several animal models. The first evidence that OX40 might be involved in the development of an immune-associated disease was obtained in a rat model of experimental autoimmune encephalomyelitis (EAE) (Weinberg *et al.*, 1994). Autoantigen-specific CD4 T cells isolated from the site of inflammation expressed high levels of OX40 (Weinberg *et al.*, 1994), whereas T cells isolated from the peripheral blood and spleen of the same animal expressed low levels of OX40. The same group also provided the first evidence that OX40 could be a therapeutic target for the autoimmune disease in the EAE model, in which

the depletion of $OX40^+$ T cells effectively suppressed the disease symptoms (Weinberg *et al.*, 1996). Since these reports, several studies have suggested that the OX40–OX40L interaction is involved in the development of EAE, IBD, graft versus host disease (GVHD), contact hypersensitivity, asthmatic airway hyperreaction, autoimmune diabetes, and atherosclerosis in mice, and rheumatoid arthritis, multiple sclerosis, Crohn's disease, ulcerative colitis, GVHD, and polymyositis in humans (Croft *et al.*, 2009; Sugamura *et al.*, 2004).

As discussed above, at least two mechanisms involving the OX40–OX40L system may mediate the development of the above diseases. One is the OX40-mediated break in T-cell tolerance caused by inhibiting Treg-cell function. Another is the sustained inflammatory response caused by the unwanted survival of effector T cells whose persistence is caused by OX40 signals. Therefore, the deliberate blockade of the OX40–OX40L interaction by using a blocking anti-OX40L mAb or OX40:immunoglobulin (OX40:Ig) could be a promising therapy for these diseases. Previous review articles have described well the possible therapeutic effect of targeting OX40L in each of these diseases (Croft *et al.*, 2009; Sugamura *et al.*, 2004), and therefore in this section we will discuss the general advantages of immunotherapy targeting OX40L, and the recent identification of polymorphisms in the OX40 and OX40L genes related to human autoimmune and inflammatory diseases.

5.1. Advantages of immunotherapy targeting OX40

Conventional immunotherapies using immunosuppressants, such as cyclosporine, tacrolimus, and methotrexate for autoimmune inflammatory diseases and organ transplantation are aimed at pathogenic T cells. However, these therapies suppress not only the functions of the T cells responsible for disease but also all the functions of the other T cells in the host. Therefore, the induced immunosuppressive state in the patient often results in the development of intractable infections, and sometimes leads to the lethal consequences of severe infections and cancer. Although second-generation immunotherapies targeting inflammatory cytokines, such as TNFα and IL-6, that are responsible for disease pathogenesis have been developed, the complications of pan-immunosuppression have not yet been resolved. In contrast, the deliberate blockade of the OX40–OX40L interaction can specifically suppress recently activated pathogenic T cells, because only activated T cells, and not resting T cells (naïve T cells and memory T cells), express OX40. Indeed, several studies in animal models of disease indicate that the blockade of the OX40–OX40L pathway is effective for tempering autoimmune, allergic, and inflammatory diseases, and for reducing tissue inflammation even during viral infection, without global immunosuppression (Croft *et al.*,

2009; Sugamura *et al.*, 2004). A study in an influenza model of lung inflammation provides convincing evidence of the relatively mild immunosuppression by OX40L blockade compared with that from an overall immunosuppressive regimen. The administration of OX40:Ig, which binds to OX40L and neutralizes OX40L function, efficiently decreased lung inflammation by suppressing effector CD4 T cells, without causing any unwanted effects on either the generation of Abs against the virus or the cytotoxicity of the killer T cells (Humphreys *et al.*, 2003).

Apart from suppressing effector T cells, the blockade of OX40L could also be therapeutic for several diseases by suppressing autoimmune responses and preventing the rejection of transplanted organs. The *in vitro* blockade of OX40L efficiently promotes the differentiation and function of iTreg cells (Vu *et al.*, 2007). Thus, if an artificial increase in Treg cells might induce $Foxp3^+$ iTreg-mediated T-cell tolerance, an established autoimmune response would be suppressed by the reinduced tolerance. Indeed, a recent study in an allo-transplantation model demonstrated that the administration of a blocking anti-OX40L mAb to the host induced long-term tolerance to an islet allograft (Chen *et al.*, 2008), although the details of the Treg cells in the host were not examined. Therefore, in addition to suppressing effector T-cell function, OX40L blockade could have a favorable effect by enhancing Treg-cell function. Since both the $OX40^+$ pathogenic effector T cells and iTreg cells are considered to be Ag-specific, the targeting of OX40L might modulate only the autoantigen-specific T-cell repertoire, without disrupting the function of the other T-cell repertoires, resulting in many fewer immunosuppressive side effects than are seen with the second-generation immunotherapies. Targeting OX40L would thus be a third-generation immunotherapy.

5.2. Genetic evidence for the involvement of the OX40–OX40L interaction in human disease

Recent gene association studies for human diseases have identified multiple genetic factors related to autoimmune and inflammatory diseases. Among them, accumulating evidence has demonstrated that polymorphisms of the OX40L and OX40 genes may be risk factors for several inflammatory diseases, including systemic lupus erythematosus (SLE) (Chang *et al.*, 2009; Cunninghame Graham *et al.*, 2008; Delgado-Vega *et al.*, 2009), atherosclerosis (Wang *et al.*, 2005), and age-dependent hypertension (Mashimo *et al.*, 2008).

The first genetic evidence implicating OX40L in human disease was obtained in a study on atherosclerosis. Atherosclerosis is an inflammatory disease in which immune mechanisms are involved. Wang *et al.* first identified the OX40L gene as a susceptible gene for atherosclerosis in mice by a conventional positional cloning method (Wang *et al.*, 2005).

In this study, OX40L-deficient mice were more resistant to diet-induced atherosclerosis, while OX40L-Tg mice, in which OX40L was ubiquitously expressed, showed a higher susceptibility to this disease compared to wild-type mice. Importantly, the same report demonstrated that the less common allele of a single nucleotide polymorphism (SNP) in the OX40L gene was significantly more frequent in patients with myocardial infarction, which is mainly caused by atherosclerosis, than in control individuals in Swedish populations (Wang *et al.*, 2005). Therefore, polymorphisms in the OX40L gene also increase the risk of atherosclerosis in humans. Based on the results from the mouse model, the indentified polymorphisms might cause an increase in OX40L expression. Since human umbilical vein endothelial cells can express OX40L *in vitro* (Imura *et al.*, 1996; Kunitomi *et al.*, 2000), the OX40L expressed on endothelial cells might interact with inflammatory effector T cells that express OX40, to induce T cell-mediated inflammatory responses on blood vessels. In support of this possibility, genetic variants in OX40 are associated with myocardial infarction and essential hypertension in Swedish and Japanese people, respectively (Mashimo *et al.*, 2008; Ria *et al.*, 2006). The identified SNPs in the OX40 gene might also affect the OX40–OX40L interaction in vascular homeostasis. Interestingly, the treatment of low-density lipoprotein receptor-deficient mice with an anti-OX40L antibody led to a 50% decrease in the formation of atherosclerotic lesions via the suppression of Th2-mediated inflammation in diet-induced atherosclerosis (van Wanrooij *et al.*, 2007). Therefore, the OX40–OX40L pathway may be an excellent target for atherosclerosis therapy.

OX40L polymorphisms have also been found in SLE. SLE is a chronic autoimmune disease characterized by the production of anti-double stranded DNA (dsDNA) auto-Ab. In patients with SLE in the UK and USA, a single risk haplotype for SLE, located in the upstream region of the OX40L gene, was demonstrated (Cunninghame Graham *et al.*, 2008). Furthermore, the association of OX40L haplotypes with SLE was observed in people from Germany, Italy, Argentina, and China in two recent studies (Chang *et al.*, 2009; Delgado-Vega *et al.*, 2009). The same SNP is correlated with an increased expression of both cell-surface OX40L and the OX40L transcript (Cunninghame Graham *et al.*, 2008). The increased expression of OX40L may predispose an individual to SLE either by quantitatively augmenting the T-cell–APC interaction or by influencing the functional consequences of T-cell activation via OX40. Although OX40 plays a dominant role in T-cell function, rather than in B-cell function, T-cell-derived cytokines may direct B cells to produce abnormal Ab levels. This notion is supported by the observation that OX40L-Tg mice have a high level of serum autoAb against dsDNA accompanied by an aberrant increase in Th2 responses (Ishii *et al.*, 2003; Murata *et al.*, 2002). The interruption of OX40L binding in OX40L-Tg mice

by using an anti-OX40L mAb successfully rescued the pathogenic Th2 response (Ishii *et al.*, 2003). This finding suggests that a therapeutic strategy for SLE that targets OX40L may be very beneficial.

These findings support the notion that ectopic or increased OX40L expression, which may induce an adverse OX40 signal, might cause inflammatory and autoimmune diseases in humans, and shed light on the possibility of clinical interventions targeting the OX40–OX40L system.

6. SUMMARY AND FUTURE PERSPECTIVES

The OX40–OX40L interaction promotes effector T-cell survival and effectively induces memory T-cell generation in a unique way. On the other hand, excess OX40 signaling inhibits the suppressive function and differentiation of Treg cells, leading to a break in T-cell tolerance. Both the enhanced effector T-cell function and the breaking of T-cell tolerance are implicated in the development of inflammatory and allergic diseases. Indeed, recent evidence from genetic studies implicates OX40L in human cardiovascular disease and SLE. An anti-OX40L blocking mAb and OX40:Ig, both of which can neutralize OX40L function, are powerful immune modulators and promising therapeutic molecules for these diseases. One emerging theme of this therapeutic approach is that pathogenic effector T cell responses and regulatory T cells are simultaneously influenced by the effects of OX40, the effector T cells by its costimulatory effects, and the regulatory T cells by its coinhibitory effects. Thus, choosing the optimal timing for blocking OX40L could improve the treatment strategy, in which a primary blockade of OX40L might suppress ongoing inflammation, and a secondary treatment might reinduce Ag-specific tolerance with minimal side effects due to immunosuppression. We hope that this attractive therapeutic approach targeting OX40L will be developed in the near future, accompanied by a more detailed understanding of the OX40–OX40L system.

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CHAPTER 4

The Family of IL-10-Secreting $CD4^+$ T Cells

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Contents

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Abstract

Regulatory T cells (Treg cells) play critical roles in the induction of peripheral tolerance to self- and foreign antigens. Naturally occurring $CD4^+CD25^+$ Treg cells, which characteristically express the transcription factor forkhead box protein P3 (Foxp3), have been studied intensively because their deficiency abrogates self-tolerance and causes autoimmune disease. However, several lines of evidence suggest that additional important mechanisms other than the Foxp3 system are required to enforce immunological self-tolerance in the periphery. Interleukin-10 (IL-10) is a regulatory cytokine that plays a central role in controlling inflammatory processes, and IL-10-secreting T cells may constitute an additional mechanism that are responsible for peripheral tolerance. Type-1 T regulatory (Tr1) cells, CD46-stimulated IL-10-secreting T cells, and IL-10-secreting T cells induced by vitamin D3 (VitD3) and dexamethasone (Dex) are induced populations with significant regulatory activities. However, assessing the detailed physiological function of these cells is difficult, because of the lack of specific markers that can reliably differentiate the population of IL-10-secreting Treg cells from other T cells. Recently, $CD4^+CD25^-LAP^+$ T cells, $CD4^+NKG2D^+$ T cells, $CD4^+IL\text{-}7R^-$ T cells, and $CD4^+CD25^-LAG3^+$ T cells have been reported as naturally present IL-10-secreting Treg cells. Although the relationship between these induced and naturally present IL-10-secreting Treg cells is unclear, elucidation of their respective roles in modulating immune responses is crucial to understand T cell-mediated tolerance. Furthermore, the identification of specific markers and molecular signatures will enable the purification or induction of IL-10-secreting Treg cells for the treatment of patients having inflammatory diseases.

1. INTRODUCTION: THE ROLE OF IL-10 IN CONTROLLING INFLAMMATION

Interleukin-10 (IL-10) was first reported as a soluble factor, cytokine synthesis inhibitory factor (CSIF), produced by murine Th2 cells capable of inhibiting activation and cytokine production in Th1 cells (Moore *et al.*, 1990). Human IL-10 was cloned from a T cell line derived from a severe combined immunodeficient patient who had developed long-term tolerance to a stem-cell allograft (Vieira *et al.*, 1991). A number of subsequent reports revealed that IL-10 is a suppressive cytokine that plays a central role in controlling inflammatory processes. Human IL-10 suppresses the expression of MHC class II, costimulatory and adhesion molecules

(de Waal Malefyt *et al.*, 1991; Willems *et al.*, 1994). IL-10 also inhibits the production of inflammatory cytokines and the T cell-stimulating capacity of antigen-presenting cells (APCs) (Allavena *et al.*, 1998; Fiorentino *et al.*, 1991a). Moreover, IL-10 regulates T cells by inhibiting their ability to produce cytokines (de Waal Malefyt *et al.*, 1993) and to proliferate (Taga *et al.*, 1993). IL-10 is actually produced by many immunological cell types, including T cells, B cells, mast cells, eosinophils, macrophages, and dendritic cells (DCs) (O'Garra *et al.*, 2008).

The impact of the suppressive function of IL-10 was highlighted by IL-10-deficient (IL-10 $^{/}$) mice, which developed severe colitis in association with commensal bacteria in the gut (Kuhn *et al.*, 1993; Sellon *et al.*, 1998). In contrast, transgenic mice expressing IL-10 under the control of MHC class II promoter were highly susceptible to infections, and were not able to mount effective Th1 or Th2 responses (Rouleau *et al.*, 1999). The colitis in IL-10 $^{/}$ mice was reported to be mediated by Th1 cells (Davidson *et al.*, 1996). In another murine model of colitis in SCID mice, cotransfer of pathogenic CD4$^+$CD45RB^{high} T cells together with a CD45RB^{low} fraction prevented the induction of disease (Powrie, 1995). However, CD45RB^{low} cells from IL-10 $^{/}$ mice were just as pathogenic as CD45RB^{high} cells (Powrie and Leach, 1995). This fact implied that T cells with regulatory activity fail to develop or function in the absence of IL-10 (Rennick *et al.*, 1997).

In 1998, Groux *et al.* demonstrated that *ex vivo* activation of human or murine CD4$^+$ T cells in the presence of high doses of exogenous IL-10 resulted in the generation of T cell clones producing IL-10 and TGF-β (Groux *et al.*, 1997). These T cell clones inhibited antigen-specific activation of autologous T cells and colitis development via IL-10, and were termed as Type-1 T regulatory (Tr1) cells. Since then, several different populations of IL-10-secreting regulatory T cells (Treg cells) have been described.

Naturally occurring CD4$^+$CD25$^+$ Treg cells, which characteristically express the transcription factor forkhead box protein P3 (Foxp3) (Hori *et al.*, 2003), has been intensively studied because their deficiency abrogates self-tolerance and causes autoimmune disease (Sakaguchi and Powrie, 2007). Mice with a frame-shift mutation of Foxp3, scurfy mice, have massive lymphoproliferation and severe inflammatory infiltration of the skin and liver (Brunkow *et al.*, 2001; Godfrey *et al.*, 1991). However, many organs including the central nervous system, the joints, and the small intestine remained unaffected in scurfy mice (Chen *et al.*, 2005). These results suggested the existence of additional important mechanisms other than the Foxp3 system to enforce immunological self-tolerance. IL-10-secreting T cells may constitute such a complementary mechanism. This chapter describes recent progress in the characterization of IL-10-secreting CD4$^+$ T cells.

2. IL-10-PRODUCING T CELLS INDUCED *IN VITRO*

2.1. Tr1 cells

2.1.1. Biological features of Tr1 cells

Tr1 cells can be induced *in vitro* upon priming of naïve T cells with an antigen in the presence of IL-10. The cytokine production profile of Tr1 cells is their key feature (Groux *et al.*, 1997). Upon activation via the T cell receptor (TCR), Tr1 cells produce large amounts of IL-10, transforming growth factor (TGF)-β and IL-5, but only small amounts of interferon (IFN)-γ and IL-2, and no IL-4. IL-10 secreted by Tr1 cells was detectable within 4 h after activation and the highest concentration was reached 12–24 h after stimulation (Bacchetta *et al.*, 1994). Tr1 clones were also isolated from mice transgenic for a TCR specific for a peptide derived from ovalbumin (OVA). In contrast to human Tr1 cells, murine Tr1 cells rarely produce IFN-γ. Tr1 cells are thought to regulate immune responses involving both naïve and memory T cells through the secretion of the immunosuppressive cytokines IL-10 and TGF-β. The suppressive effects of Tr1 cells on $CD4^+$ T cells were blocked by neutralizing anti-IL-10 or anti-TGF-β antibodies (Bacchetta *et al.*, 1994; Groux *et al.*, 1997; Kitani *et al.*, 2000; Roncarolo *et al.*, 2006). However, it is still unclear whether TGF-β secretion should be included as part of the definition of Tr1 cells, because several reports described an exclusive role for IL-10 (Battaglia *et al.*, 2006a). Battaglia *et al.* proposed that the suppressive effects mediated by IL-10-secreting Treg cells should be attributed to Tr1 cells regardless of the production of TGF-β, IL-5, and IFN-γ (Battaglia *et al.*, 2006a).

Tr1 cells proliferate poorly following TCR-mediated or antigen-specific activation (Bacchetta *et al.*, 1994; Groux *et al.*, 1997). Activation of human $CD4^+$ T cells in the presence of IL-10 results in a state of functional unresponsiveness without death, termed anergy (Groux *et al.*, 1996; Steinbrink *et al.*, 1997). The autocrine effect of IL-10 is responsible, at least in part, for the anergic state, because blocking of IL-10 partially restores proliferation (Bacchetta *et al.*, 1994; Groux *et al.*, 1997).

An OVA-specific murine Tr1 clone showed suppressive capacity if the mice were administrated the OVA peptide (Cottrez *et al.*, 2000). This fact indicated that Tr1 cells must be activated through TCR to exert regulatory effects. Once activated, they could exhibit bystander suppressive activity mediated by the local release of IL-10 and TGF-β regardless of their antigen specificities (Groux, 2003). However, contact-dependent signals, such as programmed death-1 (PD-1), glucocorticoid-induced TNF receptor (GITR), membrane TGF-β, and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), appear to be important in some situations (Akdis *et al.*, 2004; Meiler *et al.*, 2008). The precise suppressive mechanisms of Tr1 cells still need to be investigated further.

2.1.2. Mechanisms of suppression

The prominent role for IL-10 appears to be as an immunosuppressive cytokine with broad anti-inflammatory properties, in particular by its inhibition of macrophages and DCs functions (O'Garra *et al.*, 2008). IL-10 downregulates the expression of MHC class II and costimulatory molecules such as CD54, CD80, and CD86 (de Waal Malefyt *et al.*, 1991; Ding *et al.*, 1993; Moore *et al.*, 2001; Willems *et al.*, 1994). Reduced expression of these molecules significantly affected the T cell-stimulating capacity of APCs (de Waal Malefyt *et al.*, 1991; Ding and Shevach, 1992; Fiorentino *et al.*, 1991b). Moreover, IL-10 potently inhibited the secretion of IL-1α, IL-1β, IL-6, IL-10 itself, IL-12, IL-18, and tumor necrosis factor (TNF)-α by activated monocytes/macrophages (D'Andrea *et al.*, 1993; Gruber *et al.*, 1994; Moore *et al.*, 2001). IL-10 also inhibited production of CC (MCP-1, MCP-5, MIP-1α, MIP-1β, MIP-3α, MIP-3β, and RANTES) and CXC chemokines (IL-8, IP-10, MIP-2) by APCs (Kopydlowski *et al.*, 1999; Moore *et al.*, 2001; Rossi *et al.*, 1997), which influence T cell differentiation and migration (Pestka *et al.*, 2004). These observations collectively suggest that IL-10 induces differentiation of APCs which limit inflammatory responses.

The IL-10 receptor (IL-10R) complex on cells is composed of four transmembrane polypeptides (Pestka *et al.*, 2004). Two chains are ligand-binding IL-10R1 and the other two chains are signal-transducing IL-10R2. While Janus kinase 1 (JAK 1) is constitutively bound to IL-10R1, Tyrosin kinase 2 (TYK2) constitutively associates with IL-10R2. Upon activation of the IL-10 receptor complex by IL-10, JAK1 and TYK2 are activated by cross-phosphorylation of two tyrosine residues on the intracellular domain of IL-10R1 (Finbloom and Winestock, 1995). These phosphorylated tyrosines mediate the interaction of STAT3 with the IL-10R complex. As a result, phosphorylation of STAT3, STAT1, and STAT5 by JAK1 and TYK2 is induced, and activated STAT proteins translocate to the nucleus. Among genes activated by IL-10, SOCS-1 has been shown to silence JAK/STAT signaling by binding to JAK kinases (Yasukawa *et al.*, 1999). This mechanism may explain how IL-10 inhibits the effects of other cytokine signaling system. Moreover, SOCS-3 activated by IL-10 inhibited many aspects of gp130 signaling initiated by IL-6 (Niemand *et al.*, 2003). Other anti-inflammatory properties of IL-10 could be explained by its effect of stabilizing IκBα in the cytoplasm(Shames *et al.*, 1998) and preventing lipopolysaccharide (LPS)-induced NF-κB activation by inhibiting IκB kinase (Schottelius *et al.*, 1999).

2.1.3. Induction of Tr1 cells by DCs

Tr1 can be induced *in vitro* during antigen presentation. Therefore, one of important questions is whether there is a specific subpopulation or differentiated status of APCs that promotes the differentiation of Tr1 cells. Among the DCs, the professional APCs, immature DCs (iDCs) were reported to induce tolerance through deletion of antigen-specific effector

T cells (Bonifaz *et al.*, 2002; Hawiger *et al.*, 2001) and induction of T cells with regulatory activity (Dhodapkar *et al.*, 2001). Repeated stimulation of human peripheral blood $CD4^+$ T cells with allogenic iDCs resulted in the induction of IL-10-secreting Treg cells functionally similar to Tr1 cells (Levings *et al.*, 2005). In mice, a population of DCs expressing high levels of CD45RB with immature phenotype secreted IL-10 and induced Tr1 cells (Wakkach *et al.*, 2003). Plasmacytoid DCs (pDCs) is an unconventional type of APCs characterized by the ability to produce type I IFN upon activation (Asselin-Paturel *et al.*, 2001). Repetitive stimulation of naïve $CD4^+$ T cells with murine pDCs isolated from mesenteric lymph nodes led to the generation of Tr1-like cells with regulatory activity (Bilsborough *et al.*, 2003). Moreover, IL-10 itself is a potent inducer of tolerogenic DCs. DCs generated in the presence of exogenous IL-10 secreted high levels of IL-10, and IL-10 exposed DCs were more powerful than iDCs in inducing Tr1 cells (Roncarolo *et al.*, 2006).

2.1.4. Trials of *in vivo* induction of Tr1 cells

In vivo induction of antigen-specific Tr1 cells is a promising approach to treat a variety of autoimmune diseases. Based on the multimodal anti-inflammatory effect of IL-10, several clinical studies using systemic administration of recombinant IL-10 were designed in patients with inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (Asadullah *et al.*, 2003). Although early phase I and II studies showed trends toward efficacy, larger blinded studies showed only modest improvement accompanied with side effects such as headache and fever. Higher concentrations of IL-10 induced the elevation of IFN-γ levels and inflammation markers in patients with IBD (Tilg *et al.*, 2002). These results suggested the potential dual roles of IL-10 in both anti-inflammatory and immunostimulatory capacities (O'Garra *et al.*, 2008).

The addition of immunosuppressive drugs to recombinant IL-10 may enhance the anti-inflammatory properties of IL-10 by suppressing the immunostimulatory capacity of IL-10. Coadministration of IL-10 and rapamycin was reported to induce alloantigen-specific IL-10-secreting $CD4^+$ T cells *in vivo* in a murine model of pancreatic islet transplantation (Battaglia *et al.*, 2006b). Importantly, the antigen-specific tolerance was transferred to naïve mice by adoptive transfer of T cells from mice with tolerance. Administration of IL-10 alone did not prevent allograft rejection, and addition of rapamycin was required for the effective induction of tolerance. This result was intriguing considering the well-documented association between *in vivo* administration of rapamycin and a pronounced increase in the number of $CD4^+CD25^+Foxp3^+$ Treg cells (Coenen *et al.*, 2007; Noris *et al.*, 2007). PI3K-AKT-mTOR signaling, the target pathway of rapamycin, may be a common pathway for the induction of Tr1 cells and $CD4^+CD25^+Foxp3^+$ Treg cells.

2.2. CD46-stimulated IL-10-secreting T cells

CD46, initially identified as a complement regulatory receptor for C3 and as a receptor for several pathogens (Cattaneo, 2004; Kemper *et al.*, 2005), was found to be a potent costimulatory molecule in human T cells (Astier *et al.*, 2000; Zaffran *et al.*, 2001). CD46 is a ubiquitously expressed type I membrane protein. CD46 costimulated T cells in the presence of IL-2 acquired a Tr1-like phenotype and secreted large amounts of IL-10 and granzyme B (Grossman *et al.*, 2004; Kemper *et al.*, 2003). In addition, CD3/CD46-stimulated T cells secreted moderate quantities of TGF-β and IFN-γ. In contrast to poorly proliferative IL-10-stimulated Tr1 cells, CD3/CD46-stimulated T cells showed a stronger and more sustained proliferation as compared with CD3/CD28-stimulated T cells. C3b dimers, a physiological ligand of CD46, could substitute for monoclonal antibodies to CD46 in the induction of IL-10-secreting cells, thus providing a mechanism for CD46 cross-linking by antigens coated with complement fragments during antigen presentation (Kemper *et al.*, 2003). Interestingly, CD46 was also described as a ''magnet for pathogens'' (Astier, 2008). So far, several human pathogens have been found to bind to CD46, including measles virus, human herpes virus 6, adenovirus, *Streptococcus pyogenes*, and *Neisseria gonorrhoeae*. However, an endogenous ligand may be expressed on DCs or other APCs, and the ligand for CD46-mediated IL-10 secretion remains to be determined.

In multiple sclerosis (MS), a striking difference was observed between healthy donors and patients, in that little to no IL-10 was secreted by CD46-activated T cells from MS patients as compared with healthy donors (Astier *et al.*, 2006). The defect was specific to CD46 costimulation, because IL-10 secretion upon CD28 costimulation was not affected. In addition, IFN-γ secretion by CD46-activated T cells was not affected and correlated with the proliferation. Dysregulation of CD46 in MS patients was suggested because an increased expression of Cyt2 cytoplasmic isoforms was observed in T cells from MS patients with impaired IL-10 secretion. Several groups have demonstrated a defect in the CD4$^+$CD25$^+$ Tregs cells in patients with MS, type I diabetes and RA (Bacchetta *et al.*, 2005; Bluestone and Tang, 2005; Haas *et al.*, 2005; Viglietta *et al.*, 2004). Another defect in IL-10-secreting CD4$^+$ T cells is consistent with the hypothesis that there are multiple immunologic hits required to allow autoimmune disease to occur.

2.3. IL-10-secreting T cells induced by vitamin D3 and dexamethasone

Activation of naïve T cells *in vitro* in the presence of IL-10 alone, IL-10 and IL-4 (Groux *et al.*, 1997), or IL-10 and IFN-α (Levings *et al.*, 2001) has been reported to induce IL-10-secreting T cells. One important setback for the

clinical application is that these cells also secrete Th1 and/or Th2 cytokines, because they inevitably have the potential to induce or exacerbate inflammation (Hawrylowicz and O'Garra, 2005). Barrat *et al.* described that antigen-specific murine T cell stimulation in the presence of vitamin D3 (VitD3) and dexamethasone (Dex) induced IL-10-secreting Treg cells (Barrat *et al.*, 2002). VitD3 affects DCs and macrophages functions as well as lymphocyte function and inhibits the production of a variety of proinflammatory cytokines (Piemonti *et al.*, 2000). Glucocorticoids, including Dex, are among the most potent anti-inflammatory reagents in the treatment of Th1- and Th2-associated inflammatory diseases. Furthermore, VitD3 and Dex were reported to inhibit the activation of important inflammation associated transcription factors, such as nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and NF-κB (De Bosscher *et al.*, 1997; Wilckens and De Rijk, 1997). The combination of VitD3 and Dex inhibited the production of Th1- or Th2-cytokines in activated IL-10-secreting T cells (Barrat *et al.*, 2002; Vieira *et al.*, 2004). The *in vitro* induced IL-10-secreting Treg cells suppressed experimental autoimmune encephalomyelitis (EAE). Moreover, their development and function were IL-10 dependent (Barrat *et al.*, 2002). It has been demonstrated that IL-10-secreting T cells induced by VitD3 and Dex did not express Foxp3 (Vieira *et al.*, 2004). In a human study, Dex and calcitriol induced IL-10-secreting cells that inhibited proliferation and cytokine production by autologous $CD4^+$ T cells in an IL-10-dependent way (Xystrakis *et al.*, 2006).

2.4. IL-27 and IL-21 in the induction of IL-10-secreting T cells

IL-12, a heterodimeric cytokine composed of p35 and p40, was characterized as an inducer of Th1 differentiation (Trinchieri, 2003). p35 is a homolog of IL-6 and granulocyte-colony-stimulating factor (G-CSF) with a four-α-helix bundle structure, while p40 is homologous to the extracellular portion of IL-6 receptor α (IL-6Rα). Recently, IL-23, IL-27, and IL-35 were identified as heterodimeric cytokines functionally and structurally related to IL-12 (Collison *et al.*, 2007; Oppmann *et al.*, 2000; Pflanz *et al.*, 2002). IL-27 is composed of Epstein-Barr virus-induced gene 3 (EBI-3) (Devergne *et al.*, 1996), a p40 related molecule, and p28, a p35 related molecule.

In terms of T cell immunity, previous reports revealed that IL-27 has both inflammatory and immunosuppressive properties. IL-27 promoted naïve $CD4^+$ T cells to differentiate into Th1 cells by inducing IL-12Rβ2 (Pflanz *et al.*, 2002). WSX-1 is the α subunit of the IL-27R complex, and the role of WSX-1 in Th1 differentiation has been examined in WSX-1-deficient (WSX-1 $^{-/-}$) mice. WSX-1 $^{-/-}$ mice showed impaired IFN-γ production,

and enhanced susceptibility to *Leishmania major* infection (Chen *et al.*, 2000; Yoshida *et al.*, 2001). Because the reduced production of IFN-γ was observed only in the early phase of *L. major* infection, IL-27/WSX-1 signaling was only required in the initial phase of Th1 differentiation. In contrast, WSX-1 $^{/}$ mice produced increased levels of IFN-γ compared with wild-type mice and demonstrated cytokine-mediated liver damage during the *Trypanosoma cruzi* infection (Hamano *et al.*, 2003). Moreover, CD4$^+$ T cells isolated from *T. cruzi*-infected WSX-1 $^{/}$ mice showed hyperproduction of IL-6 and TNF-α. In addition, WSX-1 $^{/}$ mice produced increased amounts of various cytokines in an allergen-induced airway hypersensitivity model (Miyazaki *et al.*, 2005). These results revealed the novel role of IL-27 as an attenuator of proinflammatory cytokine production.

Recently, three groups reported the promotion of IL-10-producing T cell differentiation by IL-27 (Awasthi *et al.*, 2007; Fitzgerald *et al.*, 2007b; Stumhofer *et al.*, 2007). CD4$^+$ T cells stimulated under nonpolarizing conditions in the presence of IL-27 show significantly increased IL-10 production (Stumhofer *et al.*, 2007). Moreover, IL-27 induced IL-10 production in differentiated Th1, Th2, and Th17 cells (Awasthi *et al.*, 2007; Fitzgerald *et al.*, 2007b; Stumhofer *et al.*, 2007). TGF-β amplified the generation of IL-10-secreting T cells by IL-27 (Awasthi *et al.*, 2007). Reduction in IL-10 production was also observed in *Toxoplasma gondii* infected WSX-1 $^{/}$ mice (Hunter *et al.*, 1997). Exogenous IL-27 ameliorated EAE by suppressing Th17 responses via IL-10 (Fitzgerald *et al.*, 2007a). Therefore, IL-27 converts inflammatory effector CD4$^+$ T cells into IL-10-secreting, immunosuppressive Tr1-like cells. The phenotype of IL-27-stimulated cells was IL-10$^+$IFN-γ$^+$Foxp3 IL-17 (Batten *et al.*, 2008). Induction of IL-10 by IL-27 was dependent on the transcription factors STAT1 and STAT3 (Stumhofer *et al.*, 2007).

IL-21 is another pleiotropic cytokine that is required for normal immunoglobulin production (Monteleone *et al.*, 2009). IL-21 signals through a heterodimeric receptor containing IL-21R and the common cytokine receptor γ-chain γc, which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. Though expression of IL-21 was initially reported to be Th2 specific, subsequent studies demonstrated that IL-21 is also produced by Th1, Th2, Th17, and follicular helper T cells (TFH) (Korn *et al.*, 2009). Based on the observation that IL-21 and IL-10 levels increased with age similarly in BXSB-*Yaa* mice with lupus-like disease, Spolski *et al.* unexpectedly found that IL-21 induces IL-10 mRNA and protein expression (Spolski *et al.*, 2009). Th1 priming with IL-21 led to the accumulation of cells with immunosuppressive activity, and IL-21 overexpression decreased specific antibody production after immunization. Furthermore, Pot *et al.* showed that IL-27 was a potent inducer of three essential elements; the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS (Pot *et al.*, 2009). IL-27-driven c-Maf expression

transactivated IL-21 production, which acts as an autocrine growth factor for the expansion and/or maintenance of IL-27-induced IL-10-secreting cells. Each of these elements was essential, because the loss of c-Maf, IL-21 signaling, or ICOS decreased the frequency of IL-27-induced differentiation of IL-10-secreting T cells. Similar to IL-6, which induces IL-21 via STAT3-phosphorylation, IL-27 also induces STAT3-phosphorylation and IL-21, possibly due to the sharing of gp130 for signaling. Therefore, cooperation of IL-27 and IL-21 may be an important pathway in the generation of IL-10-secreting T cells.

2.5. IL-10-secreting anti-inflammatory Th1 cells

Interestingly, Th1 cells producing both IFN-γ and IL-10 were reported during certain infections and IL-10 produced by such Th1 cells played important regulatory roles for host protection (Anderson *et al.*, 2007; Jankovic *et al.*, 2007). In *L. major* infection to recombination activating gene (RAG)-2-deficient (RAG2 $^{/}$) mice reconstituted with T cells, IL-10 production by antigen-specific CD4^{+}CD25 Foxp3 cells, the majority of which also produced IFN-γ, was necessary for the suppression of acquired immunity (Anderson *et al.*, 2007). In mice infected with *T. gondii*, IFN-γ-secreting T-bet^{+}Foxp3 Th1 cells were the major source of IL-10 (Jankovic *et al.*, 2007). The same IL-10^{+}IFN-γ^{+} population displayed potent effector functions against the parasite. Though IFN-γ expression was imprinted and triggered with similar kinetics regardless of the state of Th1 cell activation, IL-10 secretion was induced more rapidly from recently activated than from resting cells. In human visceral leishmaniasis patients, splenic CD25 T cells had elevated expression levels of both IFN-γ and IL-10 (Nylen and Sacks, 2007). These reports suggested that IL-10 production by CD4^{+} T cells is not limited to a distinct Treg cell population but can be generated even in Th1 cells as a part of the effector response to prevent the inflammation induced pathology.

The molecular pathways leading to IL-10 expression in Th1 cells are only just beginning to be understood. While IL-10 was induced by the Th2 regulating transcription factor GATA3 in Th2 cells (Chang *et al.*, 2007), Th1 cells generated *in vitro* in the presence of IL-12 contained only a few IL-10-secreting cells. As mentioned above, another member of the IL-12 family of cytokines, IL-27, can induce IFN-γ and IL-10 production in T cells. Rutz *et al.* identified Notch as a potent inducer of IL-10 both in developing and in established Th1 cells via a STAT4-dependent process (Rutz *et al.*, 2008). Notch signaling in the presence of IL-12 and IL-27 induced Th1 cells to produce large amounts of IL-10 without diminishing IFN-γ production. Notch-exposed Th1 cells completely lost their inflammatory capacity and instead were able to actively suppress the Th1 cell induced delayed type hypersensitivity (DTH) reaction in an

IL-10-dependent manner. DCs stimulated with various Toll-like receptor (TLR) ligands acquired Delta-like 4 (DLL4) expression and concomitantly induced IL-10 production by Th1 cells *in vitro* and *in vivo*. IL-27 and Notch could be molecular switches between proinflammatory and anti-inflammatory Th1 cell function. These molecules may be a target for therapeutic intervention in inflammatory diseases.

It is likely that IL-10 production by Th1 cells is associated with conditions of high inflammation and antigenic stimulation. Saraiva *et al.* reported that development of IL-10-secreting Th1 cells required strong TCR ligation, sustained phosphorylation of ERK1 and ERK2 MAP kinases, and IL-12-induced STAT4 transcription factor activation (Saraiva *et al.*, 2009). Repeated TCR stimulation leads to enhanced IL-10 production by Th1 cells, and continued IL-12 action and high-dose TCR signaling were required for the development and maintenance of IL-10-secreting Th1 cells. Notably, while high antigen dose and IL-12 drastically downregulated GATA-3 expression which can remodel the IL-10 locus, expression of c-Maf correlated with IL-10 expression. Because the activation of ERK1 and ERK2 is a common requirement for the production of IL-10 by Th1, Th2, and Th17 cells, all Th cells may share the mechanism for controlling excessive inflammation by means of IL-10 production.

2.6. Exogenous signals in the induction of IL-10-secreting T cells

As mentioned in Section 1, the dominant suppressive function of IL-10 in mucosal immunity was clearly reinforced by the phenotype of IL-10 $^{/}$ mice (Kuhn *et al.*, 1993), which developed colitis in the presence of normal gut flora (Sellon *et al.*, 1998). In addition, IL-10 $^{/}$ mice eradicated certain intracellular pathogens efficiently. However, this was often accompanied by lethal immunopathology (O'Garra *et al.*, 2004, 2008). Therefore, IL-10 is important in limiting the inflammatory responses to pathogens and prevents damage to the host. Accordingly, there are a number of reports showing the association between pathogen-derived signals and IL-10 secretion in CD4$^+$ T cells.

Higgins *et al.* demonstrated that *Bordetella pertussis* LPS induced IL-10 production from DCs, through TLR4. Antigen-specific IL-10 production by T cells was significantly reduced and inflammatory pathology was enhanced in TLR4 defective mice, suggesting that TLR4-mediated IL-10 production promotes the generation of IL-10-secreting CD4$^+$ T cells and confers host resistance to the infection by limiting collateral damage in the lungs (Higgins *et al.*, 2003). Similarly, den Haan *et al.* described that LPS induced antigen-specific suppressive CD4$^+$ T cells that inhibit CD8$^+$ T cell priming via IL-10 (den Haan *et al.*, 2007).

TLR2 also plays a pivotal role in the recognition of pathogen-derived signals. Collaboration between TLR2 and dectin-1 resulted in the

induction of proinflammatory cytokines as well as robust IL-10 production in DCs (Underhill, 2007). Intriguingly, zymosan conditioned splenic DCs to secrete IL-10 and induced tolerogenic T cell responses (Rogers *et al.*, 2005; Slack *et al.*, 2007). Recently, Manicassamy *et al.* reported that TLR2 signaling induced splenic DCs to express the retinoic acid metabolizing enzyme retinaldehyde dehydrogenase type 2 and IL-10. TLR2-stimulated DCs also metabolized vitamin A and induced $Foxp3^+$ Treg cells (Manicassamy *et al.*, 2009). What is important was that, whereas zymosan-treated DCs in the presence of TGF-β induced both $Foxp3^+$ Treg cells and IL-10-secreting T cells, they induced mostly IL-10-secreting cells in the absence of TGF-β. These facts suggest that immune stimulus from microbes significantly contributes to the induction of IL-10-secreting and $Foxp3^+$ Treg cells.

Mazmanian *et al.* reported that the prominent human symbiont *Bacteroides fragilis* protected animals from experimental colitis induced by *Helicobacter hepaticus*, a commensal bacterium with potential pathogenesis (Mazmanian *et al.*, 2008). This protective activity required a single microbial molecule, polysaccharide A (PSA). In animals harboring *B. fragilis* not expressing PSA, *H. Hepaticus* colonization resulted in severe colitis and proinflammatory cytokine production in the colon. Administration of purified PSA to animals suppressed IL-17 production in the intestine. Moreover, PSA protection from inflammatory disease was mediated by IL-10-secreting $CD4^+$ T cells. According to the ''hygiene hypothesis,'' reduced exposure to infections in early childhood may increase the risk of allergic and autoimmune diseases (Strachan, 1989). In fact, this study showed that bacteria residing in the gastrointestinal tract produced factors mediating normal immune controls and protected the host from inflammation. Mazmanian *et al.* proposed that the mammalian genome does not encode for all functions required for immunological development but rather that mammals depend on critical interactions with their microbiome for health (Mazmanian *et al.*, 2008). This intriguing proposal should be addressed further.

Not only pathogen-derived signals but also exogenous toxins were reported to induce IL-10-secreting $CD4^+$ T cells. Meiler *et al.* reported on a multiyear study in which they examined allergen-specific T-cell responses in beekeepers during and outside of the beekeeping season (Meiler *et al.*, 2008). Continuous exposure of nonallergic beekeepers to high doses of bee venom antigens induced diminished T cell-related cutaneous late-phase swelling to beestings in parallel with suppressed allergen-specific T cell proliferation and Th1 and Th2 cytokine secretion. After multiple beestings, venom antigen-specific Th1 and Th2 cells showed a switch toward IL-10-secreting T cells. T cell regulation continues as long as antigen exposure persists and returns to initial levels within 2–3 months after the cessation of beestings. Histamine receptor 2

upregulated on specific Th2 cells displayed a dual effect by directly suppressing allergen-stimulated T cells and increasing IL-10 production. On the other hand, there was no change in the overall percentage of CD4$^+$CD25$^+$ Treg cells in response to beestings. CTLA4 and PD-1 played roles in allergen-specific T cell suppression. In contrast to its widely accepted role in mucosal allergen tolerance, TGF-β did not seem to be an essential player in skin-related allergen tolerance. Thus, the rapid switch and expansion of IL-10-secreting T cells and the use of multiple suppressive factors represent essential mechanisms in immune tolerance to a high dose of allergens in nonallergic individuals. Collectively, these findings indicate that transiently expanded IL-10-secreting T cell populations play an important role in keeping allergen-specific effector T cell responses in check, which helps to explain how skin exposure to high doses of allergen leads to a decreased immune response to subsequent allergen exposures. Moreover, the data suggest that antigen persistence is an essential condition for long-term tolerance.

3. NATURALLY PRESENT IL-10-SECRETING T CELLS

3.1. CD4$^+$CD25$^+$Foxp3$^+$ Treg cells

A unique subset of CD4$^+$ T cells known as CD4$^+$CD25$^+$ Treg cells has the role in controlling tissue damage and inflammation in the context of both innate and adaptive immune responses (Sakaguchi *et al.*, 2008; Shevach, 2009). CD4$^+$CD25$^+$ Treg cells represent a differentiated cell lineage developed in the thymus and their phenotype and function are dependent upon the expression of the transcription factor Foxp3 (Hori *et al.*, 2003). Deficiency of functional Foxp3 leads to a fetal lymphoproliferative autoimmune disorder with multiple organ inflammation (Brunkow *et al.*, 2001). In humans, individuals lacking Foxp3 have an autoimmune disorder, immunodysregulation polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome (Bennett *et al.*, 2001).

In spite of its significance, the mechanisms of suppression mediated by CD4$^+$CD25$^+$ Treg cells are poorly clarified. While a number of molecular and cellular mechanisms of active suppression have been reported, the emerging model is still confusing. It is not clear whether a single mechanism can be responsible for various manifestations of CD4$^+$CD25$^+$ Treg cell suppression. Although IL-10 was reported to play a significant role as an effector molecule of CD4$^+$CD25$^+$ Treg cell-mediated suppression of colitis (Annacker *et al.*, 2003; Asseman *et al.*, 1999, 2003), IL-10 was found to be nonessential for *in vivo* suppression of autoimmune gastritis (Shevach *et al.*, 2001). Rubtsov *et al.* analyzed mice in which the Foxp3$^+$ Treg cell-specific ablation of a conditional IL-10 allele was induced by Cre

recombinase knocked into the Foxp3 locus (IL-10$^{flox/flox}$ x Foxp3$^{YFP\text{-}Cre}$) (Rubtsov *et al.*, 2008). They found that the selective disruption of IL-10 expression in Foxp3$^+$ Treg cells resulted in spontaneous colitis. The stomach and small intestine were not affected by IL-10 deficiency in Foxp3$^+$ Treg cells. Moreover, these IL-10$^{flox/flox}$ × Foxp3$^{YFP\text{-}Cre}$ mice developed augmented immune mediated inflammation in the skin and lungs. These results are consistent with the findings in IL-10-IRES-GFP reporter mice (tiger mice) that the majority of IL-10-expressing T cells in the small intestine did not express Foxp3 (Kamanaka *et al.*, 2006). In tiger mice, anti-CD3 treatment induced Tr1-like cells in small intestinal intraepithelial lymphocytes (sIEL) and led to the accumulation of naturally occurring Treg cells in colonic lamina propria lymphocytes (cLPL). In contrast, CTLA-4 ablation in Treg cells resulted in systemic lymphoproliferative syndrome and hyperproduction of IgE, whereas the colon and skin remained largely unaffected (Rubtsov *et al.*, 2008; Wing *et al.*, 2008). These results indicated that IL-10 produced by Treg cells is required in the suppression of inflammation at environmental interfaces such as colon, skin, and lungs, but is dispensable for limiting systemic autoimmunity. Treg cells may use multiple molecular mechanisms to suppress immune responses and individual regulatory mechanisms could operate in a particular tissue and inflammatory condition.

Interestingly, a subset of Foxp3-expressing Treg cells might secrete IL-10. Maynard *et al.* reported a ''dual reporter mouse (10BiT.Foxp3^{gfp} mice)'' system of the genes encoding IL-10 and the transcription factor Foxp3 to track Treg subsets based on coordinate or differential expression of these gene (Maynard *et al.*, 2007). In 10BiT.Foxp3^{gfp} mice, secondary lymphoid tissues, lung and liver showed the enrichment of Foxp3$^+$IL-10 Treg cells, whereas the colon and small intestine exhibited the enrichment of Foxp3$^+$IL-10$^+$ Treg cells. While each Treg cells subset developed in the absence of IL-10, TGF-β blockade inhibited the development of both subsets. Therefore, Foxp3$^+$IL-10$^+$ Treg were induced by a mechanism dependent on TGF-β and independent of IL-10. In humans, Ito *et al.* found two subsets of Foxp3$^+$ Treg cells defined by the expression of the costimulatory molecule ICOS (Ito *et al.*, 2008). Whereas ICOS$^+$Foxp3$^+$ Treg cells used IL-10 to suppress dendritic cell function and TGF-β to suppress T cell function, ICOS Foxp3$^+$ Treg cells used TGF-β only. pDCs selectively promoted the proliferation of ICOS$^+$Foxp3$^+$ Treg cells in an ICOS-dependent manner, whereas myeloid DCs (mDCs) preferentially promoted the proliferation of ICOS Foxp3$^+$ Treg cells through a CD80- and CD86-dependent mechanism. Thus, DCs have been shown to play key roles in the induction and maintenance of Treg cells (Tarbell *et al.*, 2006). Foxp3$^+$IL-10$^+$ and Foxp3$^+$IL-10 Treg cells may be selected and educated by different subsets of APCs.

3.2. CD4$^+$CD25 LAP$^+$ T cells

Nakamura *et al.* showed that CD4$^+$CD25$^+$ Treg cells expressed TGF-β on their surface and exhibited their suppressive function by presenting surface TGF-β to the receptor on the target cells by cell-to-cell contact (Nakamura *et al.*, 2001). Latency-associated peptide (LAP) is the amino-terminal domain of the TGF-β precursor peptide and forms a latent TGF-β complex with the TGF-β peptide (Miyazono *et al.*, 1993). CD4$^+$CD25$^+$ Treg cells became positive for surface LAP after strong stimulation *in vitro* (Nakamura *et al.*, 2001). Weiner and colleagues reported Treg cells that expressed surface LAP and suppressed murine colitis in a TGF-β-dependent mechanism (Oida *et al.*, 2003). CD4$^+$CD25 LAP$^+$ T cells were positive for thrombospondin, which has the ability to convert latent TGF-β to the active form. CD4$^+$CD25 LAP$^+$ T cells produced high levels of TGF-β and IL-10. Notably, they exhibited regulatory activity in the CD4$^+$CD45RB^{high}-induced colitis model of SCID mice in a TGF-β-dependent manner.

Parenteral administration of CD3-specific monoclonal antibody is an approved therapy for transplantation in humans and is effective in autoimmune diabetes. Orally administrated CD3-specific antibody induced CD4$^+$CD25 LAP$^+$ Treg cells that suppress EAE and diabetes in mice by TGF-β secretion (Ishikawa *et al.*, 2007; Ochi *et al.*, 2006). Oral administration of a CD3-specific antibody may weakly signal to T cells in the gut, and enhance the regulatory function of CD4$^+$CD25 LAP$^+$ Treg cells. The exact relationship between CD4$^+$CD25 LAP$^+$ T cells and Th3 Treg cells that appear after oral administration of antigens (Chen *et al.*, 1994) is not clear. In addition to TGF-β, anti-CD3-induced CD4$^+$CD25 LAP$^+$ T cells produced significant amounts of IL-4, IFN-γ, and IL-10 (Ochi *et al.*, 2006). The contribution of IL-10 to the suppressive effect of CD4$^+$CD25 LAP$^+$ T cells remains to be determined.

3.3. CD4$^+$NKG2D$^+$ T cells

Natural killer (NK) receptors control the activation and inhibition of NK cells (Long, 1999). Among these receptors, NKG2D transduces directly activating or costimulatory signals via the paired DAP10 adaptor protein (Lanier, 2008; Wu *et al.*, 1999). In humans, NKG2D ligands, including the MHC class I-related chain A (MICA), were induced by microbial infections (Gonzalez *et al.*, 2006), and were expressed in several autoimmune conditions (Groh *et al.*, 2003; Saikali *et al.*, 2007). NKG2D also costimulated the proliferation of CD4$^+$ T cells with negative regulatory functions and reduced chronic immune activation induced by MICA expression (Groh *et al.*, 2006). Small populations of CD4$^+$ T cells with NKG2D expression showed a biased cytokine profile toward IL-10 and TGF-β. CD4$^+$NKG2D$^+$

T cells also produced Fas ligand (FasL) to cause growth arrest of bystander T cells (Groh *et al.*, 2006).

In tissues with inflammation, increased MICA expression might induce the expansion of suppressive $CD4^+NKG2D^+$ T cells. In this context, $CD4^+NKG2D^+$ T cells could have regulatory activity for inflammation. Recently, Dai *et al.* (2009) reported that normally occurring $CD4^+NKG2D^+$ T cells in healthy individuals were autoreactive and ready to produce IL-10, but lacked proinflammatory cytokines. In contrast to IL-10 production, which was associated with all T cell differentiation stages, TGF-β production was detected with central and effector memory $CD4^+NKG2D^+$ T cells, but not naïve T cells. Extensive expansions of these T cells in patients with juvenile-onset SLE were inversely correlated with disease activity, suggesting the *in vivo* regulatory effects of $CD4^+NKG2D^+$ T cells.

Though the normal $CD4^+$ T cell compartment harbored small numbers of IL-10-producing $CD4^+NKG2D^+$ T cells with regulatory activity, the concept of regulatory $CD4^+NKG2D^+$ T cells is complicated by the occurrence of effector $CD4^+NKG2D^+$ T cells in several inflammatory situations. IFN-γ and TNF-α producing cytotoxic $CD4^+NKG2D^+$ T cells occurred in RA patients (Groh *et al.*, 2003), and the frequency of perforin-expressing $CD4^+NKG2D^+$ T cells correlated with the severity of Crohn's disease (Allez *et al.*, 2007). Therefore, more specific markers for regulatory $CD4^+NKG2D^+$ T cells should be investigated.

3.4. $CD4^+CD25$ IL-7R T cells

Recently, it was reported that human $CD4^+CD25^+$ Treg cells expressed low level of CD127 (Liu *et al.*, 2006; Seddiki *et al.*, 2006), the IL-7 receptor (IL-7R) α chain that is important for survival and homeostatic maintenance of $CD4^+$ T cells (Bradley *et al.*, 2005). Based on these observation, Haringer *et al.* speculated that adaptive Treg cells might be present among $CD4^+CD25$ IL-7R fraction. They reported that human blood $CD4^+CD25$ IL-7R cells were activated, effector-like cells that coproduce IL-10 and IFN-γ but not IL-2 (Haringer *et al.*, 2009). In antigen-experienced $CD4^+CD45RA$ T cells, the majority of the cells had a $CD25^{/lo}$IL-$7R^{hi}$Foxp3 memory phenotype and $CD25^+IL\text{-}7R^{lo}$ phenotype identified natural Treg cells with Foxp3 expression. The small fraction of cells (around 1%), expressing neither CD25 nor IL-7R, were anergic and largely Foxp3-negative. They expressed low levels of Bcl-2 but high levels of Ki-67 and ICOS, suggesting that they have been recently activated *in vivo*. They responded selectively to persistent foreign (cytomegalovirus and candida) and self-antigens (Melan-A). When IL-7R cells were stimulated strongly via TCR, they suppressed proliferation of naïve and memory T cells in an IL-10-dependent manner. Based on these

observations, the CD4$^+$CD25 IL-7R cells are regarded as a subset containing human Tr1-like cells. However, the CD4$^+$CD25 IL-7R cells seems to be a heterogenous population, because only a part of CD4$^+$CD25 IL-7R cells (around 10%) produced IL-10 upon strong stimulation. Identification of more sophisticated surface and molecular markers is required for clinical evaluation and application of this population.

3.5. CD4$^+$CD25 LAG3$^+$ T cells

As mentioned above, known Treg cells are closely related to anergy. Anergy represents one of several tolerance-inducing mechanisms in T cells. T cell anergy is defined by defective proliferation and impaired IL-2 production of previously primed T cells upon restimulation, and is reversed by the addition of exogenous IL-2 (Beverly *et al.*, 1992; Schwartz, 2003). A set of functional restrictions characterizes the anergic state, including cell division, cell differentiation, and cytokine secretion. Many reports have implicated an important role for the activity of E3 ubiquitin ligases in the regulation of T cell activation (Fathman and Lineberry, 2007). The E3 ligases c-Cbl, Cbl-b, GRAIL, Itch, and Nedd4 have been linked to the promotion of T cell anergy (Heissmeyer *et al.*, 2004; Mueller, 2004). The RING-type E3 ubiquitin ligase Cbl-b promoted ubiquitination and degradation of signaling components such as phospholipase C-γ and PKC-θ. Early response gene (Egr)-2 and Egr-3 were reported to be transcription factors for the TCR-induced negative regulatory program controlling Cbl-b expression (Safford *et al.*, 2005). Egr-2 has been most widely studied in the context of nervous system, and its targeting in knockout mice resulted in early lethality concurrent to defects in hindbrain patterning, peripheral nerve myelination, and bone formation (Gillian and Svaren, 2004; Topilko *et al.*, 1994). However, the role of Egr-2 in the regulatory function of T cells has not been extensively addressed.

We have identified recently that a Treg population expresses Egr-2 and lymphocyte activation gene 3 (LAG-3) (Okamura *et al.*, 2009). LAG-3, which suppresses T cell proliferation (Workman and Vignali, 2003; Workman *et al.*, 2004), is required for maximal regulatory functioning of murine CD4$^+$CD25$^+$ T cells. Ectopic expression of LAG-3 on CD4$^+$ T cells significantly reduced their proliferative capacity and conferred on them regulatory activity toward effector T cells (Huang *et al.*, 2004). However, LAG-3 protein was hardly detected on the cell surface of CD4$^+$CD25$^+$ T cells but was expressed by a certain population of CD4$^+$CD25 T cells (Workman *et al.*, 2002). In accordance with the previous results, flow cytometric analysis revealed that more than 90% of LAG-3-expressing cells belonged to the CD4$^+$CD25 CD45RB^{low} population (hereafter called CD4$^+$CD25 LAG3$^+$ cells). These CD4$^+$CD25 LAG3$^+$ cells appeared to be

conventional $CD4^+TCR\alpha\beta^+$ T cells. The frequency of $CD4^+CD25^-LAG3^+$ cells in the $CD4^+CD25^-$ population was relatively low in the spleen (around 2%), mesenteric lymph node (around 1%), and inguinal lymph node (around 1%), but characteristically high in Peyer's patch (PP) (around 8%). Similar to most of other IL-10- secreting T cells, these cells were anergic *in vitro* upon TCR stimulation. They produced large amounts of IL-10, moderate amounts of IFN-γ, and low amounts of IL-2 and IL-4. $CD4^+CD25^-LAG3^+$ T cells exhibited weak suppressive activity in anti-CD3-stimulated cocultures of $CD4^+CD25^-LAG3^+$ T cells with $CD4^+CD25^-CD45RB^{high}$ T cells. In contrast, $CD4^+CD25^-LAG3^+$ T cells effectively inhibited colitis induced in RAG-1 $^{-/-}$ recipients by the transfer of $CD4^+CD25^-CD45RB^{high}$ T cells. The failure of colitis suppression by the transfer of $CD4^+CD25^-LAG3^+$ T cells from congenic IL-10 $^{-/-}$ mice indicated that the *in vivo* suppressive activity was IL-10 dependent.

Cytofluorometric analysis revealed that $CD4^+CD25^-LAG3^+$ T cells did not express Foxp3 protein. In addition, scurfy mice that lack functional Foxp3 protein (Brunkow *et al.*, 2001) contained significantly increased number of $CD4^+CD25^-LAG3^+$ T cells, which expressed LAG-3 and IL-10 mRNA equivalently, and exhibited distinct *in vitro* suppressive activity. The fact that $CD4^+CD25^-LAG3^+$ T cells hardly expressed CD103 (α_E integrin) and LAP on the cell surface indicated they were different from $CD103^+$ Treg cells and $CD4^+CD25^-LAP^+$ Treg cells, respectively (Ochi *et al.*, 2006; Zhu *et al.*, 2009). Collectively, these findings indicate that $CD4^+CD25^-LAG3^+$ T cells exert regulatory activity in an IL-10 dependent and Foxp3 independent manner.

Microarray analysis has revealed that the anergy-associated Egr-2 gene was significantly increased as well as the supposed signature genes for $CD4^+CD25^-LAG3^+$ such as *Lag3*, *Il10*, and *Prdm1* (B lymphocyte-induced maturation protein (Blimp)-1). Because $CD4^+CD25^-LAG3^+$ T cells were anergic in response to TCR stimulation, the increased expression of Egr-2 was particularly notable. As mentioned above, Egr-2 was recently reported as a key negative regulator of T cell activation and was necessary to induce a full anergic state through the actions of genes regulated by this transcription factor (Harris *et al.*, 2004; Safford *et al.*, 2005). The high expression levels of Egr2, LAG3, IL-10, and Blimp-1 genes in $CD4^+CD25^-LAG3^+$ T cells were confirmed by quantitative real-time PCR. These results were in line with the findings that T cell-specific Blimp-1 conditional knockout mice developed spontaneous colitis and naïve Blimp-1-deficient $CD4^+$ T cells were hyperproliferative in response to TCR stimulation and produced less IL-10 than did their wild-type counterparts (Kallies *et al.*, 2006; Martins *et al.*, 2006).

Interestingly, forced expression of Egr-2 in naïve $CD4^+$ T cells converted them to the $CD4^+CD25^-LAG3^+$ phenotype. Egr2-transduced

CD4$^+$ cells showed significant upregulation of Egr2, LAG3, IL-10, and Blimp-1 genes. In addition, Egr2-transduced CD4$^+$ cells produced significantly higher amounts of IL-10 and lower amounts of IL-2, IL-4, and IL-5 proteins. In spite of the expression of LAG-3 and IL-10 proteins, Egr2-transduced CD4$^+$ cells failed to exhibit sufficient suppressive activity in *in vitro* coculture with freshly isolated naïve CD4$^+$ responder T cells. On the other hand, in the DTH reaction of BALB/c mice against chicken OVA, Egr2-transduced BALB/c CD4$^+$ cells significantly suppressed DTH responses compared with empty-vector-transduced CD4$^+$ T cells. Moreover, OVA-specific DO11.10 CD4$^+$ T cells transduced with Egr-2 significantly suppressed DTH responses more efficiently than Egr2-transduced BALB/c CD4$^+$ T cells. These results indicated that antigen-specificity significantly contribute to the enhancement of suppressive activity in Egr2-transduced CD4$^+$ T cells.

One important question is whether CD4$^+$CD25 LAG3$^+$ T cells could develop through the thymic selection process in a similar manner to Foxp3$^+$ Tregs, which require a high-affinity agonistic interaction with self-peptide/MHCs expressed by thymic stromal cells (Coutinho *et al.*, 2005). RIP-mOVA/OT-II double-transgenic mice express a membrane-bound form of OVA in the pancreatic islets and also in the thymus together with a transgenic TCR (Vα2 and Vβ5.1) that recognizes the OVA $_{323–339}$ peptide in the context of I-A^b. In these mice, the frequency of CD4$^+$CD25 LAG3$^+$ T cells was not increased in the thymus and spleen, in contrast with an increase in the frequency of CD4$^+$CD25$^+$ Treg cells in these organs as reported previously (Coutinho *et al.*, 2005). Thus, unlike Foxp3$^+$ naturally occurring Treg cells, the development of CD4$^+$CD25 LAG3$^+$ T cells does not require high-affinity interactions with selecting peptide/MHC ligands expressed in the thymus.

On the other hand, germ free (GF) mice contained fewer CD4$^+$CD25 LAG3$^+$ T cells than specific pathogen free (SPF) mice in the spleen and PP. Though GF mice are exposed to self-antigens, to food-derived antigens, and to microbial particles from dead microorganisms in the sterilized food or bedding, the absence of viable microbiota affects the immune homeostasis (Tlaskalova-Hogenova *et al.*, 2004; Wen *et al.*, 2008). This result suggests the exposure to viable microbiota affects the development of CD4$^+$CD25 LAG3$^+$ T cells. The extrathymic development of IL-10-secreting T cells has already been reported (Maynard *et al.*, 2007). The profound decrease of CD4$^+$CD25 LAG3$^+$ T cells in the spleen and PP of GF mice showed the importance of environmental microbiota for the extrathymic development of CD4$^+$CD25 LAG3$^+$ T cells (Fig. 4.1). As discussed in Section 2.6, DCs exposed to microorganism-derived stimulation, such as PSA, TLR2-ligand, and TLR4-ligand, might be involved in the induction of CD4$^+$CD25 LAG3$^+$ T cells. The precise mechanisms

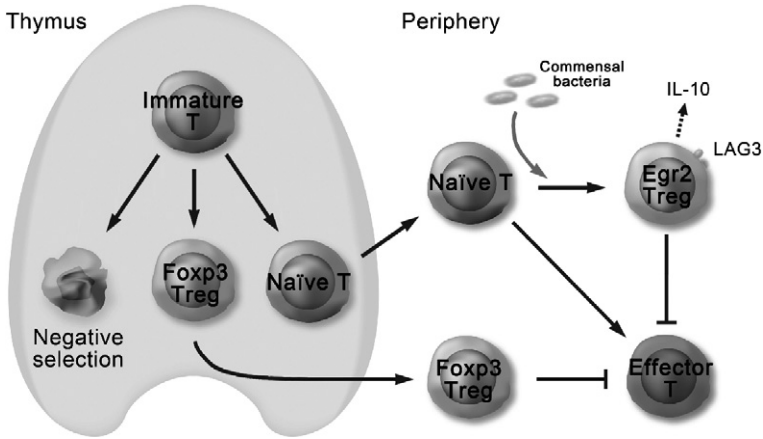

FIGURE 4.1 A model for the development of $CD4^+CD25^-LAG3^+$ Treg cells.

of the development of $CD4^+CD25^-LAG3^+$ Treg cells by environmental microbiota and APCs should be examined further.

Recently, two genome-wide association studies reported SNPs (single nucleotide polymorphisms) on chromosome 10q21 with a strong association to Crohn's disease (Rioux *et al.*, 2007; Wellcome-Trust-Case-Control-Consortium, 2007), a common form of IBD. The associated intergenic region is flanked by Egr-2, suggesting that this genetic variation could regulate Egr-2 expression. The particularly high production level of IL-10 by Egr-2-controlled $CD4^+CD25^-LAG3^+$ T cells suggests that this Treg population may contribute to the regulation of organ inflammation, especially in gut. Furthermore, T cell-specific Egr2-deficient mice showed enhanced expression of proinflammatory cytokines, increased Th1 and Th17 differentiation, and development of a late onset lupus-like autoimmune disease (Zhu *et al.*, 2008). By elucidating the function of Egr-2-dependent $CD4^+CD25^-LAG3^+$ Treg cells to produce a large amount of IL-10, they can be used for antigen-specific treatment of inflammatory disease. The features of $CD4^+CD25^-LAG3^+$ Treg cells are summarized in Table 4.1.

4. CONCLUDING REMARKS

Various kinds of IL-10-secreting $CD4^+$ T cells have been reported since the landmark report of Tr1 cells (Groux *et al.*, 1997). Some of these IL-10-secreting $CD4^+$ T cells are clearly independent from Foxp3 in their

TABLE 4.1 The features of $CD4^+CD25^-LAG3^+$ Treg cells

Characteristics of murine $CD4^+CD25^-LAG3^+$ T cells:
1. Naturally present in the spleen, lymph node, and Peyer's patch
2. Produce large amounts of IL-10 and moderate amounts of IFN-γ
3. Suppress colitis in an IL-10 dependent manner
4. Express Egr-2, an anergy associated transcription factor, which confers IL-10 production and *in vivo* suppressive activity on naïve $CD4^+$ T cells
5. Do not require high-affinity interactions with selecting peptide/MHC ligands expressed in the thymus for the development
6. Develop preferentially by the exposure to viable microbiota

function and development. Most IL-10-secreting $CD4^+$ T cells are characteristically associated with mucosal immunity, food tolerance, and antimicrobial immunity. It has been speculated that IL-10-secreting $CD4^+$ T cells, which are induced in the periphery, are important for controlling immune responses to nonself-antigens, while naturally occurring $CD4^+CD25^+$ Treg cells originating from the thymus are mainly responsible for the maintenance of self-tolerance (Roncarolo *et al.*, 2006).

The autoimmune regulator (Aire) is a gene responsible for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which influences the central induction of tolerance by regulating the clonal deletion of self-reactive thymocytes (Liston *et al.*, 2003). Aire regulates the ectopic expression of a battery of peripheral-tissue antigens, for example, insulin, fatty acid-binding protein, and salivary protein-1 (Anderson *et al.*, 2005). By an additional defect in central tolerance induction in scurfy mice, generated by crossing in a null mutation of the Aire gene, the range of affected sites was not noticeably extended, and many organs remained unaffected (Chen *et al.*, 2005). This result suggests the existence of additional important mechanisms other than central tolerance and the Foxp3 system to enforce immunological self-tolerance in the periphery. IL-10-secreting T cells may constitute such additional mechanisms that are responsible for peripheral tolerance.

However, there are lines of evidence that IL-10-secreting $CD4^+$ T cells also control immune responses to self-antigens. IL-10-secreting $CD4^+$ T cells specific for Desmoglein 3 (Dsg3) were isolated from 80% of healthy carriers of pemphigus vulgaris (PV)-associated HLA class II alleles but only 17% of PV patients (Veldman *et al.*, 2004). In the peripheral blood of patients with autoimmune hemolytic anemia, IL-10-secreting $CD4^+$ T cells specific for the red blood cell autoantigen, RhD protein, were detected (Hall *et al.*, 2002). Thus, the relationship and respective role of IL-10-secreting $CD4^+$ T cells and $CD4^+CD25^+$ Treg cells in the maintenance of self-tolerance has not been clarified sufficiently.

Recent evidence has revealed that even $CD4^+CD25^+Foxp3^+$ Treg cells show heterogeneity in their gene expression profiles (Hill *et al.*, 2007) and functional stages (Miyara *et al.*, 2009). Similarly, some IL-10-secreting $CD4^+$ T cells may represent a unique population of Treg cells arrested at different stages in development. However, assessing the *in vivo* physiological function of IL-10-secreting $CD4^+$ T cells is difficult, because of the lack of specific markers that can reliably differentiate a population of IL-10-secreting $CD4^+$ T cells from other T cells. LAP, NKG2D, IL-7R, and LAG-3 might be valuable lineage markers of IL-10-secreting $CD4^+$ T cells, which could be used in combination with molecular signatures such as Egr-2 and Blimp-1. In addition, the signaling pathways involved in the regulatory activity of IL-10-secreting $CD4^+$ T cells and the homing receptors governing their trafficking *in vivo* need to be examined. The identification of specific markers will enable us to purify and enrich IL-10-secreting $CD4^+$ T cells *ex vivo* and transfer them to the patients of inflammatory diseases.

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CHAPTER 5

Artificial Engineering of Secondary Lymphoid Organs

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Contents

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Abstract Secondary lymphoid organs such as spleen and lymph nodes are highly organized immune structures essential for the initiation of immune responses. They display distinct B cell and T cell compartments associated with specific stromal follicular dendritic cells and fibroblastic reticular cells, respectively. Interweaved through the parenchyma is a conduit system that distributes small antigens and chemokines directly to B and T cell zones. While most structural aspects between lymph nodes and spleen are common, the entry of lymphocytes, antigen-presenting cells, and antigen into lymphoid tissues is regulated differently, reflecting the specialized functions of each organ in filtering either lymph or blood. The overall organization of lymphoid tissue is vital for effective antigen screening and recognition, and is a feature which artificially constructed lymphoid organoids endeavor to replicate. Synthesis of artificial lymphoid tissues is an emerging field that aims to provide therapeutic application for the treatment of severe infection, cancer, and age-related involution of secondary lymphoid tissues. The development of murine artificial lymphoid tissues has benefited greatly from an understanding of organogenesis of lymphoid organs, which has delineated cellular and molecular elements essential for the recruitment and organization of lymphocytes into lymphoid structures. Here, the field of artificial lymphoid tissue engineering is considered including elements of lymphoid structure and development relevant to organoid synthesis.

ABBREVIATIONS

aAPC	artificial antigen-presenting cells
aLN	artificial lymph nodes
APC	antigen-presenting cells
DC	dendritic cells
ECM	extracellular matrix
ES	embryonic stem
FDC	follicular dendritic cells
FRC	fibroblastic reticular cells
HEV	high endothelial venules
HSC	hematopoietic stem cells
iBALT	inducible bronchus-associated lymphoid tissue
iPS	induced pluripotent stem cells
LN	lymph nodes
LT	lymphotoxin
LTi	lymphoid tissue inducer cells
LTo	lymphoid tissue organizer cells
LTβR	lymphotoxin-β receptor

MCS	multicellular spheroid culture
MRC	marginal reticular cells
MS	milky spot
MZ	marginal zone
PEG	polyethylene glycol
PP	Peyer's patches
RORγ	retinoic acid receptor-related orphan receptor γ
SLO	secondary lymphoid organs
TES	tissue-engineered spleen
T_{FH}	follicular B helper T cells
TLO	tertiary lymphoid organs
VEGF	vascular endothelial growth factor

1. INTRODUCTION

The adaptive immune response is orchestrated by interactions between antigen-presenting cells (APC) and effector T and B cells (Banchereau and Steinman, 1998). Generation of immune responses occurs most efficiently in secondary lymphoid organs (SLO) such as peripheral lymph nodes (LN), Peyer's patches (PP), and mucosal lymphoid tissues. Secondary lymphoid tissues display highly organized immunoarchitecture including conduit systems and T and B cell zones, which are conducive for efficient antigen distribution, screening, and effector cell proliferation. Numerous LN are strategically placed around the periphery to drain antigen from major tissue sites via the lymphatic system. A diversity of LN are present throughout the body that are distinct based on tissues sites which they drain, such as skin and mucosa. On the other hand, spleen is unique as an SLO that is not connected to the lymphatic system but is specialized for directly filtering antigens from blood (Mebius and Kraal, 2005). The development of SLO has also been well documented and is dependent on interactions between hematopoietic lymphoid tissue inducer (LTi) cells and nonhematopoietic lymphoid tissue organizer cells (LTo) (Drayton *et al.*, 2006; Randall *et al.*, 2008). Critically, SLO development depends on LTi cells which express membrane-bound lymphotoxin-$\alpha_1\beta_2$ (LT) interacting with stromal LTo cells bearing the corresponding LTβ receptor (LTβR). This signaling leads to an upregulation of adhesion molecules and chemokines on stromal organizer cells that positively enforce interactions between LTi and LTo, also recruiting new lymphocytes to the developing tissue forming organized cell clusters (Cupedo and Mebius, 2005).

An understanding of lymphoid tissue structure and development has advanced attempts to generate artificially synthesized lymphoid tissues.

Since SLO provide highly efficient platforms for initiation of immune responses, transplantation of artificially synthesized organoids is envisioned to benefit multiple immunotherapies including restoration of immune function in immunodeficient or aged patients, and enhancing specific immune responses to tumors or chronic diseases. For example, treatment of cancer is a contentious area with regards to the immunotherapeutic benefits of lymphadenectomy over LN preservation in cancer patients. Some reports suggest that specific cancer treatments, such as inoculation with *Mycobacterium bovis* Bacillus Calmette-Guérin cell wall skeleton (BCG-CWS), offers better survival to ovarian cancer patients with preserved rather than removed LN (Hayashi *et al.*, 2009). The application of artificial LN in these circumstances may augment patient immunity and afford prolonged survival. It is also known that LN inevitably degenerate with age (Pan *et al.*, 2008), such that artificial lymphoid tissues could in future replace aging LN in elderly patients. However, despite the promise for therapeutic application, the field of regenerative medicine is relatively new and only a few reports have documented the successful development of artificial lymphoid tissues in murine models (Grikscheit *et al.*, 2008; Okamoto *et al.*, 2007; Perez *et al.*, 2002; Suematsu and Watanabe, 2004). Here, we review the structure and development of SLO and consider factors important for artificial LN and spleen tissue syntheses.

2. LYMPHOID STRUCTURE AND ELICITATION OF IMMUNE RESPONSES

2.1. Stromal cell networks

The microarchitecture of secondary lymphoid tissues is critically important in response to antigen challenge, thus loss of structure is typically a hallmark of immune dysfunction (Scandella *et al.*, 2008). Segregation of cells into separate B and T cell compartments is considered dependent on distinct nonhematopoietic stromal cells which secrete specific chemokines for attracting B and T cells, respectively. Lymphocytes bearing specific receptors migrate along these chemokine gradients to assemble in discrete cellular compartments (Cyster, 1999). In the resting state, follicular dendritic cells (FDC) are located in the center of primary B cell follicles and function in both B cell organization and antigen presentation leading to a selection of B cells expressing a high-affinity antigen receptor (Cyster *et al.*, 2000). Recruitment of B cells is mediated by the homeostatic release of chemokine CXCL13 by FDC, which binds to CXCR5 expressed on B cells (Ansel *et al.*, 2000). Follicular DC are also believed to present antigen to B cells in a native, unprocessed form that is complexed to

antibody (Aydar *et al.*, 2005). T cell zones are maintained by a network of fibroblastic reticular cells (FRC) (Katakai *et al.*, 2004b). A three-dimensional reticular network in lymphoid tissues provides structural framework for migrating T cells through secretion of CCL21 and CCL19 chemokines, attracting T cells expressing the cognate CCR7 receptor (Forster *et al.*, 1999). Antigen-loaded cells such as DC that migrate from peripheral tissue sites into LN through lymphatics also express CCR7, facilitating their localization to T cell areas thus permitting antigen scanning by naïve T cells (Cyster, 1999; Gunn *et al.*, 1999).

2.2. Conduit system

A conduit system also exists in LN (Gretz *et al.*, 2000), spleen (Nolte *et al.*, 2003), and thymus (Drumea-Mirancea *et al.*, 2006) which is specialized for transporting small antigens rapidly to T and B cell zones (reviewed by Roozendaal *et al.*, 2008). The conduit system, which corresponds with the FRC network, transports small antigens and chemokines through a lumen formed from extracellular matrix (ECM) and collagen fibers enclosed in ER-TR7^{+} FRC (Nolte *et al.*, 2003). Only low-molecular weight antigens gain access to the conduit system (Nolte *et al.*, 2003; Roozendaal *et al.*, 2009), whereas larger molecules are selectively excluded and captured in the spleen marginal zone (MZ) or subcapsular sinus of LN by APC. The reticular network and conduit system extend mainly throughout the T cell area of lymphoid tissue eventually connecting to high endothelial venules (HEV) in LN (Katakai *et al.*, 2004a), however, a less-dense network of conduit also reaches B cell follicles transporting antigen directly to FDC and B cells (Roozendaal *et al.*, 2009). The inner lumen of the conduit system is largely isolated from the external lymphoid microenvironment, however, at intermittent intervals myeloid DC interrupt by extending dendritic projections into the conduit, actively sampling and processing antigen that is presumably presented directly to T cells (Sixt *et al.*, 2005).

2.3. Lymphatics and blood vessels in lymph nodes and spleen

Cell entry into LN is regulated by unique vasculature deriving from both blood and lymphatics. Naïve lymphocytes circulating in the bloodstream typically extravasate across specialized blood vessels called HEV that open into the cortical ridge of LN surrounded by T and B cell zones (Miyasaka and Tanaka, 2004; Mueller and Germain, 2009). Lymphocyte migration across HEV is facilitated by the expression of vascular addressins such as peripheral node addressin (PNAd) and chemokines including CCL19 and CCL21, which attract lymphocytes expressing L-selectin and CCR7 cognate receptors, respectively (Butcher *et al.*, 1999). In mucosal

lymphoid tissues and PP, HEV express mucosal addressin cell adhesion molecule-1 (MAdCAM-1) enabling specific migration of lymphocytes expressing $\alpha_4\beta_7$ integrin (Nakache *et al.*, 1989). In contrast, APC and antigen can enter LN through afferent lymphatics which channel directly into the subcapsular sinus of LN (Mueller and Germain, 2009).

Spleen is an organ which serves dual purpose in filtering blood for damaged and aging erythrocytes, and in surveillance for blood-borne foreign pathogens (Mebius and Kraal, 2005). These functions are reflected in the structure of spleen which is grossly divided into red and white pulp areas, the latter corresponding to the more immunologically active area of spleen. Accordingly, spleen white pulp shares a high structural similarity with LN, displaying organized T and B cell zones and a conduit system (Mebius and Kraal, 2005; Nolte *et al.*, 2003). However, in terms of cell entry, differences emerge as spleen lacks afferent lymphatics and HEV, but contains a distinct vasculature upon which immune cells and particulates enter via blood. A central artery entering spleen divides progressively into smaller branches and terminates in central arterioles in the MZ and red pulp (Mebius and Kraal, 2005). The MZ interfaces between the red pulp and white pulp and consists abundantly of phagocytic macrophages, MZ B cells and DC, serving as a prominent site for uptake and surveillance of blood-borne antigens that drain into spleen (Kraal and Mebius, 2006). This is important since spleen resident APC typically collect and present blood antigens directly to T cells in the white pulp (Villadangos and Heath, 2005), in contrast to LN-based antigen presentation where APC migrate from peripheral sites to regional LN to induce T cell activation (Henri *et al.*, 2001). Thus, while LN and spleen white pulp share similar lymphoid architecture, subtle differences exist in cell entry and antigen presentation which may be important to consider in design and artificial construction of each tissue type.

3. ORGANOGENESIS OF SECONDARY LYMPHOID TISSUES

3.1. Organogenesis in embryonic stages

Lymphoid structures which mimic SLO are highly desirable in artificially engineered tissues. Synthesis of lymphoid tissues therefore benefits from an understanding of SLO development that essentially relies upon cell organization into discrete compartments. The molecular signaling events leading to secondary lymphoid tissue organogenesis have been well covered in a series of reviews (Blum and Pabst, 2006; Cupedo and Mebius, 2005; Mebius, 2003; Randall *et al.*, 2008) and will be briefly discussed here, with a larger focus on adult lymphoid tissue development. In general, embryonic SLO organogenesis depends on two fundamental cell types,

hematopoietic $CD45^+CD3^-CD4^+ckit^+LT^+$ LTi cells (Finke, 2005; Mebius *et al.*, 1997) and nonhematopoietic $CD45^-VCAM^+ICAM^+LT\beta R^+$ stromal LTo cells (Cupedo *et al.*, 2004b). Clustering of both cell types in LN anlagen and ligation of LTβR on stromal LTo by LT-$\alpha_1\beta_2$ lead to activation of two NF-κB signaling cascades that result in the stromal expression of adhesion molecules such as VCAM-1 and homeostatic chemokines including CCL19, CCL21, and CXCL13 (Dejardin *et al.*, 2002; Honda *et al.*, 2001; Ngo *et al.*, 1999). Chemokine feedback further enforces the expression of LT-$\alpha_1\beta_2$ on LTi creating a positive feedback loop between organizer and inducer cells, also recruiting B and T cells to the developing LN anlagen via chemotactic attraction. Endothelial cell differentiation forming HEV and segregation of migrating lymphocytes into T and B cell clusters then form the basis of secondary lymphoid structure.

Interactions leading to SLO organogenesis have been largely delineated with the aid of gene-targeted mouse models. Both LT-α and LT-β subunits of LT were established as essential molecules for peripheral LN and PP development, with mice deficient in either subunit lacking both LN and PP (Banks *et al.*, 1995; De Togni *et al.*, 1994; Koni *et al.*, 1997). In LT-β deficient mice, less severe defects were apparent with normal development of mesenteric and cervical LN, in contrast LT-α deficient mice largely lacked these tissues. In contrast, spleen was present in both mouse models but displayed in abnormal white pulp structure with loss of MZ and T and B cell segregation, defects which were less pronounced in LT-$\beta^{-/-}$ mice (Banks *et al.*, 1995; De Togni *et al.*, 1994; Koni *et al.*, 1997). Consistent with these findings, mice deficient in LT receptor, LTβR, also lacked LN and PP, and showed abnormal spleen white pulp organization (Futterer *et al.*, 1998). Other signaling molecules such as TRANCE are also required for LN development. It has been reported that peripheral and mesenteric LN are absent in TRANCE$^{-/-}$ mice (Kim *et al.*, 2000). However, spleen exhibits less structural defects compared to LT- or LTβR-deficient mice which possess typical T and B cell organization, but disruptions in MZ formation. Deficient TRANCE expression also correlates with a reduction in LTi cells, which results in a lack of LN (Kim *et al.*, 2000). Transcription factors have also been identified which are crucial for LN and PP development. Mice deficient in retinoic acid receptor-related orphan receptor (RORγ) completely lack LN and PP, however, spleen white pulp structure is largely unaffected (Eberl *et al.*, 2004; Sun *et al.*, 2000). Similarly, Id2$^{-/-}$ mice also show an absence of LN and PP, while spleen displays normal immunoarchitecture (Fukuyama *et al.*, 2002; Yokota *et al.*, 1999). In both models, lack of LN and PP development is associated with a failure to produce embryonic LTi cells which normally express both RORγ and Id2. Milky spots (MS) represent a unique, omentum-based SLO mediating peritoneal immune responses (Rangel-Moreno *et al.*, 2009). Not only are MS distinct to LN and PP in terms of structure and

function, but also they develop independently LTi cells as demonstrated by their presence in RORγ $^{-/-}$ and Id2 $^{-/-}$ mice (Rangel-Moreno *et al.*, 2009). However, MS require both LT and CXCL13 expressions for development, as they are smaller or even absent in CXCL13 $^{-/-}$ or LTα $^{-/-}$ mouse models (Rangel-Moreno *et al.*, 2009).

3.2. Adult-stage generation of lymphoid tissues

Organogenesis of adult lymphoid tissues appears to occur by the same general mechanisms displayed in embryonic development (Cupedo and Mebius, 2003). Adult CD3 CD4^{+} LTi cells have been identified in spleen, which promote organization of T and B cell structures in secondary lymphoid tissues (Kim *et al.*, 2005, 2006, 2007). Compared to neonatal LTi, expression of OX40L and CD30L which supports CD4^{+} T cell survival is evident on adult LTi counterparts (Kim *et al.*, 2003, 2005, 2006). Moreover, in contrast to the common view that LTi are characteristically CD3 CD4^{+} cells (Finke, 2005; Kim *et al.*, 2007), two populations of CD4 and CD4^{+} adult LTi have now been identified (Kim *et al.*, 2008). Apart from CD4 expression, both adult LTi populations share similar gene and cell surface marker expressions, characterized as lineage negative (CD3 CD8 CD27 B220 CD11c) Thy^{+}CD4 $^{-/+}$ cells, expressing ckit^{+}OX40L^{+}CD30L^{+}CD69^{+} markers (Kim *et al.*, 2008). Adult organizer-like stromal cells have also been identified in multiple SLO including LN, spleen, and PP (Katakai *et al.*, 2008). Designated marginal reticular cells (MRC), these cells localize to a layer underneath the subcapsular sinus of LN or MZ of spleen, often adjacent to B cell follicles. MRC express VCAM-1, ICAM-1, MAdCAM-1, and TRANCE markers, sharing high similarity to embryonic LTo. Histological analysis of developing neonatal LN and spleen white pulp also demonstrated an outward expansion of MRC from LTo stromal cells in newborn tissue (Katakai *et al.*, 2008). However, the function of adult MRC is less clear and is presumably involved in lymphoid structure maintenance rather than organogenesis. Furthermore, MRC differ between SLO since inhibition of LTβR signaling by LTβR-Fc treatment disrupts MRC structure in spleen white pulp, whereas MRC in LN are largely unaffected (Katakai *et al.*, 2008). Therefore, the functional role of MRC between different lymphoid organs may also differ.

3.3. Ectopic tertiary lymphoid development

Tertiary lymphoid organs (TLO) are adult-stage temporary immune structures that display SLO organization but can develop in nonlymphoid tissue sites (Drayton *et al.*, 2006). Tertiary lymphoid development can be

mimicked by ectopic expression of certain factors such as CXCL13, CCL19, CCL21, CXCL12, and IL-7 (Fan *et al.*, 2000; Luther *et al.*, 2000, 2002; Meier *et al.*, 2007). Transgenic mice expressing CXCL13 in the pancreas attract B cells and lead to development of lymphoid-like tissue with T and B cell clusters, HEV, and stromal cell networks (Luther *et al.*, 2000). A role for LT in ectopic lymphoid development was further demonstrated by blocking signaling via LTβR-Ig fusion protein which reversed formation of LN-like structures, a signal likely provided by B cells since crossing mice to a B-cell deficient mouse strain also led to a reduction in LN development (Luther *et al.*, 2000). Expression of T cell chemokine CCL21 in the pancreas also induced formation of ectopic lymphoid structures (Fan *et al.*, 2000; Luther *et al.*, 2002), a phenomenon also observed with CCL19 expression in the pancreas albeit to a lesser extent (Luther *et al.*, 2002). Interestingly, expression of CXCL12, which induced only small and infrequent pancreatic lymphoid infiltrates, displayed a preferential attraction for DC and plasma cells. A key cytokine for LTi cell function is IL-7, which signals through IL-7R expressed on LTi to initiate SLO organogenesis (Yoshida *et al.*, 2002). Overexpression of IL-7 in transgenic mice led to enhanced survival of LTi and increased formation of PP and ectopic LN (Meier *et al.*, 2007). In addition, transfer of neonatal LN single-cell suspensions into adult recipients also induced ectopic tertiary lymphoid formation in skin, however, T and B cell segregation only occurred after immune activation (Cupedo *et al.*, 2004a). Consistent with ectopic expression studies, hematopoietic cells present in tertiary lymphoid structures emigrated from the host, in contrast stromal elements such as VCAM-1$^+$ICAM-1$^+$ cells and MAdCAM-1$^+$ HEV remained of donor origin (Cupedo *et al.*, 2004a).

3.4. Spleen development

Insight into the cellular requirements for spleen development has come from transplantation of embryonic day 15 (E15) spleen into the kidney capsule of adult mice (Glanville *et al.*, 2009). Following transplantation, normal white pulp formation was evident after 4 weeks containing hematopoietic cells segregated into distinct T and B cell zones. Interestingly, comparable transplant of E15 inguinal LN into adult kidney capsules failed to show lymphoid tissue development (White *et al.*, 2007), indicating distinct host-derived cellular or molecular requirements between spleen and LN organogenesis. To determine the requirement for LTi cells and expression of LT for spleen development, embryonic spleens from mutant LTα$^{-/-}$ mice were transplanted into wild-type hosts. E15 LTα$^{-/-}$ spleen grafts displayed splenic development comparable to wild-type grafts, indicating that LT signaling from adult host-derived

inducer-like cells was sufficient to promote spleen organogenesis which is initiated by embryonic stromal cells (Glanville *et al.*, 2009). In terms of artificial spleen engineering, donor spleen stromal cells alone appear to be adequate to support spleen organogenesis, driven by host-derived, LT-expressing adult inducer-like cells.

The adult inducer cells that promote spleen organization are likely to correspond to CD11c CD3 $CD4^{+}OX40L^{+}CD30L^{+}$ adult LTi-like cells (Kim *et al.*, 2007, 2008), rather than B cells which also express LT. Transfer of LTi-like cells but not splenocytes (including B cells) induces formation of T and B cell compartments in spleen of $LT\alpha^{-/-}$ mice (Kim *et al.*, 2007). Similarly, transfer of $LT\alpha^{-/-}$ splenocytes into $Rag^{-/-}$ mice leads to B and T cell segregation, indicating B cells expressing LT are not required for initial cellular organization (Withers *et al.*, 2007). In contrast, the maintenance of spleen structure once assembled may differ in LT requirements. In this case, a function for B cells expressing LT-$\alpha_1\beta_2$ has been demonstrated in maintaining signaling required for cellular organization. In particular, spleen MZ organization is critically regulated by LT expressed on B cells as demonstrated by mice with a B cell-specific deletion of the LTβ gene (B-LTβ KO mice) (Tumanov *et al.*, 2002). In these mice, reductions in B cells, MOMA-1^{+} metallophilic macrophages, and ER-$TR9^{+}$ MZ macrophages were observed compared to wild-type mice. A later study also supported the importance of B cells in both MZ development and maintenance (Nolte *et al.*, 2004). Additionally, the presence of FDC was also largely reduced in B-LTβ KO mice, demonstrating a role for LT^{+} B cells in FDC development. In contrast, B-LTβ KO mesenteric LN immunized with sheep red blood cells (SRBC) showed FDC clustering comparable to wild-type mice, indicating divergent roles for LT^{+} B cells in spleen and LN FDC development. Furthermore, lethally irradiated $LT\alpha^{-/-}$ mice reconstituted with a mixture of bone marrow (BM) from $LT\alpha^{-/-}$ and either $BCR^{-/-}$ or $TCR^{-/-}$ mice showed that B cells expressing LTα, but not T cells are required for the development of spleen FDC clusters (Fu *et al.*, 1998).

The tissue distribution of adult LTi-like cells has been reported in various mouse models. Adult LTi-like cells are normally present in spleen (Kim *et al.*, 2008) but are absent in BM of $Rag^{-/-}$ mice and blood of CD3ε-transgenic mice (Glanville *et al.*, 2009), mouse models that lack mature lymphocyte subsets allowing more sensitive detection of LTi. Despite an absence of LTi-like cells in BM, reconstitution of irradiated CD3ε mice with $Rag^{-/-}$ BM cells results in differentiation of donor adult LTi-like cells, suggesting that they indeed derive from BM progenitors. These observations can be interpreted through a model whereby adoptively transferred BM progenitors engraft into spleen hematopoietic niches where they are directed to develop by local signals into LTi-like cells. Precedent exists for spleen microenvironments supporting hematopoiesis from BM progenitors (Despars and O'Neill, 2006b; Periasamy *et al.*, 2009)

which could explain why adult LTi-like cells are selectively present in spleen, but not BM or circulating in blood. Furthermore, E15 inguinal LN grafted into adult mouse kidney capsules, which normally colonized during embryogenesis by LTi (White *et al.*, 2007), may not be supported in adult mice due to a lack of circulating LTi-like cells and absence of hematopoietic niches which sustain LTi progenitor development. In contrast, spleen posseses a hematopoietic microenvironment (Dor *et al.*, 2006) that could support LTi differentiation.

4. ENGINEERING ARTIFICIAL SECONDARY LYMPHOID TISSUE

4.1. *In vitro* synthesis of lymphoid structures

Tissue culture systems have provided an accessible means for studying cell development and function *in vitro*. In this respect, traditional two-dimensional tissue culture in plates or flasks has been successfully used for maintenance of specific cell lineages (O'Neill *et al.*, 2004). On the other hand, recreation of complex multicellular interactions evident in secondary lymphoid structures requires development of alternative culture systems. *In vitro* culture systems for this purpose are largely concerned with two main features, three-dimensional culture systems that promote more physiologically relevant cell-to-cell interactions, and scaffold materials that mimic ECM networks upon which cells can attach and migrate. Multicellular spheroid culture (MCS) is a three-dimensional culture technique that takes advantage of the tendency for some mammalian cell types to aggregate when cultured in suspension (Lin and Chang, 2008). Cells grown in MCS display features distinct from equivalent monolayer cultures in terms of gene expression and cell function, which more accurately represent physiological tissue (Lin and Chang, 2008). A bioreactor system has also been developed that comprises central and outer culture spaces, the central culture space supporting matrix sheets that allow three-dimensional cell adherence and migration. Using DC-loaded matrices and following leukocyte inoculation, bioreactor-assisted cell culture promotes effective leukocyte clustering that is responsive to LPS treatment, demonstrating the development of functional lymphoid-like structure (Giese *et al.*, 2006).

4.2. Scaffold materials

The scaffold material used in culture systems including bioreactors has been subject to much consideration. Scaffold materials tested *in vitro* in cell culture include agarose, alginate, polyamide, polyethylene glycol

(PEG)-based hydrogels, CellFoam, and collagen (Clark *et al.*, 2005; Giese *et al.*, 2006; Gonen-Wadmany *et al.*, 2007; Irvine *et al.*, 2008; Poznansky *et al.*, 2000; Shapiro and Cohen, 1997; Stachowiak and Irvine, 2008; Zimmermann *et al.*, 2007). For bioreactor tissue culture, Giese *et al.* (2006) reported that collagen and polyamide materials best supported cell attachment, growth, and stability. The use of composite biosynthetic scaffolds combining biological macromolecules with synthetic polymer scaffolds is now being investigated (Dikovsky *et al.*, 2006). One approach combining PEG hydrogels with fibrillar collagen matrix infused into the pores has proven efficient for supporting T cell and DC migration (Stachowiak and Irvine, 2008). Moreover, naïve T cell migration was improved with CCL21 anchored to the PEG scaffold. PEG-conjugated collagen and fibrin, but not albumin, were also shown to sustain smooth muscle cell migration within a hydrogel network (Dikovsky *et al.*, 2006; Gonen-Wadmany *et al.*, 2007). Composite scaffolds appear advantageous for *in vivo* application since the biological activity of ECM proteins, important for cell adhesion and migration, is coupled with the mechanical strength of synthetic scaffolds prolonging otherwise rapid ECM biodegradation.

The design and fabrication of scaffolds are achieving greater control with the aid of computers and automated bioprinting techniques (Hollister, 2005). Three-dimensional scaffold fabrication using layer-by-layer nozzle-based printing has in some applications shown superior efficacy to older processing techniques, such as murine cartilage regeneration using Bioplotter-fabricated PEG/PBT scaffolds compared with the same scaffold fabricated by porogen leaching (Hollister, 2005). Organ printing is a further approach to layer-by-layer scaffold fabrication using tissue spheroid units rather than synthetic molecules as building blocks. This process forgoes synthetic scaffolds altogether, instead relying on close placement of cells and subsequent cell–cell fusion to form three-dimensional structures (Boland *et al.*, 2006; Mironov *et al.*, 2003, 2009). An advantage of direct cell printing is that controlled placement of multiple cell types may in future facilitate construction of intraorgan blood vessels, providing a possible option for vascularization of artificial lymphoid tissues.

At present, an elegant study has demonstrated the immense potential for synthetic scaffolds to encourage vascularization and development of artificial tissue *in vivo* (Richardson *et al.*, 2001). A polymer (polylactide-coglycolide) scaffold was engineered to incorporate multiple vascular growth factors that were released into the tissue microenvironment at different rates. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are distinct angiogenic growth factors, supporting early and late stages of blood vessel formation, respectively (von Tell *et al.*, 2006; Yancopoulos *et al.*, 2000). To incorporate these

growth factors into scaffolds, polymer particles were mixed with both lyophilized VEGF which permitted rapid release of growth factor, and premicrosphere encapsulated PDGF designed to delay growth factor release. Once scaffolds were processed and implanted into rats, controlled release of each growth factor led to a stable and mature vasculature formation, an outcome which could not be replicated by bolus injection of both growth factors simultaneously, or by scaffold-released delivery of either growth factor alone (Richardson *et al.*, 2001). Therefore, artificially engineered lymphoid tissues which, like other avascular tissue implants, are restricted by size due to limited oxygen diffusion, could benefit greatly from induced blood vessel formation provided by scaffold-incorporated controlled growth factor release (Griffith and Naughton, 2002).

4.3. Trials to generate *in vivo* artificial lymphoid organs

The ability to engineer artificial lymphoid tissue is an attractive prospect for immunotherapy, however, few reports have described successful *in vivo* lymphoid tissue synthesis. In extension to *in vitro* culture systems, one strategy for constructing *in vivo* artificial tissue involves the use of biocompatible three-dimensional scaffolds, which provide a structural basis for predonor cell attachment as well as subsequent space and framework for lymphocyte emigration and tissue formation. Artificial tissues successfully generated utilizing this technique include LN (Okamoto *et al.*, 2007; Suematsu and Watanabe, 2004), mucosal immune tissues (Perez *et al.*, 2002), and spleen (Grikscheit *et al.*, 2008).

Synthesis of *in vivo* primary lymphoid tissue has yet been reported, however, tissue-engineered thymic organoids have been successfully generated *in vitro* (Clark *et al.*, 2005; Poznansky *et al.*, 2000). Artificial thymic microenvironments were developed by seeding mouse thymic stromal cells (Poznansky *et al.*, 2000) or human skin-derived fibroblasts and keratinocytes (Clark *et al.*, 2005) onto a CellFoam matrix, a biodegradable three-dimensional carbon matrix fabricated by high-temperature precipitation of tantalum. These matrices possess an open pore structure permissive for infiltration and integration of cells into engraftment sites. Coculture with human BM-derived $CD34^+$ hematopoietic progenitor cells generate mature and functional $CD4^+$ or $CD8^+$ T cells after 2–3 weeks, which importantly express a large repertoire of T cell receptors. In this system, cell–cell associations provided by three-dimensional thymic architecture critically gave rise to efficient T cell differentiation from human hematopoietic stem cells (HSC). These studies offer promise for artificial generation of diverse and functional T cell populations that can be applied therapeutically in immunosuppressed patients.

4.4. Artificial mucosal tissue

Tissue-engineered neogenesis of small intestine has previously been investigated as a potential therapy for treating short bowel syndrome (Choi and Vacanti, 1997), however, concurrent development of mucosal immune tissue has also been demonstrated through neointestine synthesis (Perez *et al.*, 2002). This technique involves disaggregation of neonatal 3-day-old rat small bowel followed by seeding onto biodegradable polymer tubes created from polyglycolic acid fibers coated with collagen. Constructs are then implanted into the omentum of adult recipients and anastomosed after 4 weeks to the native jejunum. After 20 weeks, the presence of $CD3^+$ T cells, $CD32^+$ B cells, $CD56^+$ NK cells, and $CD68^+$ macrophages was detected in tissue-engineered neointestines comparable to that of native jejunum, confirming artificial mucosal tissue with immune potential had developed.

4.5. Artificial spleen

Construction of artificial splenic tissue is a highly attractive prospect as spleen autotransplantations are widely performed in splenectomized individuals to preserve partial immune function to blood-borne infections (Yamataka *et al.*, 1996). However, transplantation of spleen fragments is highly inefficient, associated with mass tissue necrosis followed by gradual tissue regeneration (Pabst *et al.*, 1991). To gain a better understanding of the cellular processes involved in spleen regeneration, spleen transplantations have been widely studied in animal and murine models (Marques *et al.*, 2002; Miko *et al.*, 2007). Construction of tissue-engineered spleen (TES) is a recent advancement in murine spleen transplantation technology, where disaggregated spleen units from neonatal 6-day-old rats are loaded onto a polyglycolic acid scaffold coated with collagen and transplanted into the omentum (Grikscheit *et al.*, 2008). Unlike spleen fragment transplantation, organogenesis of TES proceeds without early phase necrosis yet results in formation of normal spleen structure, including red and white pulp areas. Furthermore, the immunological function of TES has been demonstrated by higher survival rates of TES-transplanted recipients upon challenge with pneumococcal infection compared with splenectomized controls (Grikscheit *et al.*, 2008).

The protective function of spleen autotransplants is a somewhat contentious area although it is generally assumed to associate with donor age, which in turn correlates with the successful development of organized splenic white pulp structure (Willfuhr *et al.*, 1992). Autotransplants of neonatal origin that display immune function including tissue-engineered (Grikscheit *et al.*, 2008) and fragmented spleen (Willfuhr *et al.*, 1992) are associated with normal white pulp structure, whereas

spleen transplants from older donors display poorly developed white pulp structure and loss of immunological function (Willfuhr *et al.*, 1992). Therefore, it is important to dissect which cellular components in neonatal spleen tissue efficiently initiate white pulp formation, and translate these findings to adult spleen tissue. Clearly, the majority of splenocytes present in spleen transplants are not required for tissue regeneration. This is evidenced by mass necrosis of spleen fragments after initial transplantation, leaving behind a few surviving stromal cells, lymphocytes, and macrophages (Westermann and Pabst, 1997). Similarly, transplanted-LN undergo initial tissue destruction before regeneration of lymphoid microarchitecture (Hammerschmidt *et al.*, 2008). Transplantation with GFP^+ donor LN further demonstrated that tissue regenerates with host-derived GFP hematopoietic lymphocytes, but importantly with donor origin stromal cells as shown by GFP^+ colocalization with FRC markers glycoprotein podoplanin (gp38) and ER-TR7 (Hammerschmidt *et al.*, 2008). Furthermore, transplantation of embryonic spleen also suggests that spleen stromal cells alone are sufficient to promote lymphoid regeneration via host-derived hematopoietic cells (Glanville *et al.*, 2009). As hematopoietic cells comprise an overwhelming proportion of whole splenocytes, and considering that current artificial and autologous spleen transplantation techniques are heavily restricted by graft size such that only a fraction of spleen can be successfully transplanted, it is tempting to speculate a technique which enriches total nonhematopoietic spleen cells followed by implantation could improve the outcome of lymphoid tissue development, especially from adult spleen. Considering the potential role for stromal cells in spleen organogenesis, investigation of characterized spleen stromal cell lines which support hematopoiesis (Despars and O'Neill, 2006a) may be beneficial for future development of artificial spleen.

4.6. Artificial lymph nodes

A more calculated approach to artificial LN construction that recognizes the roles of stromal and hematopoietic cells in lymphoid tissue organogenesis was performed by Suematsu and Watanabe (2004). A thymic stromal cell line, TEL-2, was initially selected as a stromal backbone for supporting thymic organoid development (Nakashima *et al.*, 1990). TEL-2 cells, which express LTβR and adhesion molecules such as VCAM-1, were further transfected to express LT-α, mimicking signaling performed by LTi cells in SLO neogenesis. TEL-2–LTα stromal cells embedded into a collagenous scaffold prepared from freeze-dried bovine Achilles tendon and implanted into the kidney subcapsule space of recipient mice successfully generated a microenvironment conducive for attracting hematopoietic cells and forming secondary lymphoid structure (Suematsu and

Watanabe, 2004). Moreover, inclusion of activated BM-derived DC into the scaffold further augmented formation of lymphoid structure. Artificial organoids resembled normal SLO containing organized B and T cell clusters, germinal centers, FDC networks, FRC networks (including conduit systems), and HEV-like structures. They also displayed effective immunological function demonstrated by the production of high-affinity antigen specific IgG_1 antibody following inoculation and antigen restimulation (Suematsu and Watanabe, 2004). Artificial LN were also transplantable to immunodeficient SCID mice where they elicited strong secondary immune responses, resulting in the enrichment of memory B cells and production of large amounts of high-affinity antigen-specific IgG class antibodies (Okamoto *et al.*, 2007). In addition, a population of $CD44^{hi}CD62L^{lo}$ memory-type $CD4^+$ T cells was also highly enriched in aLN (Okamoto *et al.*, 2007), which, by gene expression profile and flow cytometry analysis, appeared to correspond with follicular B helper T (T_{FH}) cells (Akiba *et al.*, 2005; Chtanova *et al.*, 2004; Fazilleau *et al.*, 2009; Nurieva *et al.*, 2008; Reinhardt *et al.*, 2009; Yu *et al.*, 2009). Overall, these results demonstrate the feasibility of constructing artificial lymphoid tissues from biocompatible scaffolds embedded with stromal cells and DC which serve to attract host cells and form secondary lymphoid structures.

Organogenesis of aLN may be further improved with growth factor therapy to enhance lymphatic and blood vessel formation. Injection of platelet-rich plasma was shown to enhance viability and regeneration of transplanted LN fragments in rats, presumably due to undefined growth factors which promote lymphangiogenesis (Hadamitzky *et al.*, 2009). Moreover, the specific expression of VEGF-C on LN prior to transplantation demonstrated a significant improvement in connections of transplanted LN to the host lymphatic vasculature (Tammela *et al.*, 2007). Scaffolds which control release of various angiogenic growth factors could also find application in aLN organogenesis to promote blood vessel formation (Richardson *et al.*, 2001). The nature of stromal cells used in aLN may also require consideration since stromal elements that direct immune responses are distinct between different LN (Ahrendt *et al.*, 2008; Hammerschmidt *et al.*, 2008). Finally, while stromal cells are well recognized for providing a cellular backbone for lymphoid neogenesis and cell organization, more consideration may in future be given to the role of DC in promoting T and B cell clusters in artificial tissue (Suematsu and Watanabe, 2004). Recently, TLO such as inducible bronchus-associated lymphoid tissue (iBALT) were shown to require DC for structural maintenance, with selective depletion of $CD11c^+$ DC using a diphtheria toxin receptor (DTR) transgenic mouse model resulting in abolition of iBALT (GeurtsvanKessel *et al.*, 2009). Myeloid DC present in iBALT expressed high levels of homeostatic chemokine mRNA CXCL12, CXCL13, CCL19,

and CCL21 (GeurtsvanKessel *et al.*, 2009), suggesting they play a role in B and T cell organization, a function typically associated with stromal cells in SLO. Moreover, these myeloid DC expressed high levels of LT-β indicating that they may also signal to stromal organizer cells via LTβR ligation, further supporting a role for DC in TLO organization. Consistent with these observations, repeated intratracheal injections of GM-CSF-cultured BM-DC also induced formation of iBALT structures (GeurtsvanKessel *et al.*, 2009). In light of this broader role for DC in lymphoid organogenesis, multiple DC subsets and activation states have been characterized (Tan and O'Neill, 2005) which may provide further scope for optimization of artificially synthesized lymphoid tissue.

5. FUTURE PROSPECTS

Current progress into the generation of artificial lymphoid tissues is promising highlighted by the development of artificial LN from highly characterized stromal components and factors (Suematsu and Watanabe, 2004). Furthermore, there appears to be excellent promise for generation of alternate lymphoid tissue such as artificial spleen (Grikscheit *et al.*, 2008). A translational step into the clinical setting is now timely but may require several intermediate studies such as large animal modeling to determine proper feasibility. However, one model to assess the development and function of human artificial lymphoid tissues is transplantation into ''humanized mice'' (Manz and Di Santo, 2009). Humanized mice are murine chimeras possessing human hematopoietic cells generated by the adoptive transfer of human stem cells into various immunodeficient mouse strains (Ishikawa *et al.*, 2005; Ito *et al.*, 2002; Shultz *et al.*, 2005; Traggiai *et al.*, 2004). These mouse models have historically suffered from low levels of human leukocyte chimerism, however, in recent years the efficiency has improved dramatically allowing humanized mice to become an effective model for studying several aspects of human immunology (Ishikawa *et al.*, 2007; Legrand *et al.*, 2009; Shultz *et al.*, 2007; Unsinger *et al.*, 2009; Zhang *et al.*, 2007). Development of human artificial lymphoid tissue in these models may be one approach to demonstrate immunological efficacy prior to consideration in human trials.

New developments in stem cell technology could facilitate the use of induced pluripotent stem (iPS) cells for human artificial lymphoid tissue engineering, owing to the potential for iPS to differentiate into multiple cell lineages (Takahashi *et al.*, 2007). Embryonic stem (ES) cells can also generate multiple cell lineages (Cho *et al.*, 1999; de Pooter *et al.*, 2003; Nakano *et al.*, 1994; Nakayama *et al.*, 1998; Schmitt *et al.*, 2004), however, iPS can be induced from adult cells ranging from HSC to differentiated lymphocytes avoiding ethical concerns surrounding the use of ES cells

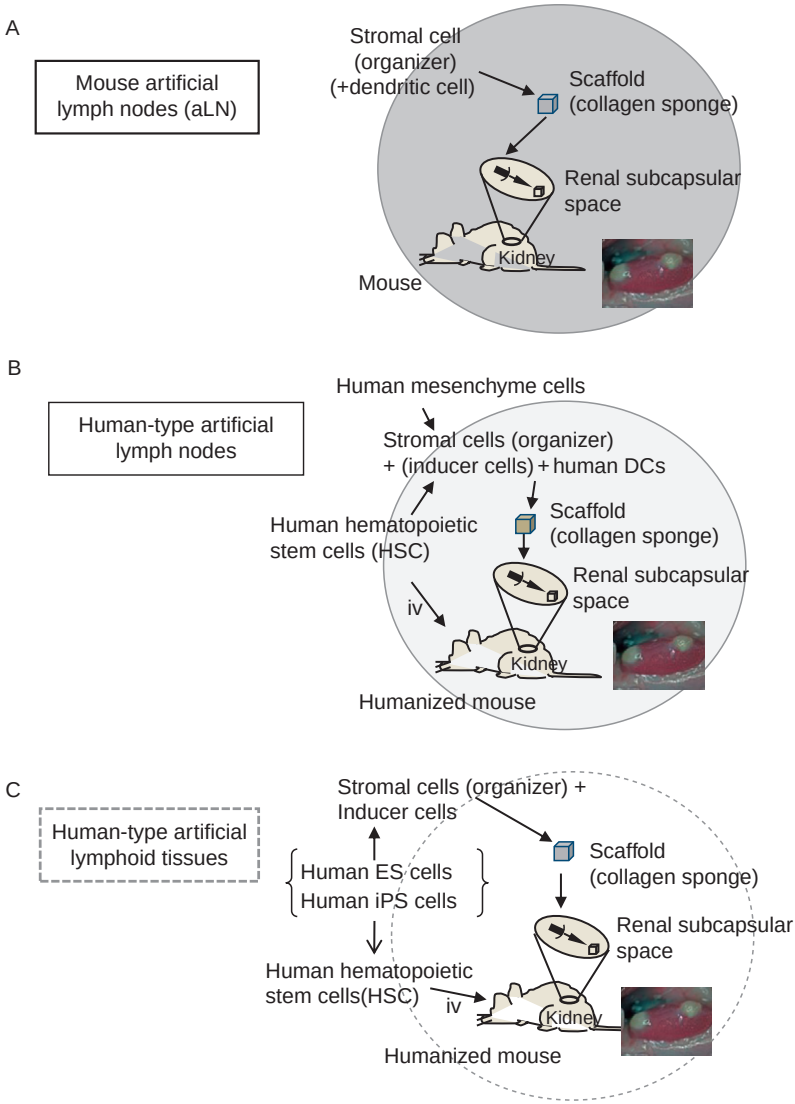

FIGURE 5.1 Future development of artificial lymph nodes progressing into humanized mouse models. (A) Artificial lymph nodes (aLN) have been successfully synthesized in mice (Suematsu and Watanabe, 2004) requiring stromal organizer cells and mature DC embedded into a collagen sponge, which is subsequently implanted into the renal subcapsular space of murine recipients. (B) The next phase of aLN engineering attempts to synthesize human aLN from human stromal and dendritic cell lines, implanted into "humanized" mouse models. (C) It is envisioned that human aLN synthesis will eventually be streamlined by the use of human ES or iPS cells to differentiate all human cell lineages necessary for organoid engineering.

(Eminli *et al.*, 2009; Takahashi and Yamanaka, 2006). It is envisioned that iPS can be induced from the patient and differentiated into all stromal and hematopoietic components required for artificial tissue organogenesis, bypassing issues with tissue incompatibility. However, iPS represent a relatively new technology such that clinical use in human immunotherapy will likely take several years (Yamanaka, 2009). Alternatively, established human cell lines may be useful in the interim for trialling construction of human artificial lymphoid tissue. APC, which have been shown to be important for development of murine aLN along with stromal organizer cells (Fig. 5.1A; Suematsu and Watanabe, 2004) are presently available as a human cell line (Butler *et al.*, 2007).

Human artificial (aAPC) represent a K562 parental cell line engineered to express HLA-A2, CD80, and CD83. Functionally, aAPC are capable of inducing antigen specific cytotoxic T cell responses comparable to DC differentiated and matured from human monocytes using GM-CSF/IL-4, TNF-α, and double-stranded RNA (Butler *et al.*, 2007). The future availability of human stromal cell lines could facilitate development of artificial human LN constructed from human stromal cells and APC, transplanted into ''humanized'' mice (Fig. 5.1B). Eventually, the use of ES or iPS cells is anticipated to supply both stromal organizer and hematopoietic inducer cells for human artificial lymphoid tissue development (Fig. 5.1C).

6. CONCLUSION

SLO are specialized tissues that facilitate interactions between APC and effector lymphocytes. Tissue-engineered lymphoid organs attempt to recapitulate this structure by providing artificial tissue upon which adaptive immune responses can be initiated. The success of engineering artificial lymphoid tissue has benefited from a better understanding of lymphoid organogenesis and molecular signaling events which are required for lymphoid structure formation. Future challenges now lie in the development of a diversity of secondary lymphoid tissues and application of this knowledge to clinical immunotherapy.

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CHAPTER 6

AID and Somatic Hypermutation

Robert W. Maul and **Patricia J. Gearhart**

Contents

Abstract

In response to an assault by foreign organisms, peripheral B cells can change their antibody affinity and isotype by somatically mutating their genomic DNA. The ability of a cell to modify its DNA is exceptional in light of the potential consequences of genetic alterations to cause human disease and cancer. Thus, as expected, this mechanism of antibody diversity is tightly regulated

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and coordinated through one protein, activation-induced deaminase (AID). AID produces diversity by converting cytosine to uracil within the immunoglobulin loci. The deoxyuracil residue is mutagenic when paired with deoxyguanosine, since it mimics thymidine during DNA replication. Additionally, B cells can manipulate the DNA repair pathways so that deoxyuracils are not faithfully repaired. Therefore, an intricate balance exists which is regulated at multiple stages to promote mutation of immunoglobulin genes, while retaining integrity of the rest of the genome. Here we discuss and summarize the current understanding of how AID functions to cause somatic hypermutation.

1. INTRODUCTION

Diversity in antibodies is produced during two stages in B cell development. In pre-B cells, rearrangement of variable (V), diversity (D), and joining (J) gene segments occurs to produce the primary repertoire of immunoglobulin (Ig) receptors. In mature B cells, Ig receptors undergo affinity maturation (AM) and class switch recombination (CSR) to produce the secondary, or memory, repertoire of antibodies. The latter event occurs after antigen binds to the receptor, which initiates a dynamic cascade of cell signaling events to cause cellular activation (Gauld *et al.*, 2002; Kurosaki, 2002; Niiro and Clark, 2002). The result of this activation is the differentiation of B cells into plasma or memory cells, which now express a large repertoire of antibodies to clear a plethora of different foreign antigens.

Diversity in the secondary repertoire is created by modifying rearranged V(D)J sequences and switching heavy chain constant genes (C_H). Alteration of the V gene sequence is achieved by either direct mutagenesis or DNA strand breaks during gene conversion (GC), where strand breaks are repaired using different pseudo-V gene segments in a templated recombination mechanism. In either case, cells containing mutations that increase antibody affinity will be selected to divide and further mutate, while mutations that decrease affinity will be lost through apoptosis. Alteration of the C_H gene occurs by DNA strand breaks in the switch (S) regions flanking the different C_H gene exons. Breaks in two different S regions are then repaired by nonhomologous end joining to remove the intervening introns and exons. This recombination event allows of production of a defined VDJ exon with different C_H gene isotypes to regulate antibody function.

A single enzyme is responsible for initiating diversity in V(D)J and C_H genes: activation-induced deaminase (AID), which is a cytosine deaminase that enzymatically converts cytosine to uracil. Uracil is mutagenic

when paired with guanosine in DNA, since dU mimics dT during replication, and the U:G mismatch triggers error-prone DNA repair in B cells. Thus, AID introduces somatic hypermutation (SHM) by converting dC to dU. In this chapter, the initiating events caused by AID are referred to as SHM, regardless of whether dU is found in the V or S regions. If dU occurs in V(D)J genes, SHM can produce AM or GC. If dU occurs in S regions, SHM can produce CSR. Furthermore, the proteins that process dU, such as UNG, MSH2, MSH6, and DNA polymerases, have the same activity whether dU is located in the V(D)J or S regions. Therefore, SHM, caused by AID-generated dU, underpins the three mechanisms of AM, GC, and CSR.

One key aspect of AID biology is the balance between mutagenic diversity and genomic integrity. When AID functions at non-Ig loci, both mutation and translocations can promote carcinogenesis (Ramiro *et al.*, 2007). Thus, it is imperative to the organism that AID activity will be tightly controlled to inhibit possible oncogenic transformation, while still allowing the production of a wide diversity of antibodies. In this chapter, we highlight the intricate aspects of AID biology and regulation.

2. AID, THE MASTER CATALYST

The mechanisms of AM, GC, and CSR were significantly advanced by the ground-breaking discovery of AID (Muramatsu *et al.*, 1999) and its subsequent genetic analysis in humans, mice, and chickens (Arakawa *et al.*, 2002; Rada *et al.*, 2002b; Revy *et al.*, 2000). Broader analysis of AID indicates that an intricate network of regulatory mechanisms controls its expression at the levels of gene transcription, mRNA stability, protein localization, protein phosphorylation, and cell signaling.

2.1. Gene transcription

The *Aicda* locus, which encodes AID, is comprised of four regions which control transcription (Yadav *et al.*, 2006). Starting at the 5′ end of the locus, the first region is located about 8 kb upstream of exon 1 in the mouse, and contains potential motifs for NF-κB, STAT6, C/EBP, and Smad3/4 proteins (Tran *et al.*, 2010; Yadav *et al.*, 2006). This region may respond to stimulation by the mitogen lipopolysaccharide (LPS) and the T-cell mimic anti-CD40 antibody to upregulate expression of AID after stimulation. The second region is located about 1 kb upstream of exon 1 and has sites for NF-κB, Stat 6, Sp transcription factors, HoxC4, and Pax5 (Dedeoglu *et al.*, 2004; Gonda *et al.*, 2003; Park *et al.*, 2009; Yadav *et al.*, 2006). The third region is found in the intron between exon 1 and exon 2 and contains sites for NF-κB, E proteins, Pax5, and several other factors (Gonda *et al.*, 2003;

Sayegh *et al.*, 2003; Tran *et al.*, 2010; Yadav *et al.*, 2006). The fourth region is located about 6 kb downstream of exon 5 in the mouse (Tran *et al.*, 2010; Yadav *et al.*, 2006) and appears to function as an enhancer (Crouch *et al.*, 2007). Many of the sites in the first three regions bind to transcription factors that are upregulated after B cell stimulation, so they likely play a role in inducing AID *in vivo*.

Conversely, proteins Id1, Id2, and Id3 reduce CSR (Goldfarb *et al.*, 1996; Quong *et al.*, 1999), potentially by inhibiting AID transcription (Gonda *et al.*, 2003; Sayegh *et al.*, 2003). The Id proteins function by binding to stimulatory factors such as E47 and Pax5, which prevents their binding to DNA. Other factors inhibit transcription by binding to sites in the third region (Tran *et al.*, 2010), and may play a role in restricting AID expression to B cells, and not to other cell types.

Additional proteins that appear to function independently of antigen stimulation bind to sites in the second region. For example, Sp1 and Sp2 proteins bind to sites *in vitro* (Yadav *et al.*, 2006), but their *in vivo* role is not known. Recently, the sex hormones estrogen and progesterone have been found to regulate AID expression (Pauklin and Petersen-Mahrt, 2009; Pauklin *et al.*, 2009). Both estrogen and progesterone response elements have been found within the second region, and they could have a potential role in upregulating AID in hormone-based cancers and autoimmunity (Maul and Gearhart, 2009; Petersen-Mahrt *et al.*, 2009). However, as with the Sp-binding sites, further research is required to understand the role these factors play during normal B cell development and activation. Finally, B cells from old mice and humans have less AID and reduced CSR compared to B cells from young mice and humans. This may be due, in part, to degradation of E47 mRNA, which encodes molecules that stimulate AID transcription (Frasca *et al.*, 2008). In B cells from old mice, tristetraprolin binds to E47 mRNA and degrades it, whereas in B cells from young mice, tristetraprolin is phosphorylated and cannot bind to the mRNA (Frasca *et al.*, 2007). Thus, by defining the factors that limit antibody diversity with age, it may be possible to increase the efficacy of vaccines in the elderly.

2.2. mRNA transcripts

Once the *Aicda* gene is transcribed, the level of transcripts can be controlled by regulation though microRNA molecules. Specifically, miR-155 binds to the 3′ untranslated region of AID mRNA and destabilizes the message to reduce SHM and CSR. However, *in vivo* analysis of miR-155 function in SHM is complicated by the global defects of miR-155 on other cells that alter germinal center cell number and function (Kohlhaas *et al.*, 2009; Thai *et al.*, 2007; Vigorito *et al.*, 2007). To overcome the global effect, specific mutants of the 3′ untranslated region of AID, which prevent

binding of miR-155, were utilized to examine SHM and CSR *in vivo*. As predicted, AID expression was increased in splenic and Peyer's patch mutant B cells (Dorsett *et al.*, 2008; Teng *et al.*, 2008), and there was a dramatic increase in chromosomal translocations between *Myc* and *Igh* genes (Dorsett *et al.*, 2008). However, the increased AID protein only modestly elevated the level of SHM in the V or S regions and decreased AM, suggesting that excess AID was not specifically targeted to the *Ig* locus. This is consistent with earlier studies showing that overexpression of AID does not always produce increased SHM or CSR, perhaps because of inactivation of the protein by an unknown mechanism (Muto *et al.*, 2006). Another molecule, miR-181b, regulates AID expression in a similar manner, by binding to the 3′ untranslated region and lowering the levels of AID and CSR (de Yebenes *et al.*, 2008). Nonetheless, since both of these miR molecules affect multiple genes as well as *Aicda*, their biological role in AID expression remains unclear.

2.3. Protein localization

Extensive analyses have identified posttranslational mechanisms that coordinate AID subcellular localization. Surprisingly, AID protein is far more abundant in the cytoplasm than in the nucleus, as first seen in the Ramos cell line using artificial AID-GFP constructs (Rada *et al.*, 2002a) and in primary B cells looking at endogenous AID (Schrader *et al.*, 2005). Three mechanisms appear to be involved in actively moving AID in and out of the nucleus, and their respective amino acid residues are illustrated in Fig. 6.1. First, high levels of AID may be retained in the cytoplasm through an anchor sequence in the C-terminal region of AID (Patenaude *et al.*, 2009). This would be advantageous, as the protein is quickly degraded when it is in the nucleus through polyubiquitination (Aoufouchi *et al.*, 2008). Second, AID is actively imported into the nucleus through the use of an N-terminal nuclear localization signal (NLS); however, the exact amino acids that form the NLS are currently unclear. Di Noia and colleagues speculated that a nonclassical NLS exists in AID (Fig. 6.1, NLS^a) (Patenaude *et al.*, 2009), while Honjo and colleagues have identified a classical bipartite NLS (Fig. 6.1, NLS^b) (Ito *et al.*, 2004; Shinkura *et al.*, 2004). In addition, it has also been proposed that AID may passively diffuse into the nucleus (Brar *et al.*, 2004; McBride *et al.*, 2004). However, the identification of an interaction of AID with importin-α suggests that AID does contain a NLS for active nuclear import (Patenaude *et al.*, 2009). Third, a conserved nuclear export signal (NES) in the C-terminal residues 189–198 transports most of the protein out of the nucleus (Brar *et al.*, 2004; Ito *et al.*, 2004; McBride *et al.*, 2004). Treatment of B cells with leptomycin B, a potent inhibitor of the CRM1 export receptor, increased the abundance of AID in the nucleus. Further dissection of the C-terminal domain

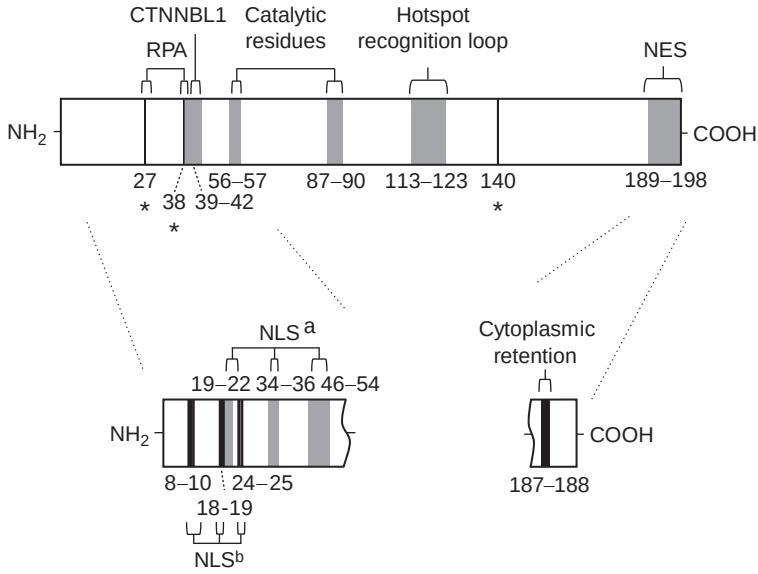

FIGURE 6.1 *Amino acids defining AID activity*. Amino acids have been identified that bind cofactors, cause catalysis, recognize the hotspot motif, and shuttle the protein in and out of the nucleus. Confirmed residues that function in these activities are shown in the upper diagram. Amino acids that are phosphorylated are marked by asterisks and black bars; NES, nuclear export signal. Putative residues that may function are shown in the lower diagram. These include nuclear localization signals (NLS) in the N-terminal region identified by two groups, NLS[a] (Patenaude *et al.*, 2009), in gray bars, and NLS[b] (Ito *et al.*, 2004) in black bars. Two residues that may cause retention of AID in the cytoplasm are shown in the C-terminal region (Patenaude *et al.*, 2009).

showed that export can be abolished by the single point mutation F198A, resulting in increased SHM and CSR activities (McBride *et al.*, 2004). Therefore, these three mechanisms exquisitely regulate the amount of AID in the nucleus, ensuring that there will be only low levels of the mutagenic protein after cell activation. Perturbing any one of these pathways can affect the fine balance between antibody diversity and chromosomal mutagenesis.

2.4. AID phosphorylation

While subcellular localization and degradation coordinate the access of AID to genomic DNA, phosphorylation regulates the activity of the protein. AID phosphorylation was first identified by examining catalytic differences between protein purified from B cells (AID^{Bcell}) or from 293 kidney cells (AID^{293}). Alt and colleagues reported that AID^{293} was less

active than AID^{Bcell} when tested for deamination activity using an *in vitro* transcription-based assay (Chaudhuri *et al.*, 2004). Subsequent analysis by mass spectroscopy identified four phosphorylation sites in AID: T27, S38, T140, and Y184 (Basu *et al.*, 2005; McBride *et al.*, 2006, 2008; Pasqualucci *et al.*, 2006). Residues T27 and S38 are phosphorylated in a coordinated fashion by protein kinase A (PKA), and they regulate protein–protein interaction between AID and replication protein A (RPA) (Basu *et al.*, 2005; Pasqualucci *et al.*, 2006). Mutation of these residues in B cells stimulated *ex vivo* or in DT40 cells inhibits SHM, GC, and CSR (Basu *et al.*, 2005; Chatterji *et al.*, 2007; McBride *et al.*, 2006; Pasqualucci *et al.*, 2006; Vuong *et al.*, 2009). Indeed, S38 appears to be the key phosphorylation site *in vivo*, as mice with a mutation of this residue had reduced SHM and CSR (Cheng *et al.*, 2009; McBride *et al.*, 2008). Recently Nussenzweig and colleagues reported phosphorylation of AID residue T140 in mouse B cells after activation with LPS and IL-4 (McBride *et al.*, 2008). Unlike S38, T140 is not a substrate for PKA phosphorylation but rather for protein kinase C (PKC). Furthermore, mutation of T140 to alanine in mice showed that SHM was affected more significantly than CSR, suggesting that differential phosphorylation of S38 and T140 can produce different biological outcomes. In contrast to residues T27, S38, and T140, phosphorylation of Y184 may not play a significant role in AID function, since replacement of the amino acid in B cells did not have an effect on CSR (Basu *et al.*, 2005).

2.5. Cell signaling

As implied in its name, AID is induced after activation by exogenous stimuli, such as bacterial or viral molecules, CD40 ligand, and antigen or anti-Ig. To study the signaling pathways for these activators, murine splenic B cells can be conveniently stimulated *ex vivo*, and the levels of AID and CSR can be measured. Abundant AID expression and CSR occur after stimulation with LPS, which binds to toll-like receptor 4, or with anti-CD40 antibody, which binds to the CD40 receptor. In contrast, AID expression is delayed and CSR is ablated when cells are stimulated with anti-IgM, which binds to the Ig receptor (Heltemes-Harris *et al.*, 2008; Jabara *et al.*, 2008). Furthermore, AID expression and CSR are actually suppressed when anti-IgM is added to cells stimulated with LPS or anti-CD40 (Heltemes-Harris *et al.*, 2008; Jabara *et al.*, 2008; Rush *et al.*, 2002). This inhibition has been linked to upregulation of the phosphatidyl inositol 3 kinase (PI3K) pathway (Doi *et al.*, 2008; Heltemes-Harris *et al.*, 2008; Omori *et al.*, 2006). Indeed, balancing the levels of PI3K activation may determine whether CSR is induced or suppressed. B cells treated with LPS and IL-4 activate PI3K signaling, which phosphorylates AKT to a low-enough concentration to allow CSR. In contrast, the addition of anti-IgM along

with LPS and IL-4 enhanced AKT phosphorylation to a greater extent to inhibit CSR (Heltemes-Harris *et al.*, 2008). A recent report shows that anti-IgM also inhibits AID expression through the calcium-signaling pathway (Hauser *et al.*, 2008). Taken together, these reports suggest that IgM cross-linking *ex vivo* mimics the end of a germinal center response, where B cells with high-affinity receptors stop SHM and are converted into memory and plasma cells. However, the complete story has yet to emerge, as another stimulator, 8-mercaptoguanosine, which binds to toll-like receptor 7 located in endosomes, has the ability to work synergistically with IgM cross-linking to promote AID expression and CSR (Tsukamoto *et al.*, 2009). Perhaps after B cell receptor cross-linking, stimulation from different cellular microenvironments regulates the outcome of B cell differentiation.

3. THE TARGETING ENIGMA

3.1. Global targeting to the *Ig* loci

The Ig loci are mutated in well-defined regions encoding rearranged V genes on the heavy and light chain loci, and S regions on the heavy chain locus. Sequence analysis has shown that mutation occurs in a 2-kb region around V(D)J genes (Lebecque and Gearhart, 1990) and in a 4–7-kb region around S regions (Xue *et al.*, 2006). Thus, it can be assumed that AID functions on 10^{5} to 10^{6} of the genome at a given time, suggesting that precise levels of regulation target AID to such a small percentage of the genome. Examination of the mutational pattern in both the V(D)J and the S regions shows that mutation occurs in close proximity to either the V gene promoter or S intron promoters, respectively. This has led to the hypothesis that AID is recruited to the *Ig* region in association with the transcriptional machinery. In fact, mice with transgenes that have been altered to express different amounts of transcripts have mutation frequencies that correlate with the level of transcription (Bachl *et al.*, 2001; Fukita *et al.*, 1998; Sharpe *et al.*, 1991). Transcription of the heavy and light chain loci is coordinated by three well-characterized promoters/enhancers shown in Fig. 6.2: V gene promoter, intronic enhancer (iE), and downstream enhancers (3′E and hypersensitive (HS) sites). While all the three elements and the V/S regions are involved in transcription and SHM, the specific role for each has been harder to elucidate.

The V gene promoter can be replaced with other non-Ig promoters in transgenes and still promote SHM, suggesting that the promoter may serve to only maintain adequate levels of transcription (Betz *et al.*, 1994; Fukita *et al.*, 1998; Shen *et al.*, 2001; Tumas-Brundage and Manser, 1997). However, this may not be the whole story since at least one promoter,

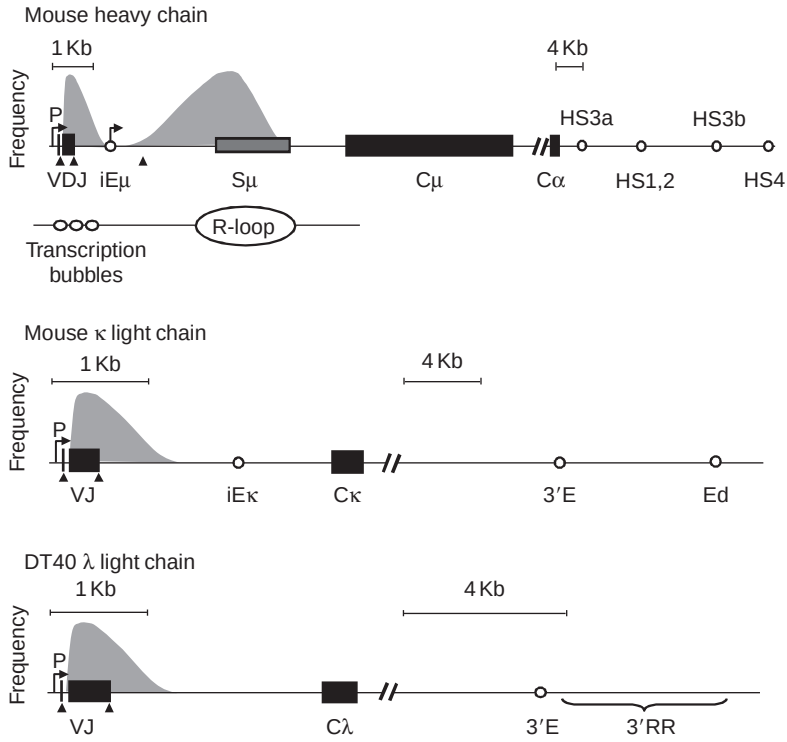

FIGURE 6.2 *Targeting elements for SHM in the Ig loci.* For all loci, the relative mutation frequency is shown by shaded peaks over the V region or S region. P, promoter; arrows, start of transcription; iE, intronic enhancer; triangles, location of donor splice sites in the leader exon, V(D)J exon, and iEμ intronic exon. AID may be active on single-strand DNA in the V regions by transcription bubbles, and in the S regions by R-loops. 3′ enhancers downstream of the C genes are shown on a different scale. For the mouse *Igh* chain locus, 3′ enhancers include four hypersensitive (HS) sites. For the mouse *Igκ* locus, there are two enhancers, 3′E and Ed. For the chicken DT40 *Igλ* locus, identified enhancers are 3′E and a regulatory region, 3′RR.

human elongation factor 1-α, can induce high levels of transcription without supporting SHM (Yang *et al.*, 2006). Furthermore, substitution of the V gene promoter has not been studied in the endogenous locus with knock-in mice, so its requirement is not fully resolved.

The seeming lack of specificity for promoters would suggest that the V(D)J sequence contains the information required for targeting. However, when the VJ sequence is removed and replaced by different sequences in *Igκ* transgenes, the new sequence is still subject to SHM (Peters and Storb, 1996; Yelamos *et al.*, 1995). Additionally, when a 750-bp insert is introduced between the leader sequence and the VDJ sequence, the mutation

window shifts ~750 bp toward the promoter (Tumas-Brundage and Manser, 1997). Interestingly, when the promoter is removed from the leader sequence by insertion of 2 kb of λ DNA, SHM is lost, indicating that targeting may be linked by the proximity of the promoter to the leader sequence and/or the leader splice site (Winter *et al.*, 1997). However, the caveat still remains that replacement of the V(D)J sequence in the endogenous context has not been studied, so its requirement is not certain.

Switch regions are also a target for SHM and sustain as high a frequency of mutation as does the V region. Knockout mice with a partial deletion of Sμ tandem repeats had decreased CSR (Luby *et al.*, 2001; Schrader *et al.*, 2007), and when the entire 4.6 kb region containing Sμ tandem repeat sequences was deleted, SHM and CSR were ablated (Khamlichi *et al.*, 2004). Thus, the repetitive sequences in Sμ are a magnet for AID activity and are required.

iEs are located in the intron sequence between either the VDJ sequence and the S region for Cμ, or the VJ sequence and C gene in the *Igκ* locus. A plethora of papers in the 1990s addressed the role of iE for SHM in murine transgenes encoding rearranged V and C genes from the *Igh* and *Igκ* loci (Odegard and Schatz, 2006). Interpretation of the varying results was complicated by the random location of transgenes in the genome, which could affect transcription levels. As technology advanced, it became possible to directly delete iE from the endogenous locus in knockout mice. These studies consistently showed that iE had no effect on SHM in the *Igh* or *Igk* loci (Inlay *et al.*, 2006; Perlot *et al.*, 2005).

3′ Enhancers are located downstream of the C genes on the heavy and light chain loci and are important for transcription of rearranged V genes. Interestingly, it now appears that all three loci have multiple 3′ enhancers which play a role in SHM. As in the iE studies, conflicting results were obtained from transgenic mice, whereas more reliable data were found in germline knockout mice. The IgH 3′E is characterized by the presence of four DNaseI HS sites downstream of the Cα gene (Dariavach *et al.*, 1991; Lieberson *et al.*, 1991; Madisen and Groudine, 1994; Matthias and Baltimore, 1993; Pettersson *et al.*, 1990). Partial deletion of the HS sites did not affect SHM (Le Morvan *et al.*, 2003), whereas deletion of the entire four sites in a 230-kb bacterial artificial chromosome mouse model showed reduced transcription and SHM (Dunnick *et al.*, 2009). The *Igκ* locus has a 3′E (Meyer and Neuberger, 1989) and a downstream enhancer, Ed (Liu *et al.*, 2002). Deletion of 3′E reduced transcription but did not affect SHM (Inlay *et al.*, 2006; van der Stoep *et al.*, 1998), whereas deletion of Ed reduced both transcription and SHM (Xiang and Garrard, 2008). Likewise, the chicken *Igλ* locus has a defined 3′E (Bulfone-Paus *et al.*, 1995) and another downstream enhancer, 3′RR (Kothapalli *et al.*, 2008). In the DT40 cell line, deletion of the 3′E had no effect on SHM (Yang

et al., 2006), but deletion of 3′RR ablated transcription and SHM (Kothapalli *et al.*, 2008). Furthermore, the 3′RR appears to contain a site that recruits AID to the *Ig*λ locus (Blagodatski *et al.*, 2009; Kothapalli *et al.*, 2008).

To summarize, transcription is necessary for SHM. The V gene promoter may not be required; the V(D)J sequence may not be required; the S region sequence is required; the iE is not required; and both the 3′ E and downstream enhancers are required. Finally, the downstream enhancer may contain a motif that recruits AID to the *Ig* loci, although more work is needed to establish this.

In addition to AID being recruited to the *Ig* loci, it has become increasingly clear that AID is erroneously targeted to non-Ig genes throughout the genome. In the absence of DNA repair proteins uracil DNA glycoslyase (UNG or UDG) and mismatch repair protein MSH2, Schatz and colleagues found a high mutation frequency for several non-Ig genes that was only 10-fold lower than in V genes (Liu *et al.*, 2008). The handful of different genes that might be targets for AID suggest that the promoters/enhancers of Ig genes are not the only elements that attract AID. Interestingly, the recent finding that *Ig*λ, *Igh*, and *Myc* are spatially contained in close proximity in the nucleus suggests that nuclear organization affects both *Ig* and non-*Ig* targeting (Wang *et al.*, 2009a).

Other transcriptional events such as chromatin acetylation may play a role in coordinating AID activity. Examination of the abundance of histone acetylation upon stimulation *in vivo* or *ex vivo* suggests that the V and Sμ regions are maintained in a hyperacetylated (open) state independent of cellular activation, and the C region had a lower level of acetylation (Li *et al.*, 2004a; Odegard *et al.*, 2005; Wang *et al.*, 2006, 2009b). Furthermore, downstream S regions are maintained at a low level of acetylation until stimulated with specific cytokines to promote transcription (Li *et al.*, 2004a; Nambu *et al.*, 2003; Wang *et al.*, 2006, 2009b; Woo *et al.*, 2003). While these events seem to be independent of AID, the difference in the acetylation state might coordinate a functional window for AID activity in the V and S regions, and block activity in the C region.

3.2. Regional targeting to V and S regions

Once recruited to the *Ig* loci, AID-generated mutations show a distinctive bell-shaped pattern, suggesting increased activity in particular regions (Fig. 6.2). This pattern of mutation is advantageous to antibody diversity in that the peak of mutation is over either the V exon or the repetitive core in the S regions. Thus, in the V region, SHM promotes AM and GC, and in the S region, SHM initiates double-strand breaks for CSR. This focusing of

mutation in a defined region is determined by the nucleotide sequence of the loci which coordinates transcription and AID activity.

Targeting to the V region is similar for rearranged V genes on the *Igκ*, *Igλ*, and *Igh* loci. Mutations start just downstream of the V promoter, proceed for about 2 kb, and then trail off (Lebecque and Gearhart, 1990). The pattern is the same regardless of which J gene segment is being used. For example, if a V gene rearranges to J_H1, mutations cease 1550 bp before the iEμ, whereas if a V gene rearranges to J_H4, mutations end just before iEμ. This indicates that the V gene promoter determines the start of mutation, and the iE does not stop mutation. Rather, AID appears to be recruited to the promoter, proceeds for 2 kb and then may dissociate from the ongoing transcription complex. Indeed, one of the most perplexing questions is ''why do mutations start and why do they stop?'' The frequency of mutations, about 10^{-2} to 10^{-3} mutations per bp, is similar in both coding and flanking sequences around the V gene, and is higher in the complementarity-determining regions because of selection during AM for amino acids giving high-affinity interactions with the cognate antigen.

Targeting to the intronic S region promotes a high level of deamination events in close proximity, which lead to double-strand break formation. Mutations start downstream of the intronic exon promoter, accumulate for about 4–7 kb depending on the length of the S region, and then fall off (Xue *et al.*, 2006). The pattern, although longer, is thus similar to that in the V region, in that AID may assemble at the intronic promoter, proceed through the S region, and then dissociate. The S regions in mammals are unique in that they contain clusters of G nucleotides on the nontranscribed strand, and repetitive hotspot motifs for AID deamination, WGCW (W = A or T). The G clusters have been shown to form R-loops, or RNA–DNA hybrids, *in vivo* (Yu *et al.*, 2003) and *in vitro* (Roy and Lieber, 2009). The corresponding C clusters on the transcribed strand can stably hybridize to G-rich RNA. One effect of R-loop structure is that the nontranscribed strand becomes single stranded in regions larger than transcription bubbles to maximize deamination by AID. In contrast to mice, switching in frogs is not all that efficient, since the Sμ regions do not have G clusters or R-loops (Zarrin *et al.*, 2004).

A second effect of R-loop structure is to slow down RNA polymerase II molecules as they move through the S region. It has been shown *in vitro* that R-loops block transcription (Canugovi *et al.*, 2009; Tornaletti *et al.*, 2008), because the polymerases may have difficulty in unwinding the stable RNA–DNA hybrid. A recent study (Rajagopal *et al.*, 2009) measured the density of polymerases *in vivo* by nuclear run-on, and found a 5-10-fold increase in polymerases located in close proximity to the Sμ repetitive core. These polymerases appeared to be piling up because they encountered a road-block ahead caused by the RNA–DNA hybrids. Once the polymerases slowly make it through the repetitive core,

they speed up again as the R-loop density is lower. In addition to Sμ, R-loops are present in other S regions (Huang *et al.*, 2006; Yu *et al.*, 2003), and RNA polymerase II has been shown to accumulate in Sγ3 (Wang *et al.*, 2009b), which suggests a conserved mechanism for deamination in S regions. If AID is associated with transcription, this model of paused polymerases may allow AID more opportunity to deaminate DNA, producing more mutations and strand breaks.

Is AID differentially targeted to V or S regions during certain cellular responses? This may seem to occur during stimulation of B cells *ex vivo* with mitogens, where mutations are found only in S regions and not in V regions (Reina-San-Martin *et al.*, 2003). Conversely, in B cell lines such as Ramos and DT40, mutations accumulate in V regions and apparently not in S regions, as the cells do not undergo switching. In addition, IgM memory cells from humans (Klein *et al.*, 1998; Rosner *et al.*, 2001) and mice (Dogan *et al.*, 2009) have mutations in the V region, but have not switched isotypes. This suggests that specific cofactors may guide AID to the V or S regions; however, other interpretations are possible. Mutations may occur first in S regions in cells stimulated *ex vivo* because of the formation of R-loops and RNA polymerase II pausing, which magnifies AID activity. Likewise, B cell lines or memory IgM cells may have mutations in S regions but not switch because some proteins involved in NHEJ are not functioning. It would be interesting to compare the time course of mutations in V and S regions following immunization *in vivo*, to see whether they occur simultaneously or differentially.

3.3. Local targeting to hotspots

As mentioned earlier, SHM occurs at a greater frequency in a defined sequence motif, WRC (W = A/T, R = A/G) (Rogozin and Kolchanov, 1992). Prior to the discovery of AID, sequence analysis of V genes indicated that the complementarity-determining regions are heavily biased in using the serine codons AGC or AGT, while framework regions utilized the TCN serine codons (Wagner *et al.*, 1995). Additionally, SHM occurred in AGY (Y = C/T) codons at a higher rate than in TCN, indicating a bias to focus mutation within the complementarity-determining regions for AM. Further analyses using *in vivo* mouse models deficient for different DNA repair enzymes have defined WGCW in V and S regions as the mutational hotspot for SHM (Delbos *et al.*, 2007; Ehrenstein and Neuberger, 1999; Martomo *et al.*, 2004; Rada *et al.*, 1998). Characterization of AID activity *in vitro* indicates that deamination events occur with a high frequency within the WRC context (Bransteitter *et al.*, 2004; Larijani *et al.*, 2005; Yu *et al.*, 2004). As with AM in V genes, CSR has evolved to utilize these hotspots to focus AID activity to the WGCW motif within the repetitive core repeat (Davis *et al.*, 1980; Dunnick *et al.*, 1980; Kataoka *et al.*,

1981; Sakano *et al.*, 1980). Additionally, the frequency and palindromic nature of the motif allows deamination events on both strands to occur in close proximity to promote double-strand break formation versus mutagenic repair.

In addition to the catalytic residues in the AID protein, a loop at residues 113–123 has recently been identified as the main determinant in directing activity to the WRC hotspot (Fig. 6.1). Altering this loop to resemble the homologous loop from APOBEC3 family members switches the hotspot motif to that of the APOBEC3 enzymes (Kohli *et al.*, 2009; Wang *et al.*, 2010). Taken together, this indicates a coevolution of both the *Ig* loci sequence and the AID enzyme.

3.4. Access to both DNA strands

An additional phenomenon of AID activity within the *Ig* loci is the ability of AID to access and mutate both the strands. Mutational analysis in *Ung*$^{-/-}$*Msh2*$^{-/-}$ and *Ung*$^{-/-}$*Msh6*$^{-/-}$ mice indicates that both the transcribed and nontranscribed strands are mutated at an equal frequency (Rada *et al.*, 2004; Shen *et al.*, 2006; Xue *et al.*, 2006). Most models for AID deamination suggest that AID can access single-strand DNA within transcription bubbles and R-loops. However, these models would only allow deamination of the nontemplate strand as the template strand would be either associated with the RNA polymerase or contained within an RNA–DNA complex. To achieve access to both strands, it has been proposed that antisense transcription occurs throughout the *Ig* loci. In support of this model, RT-PCR has been utilized to identify low levels of antisense transcripts in V and S regions (Chowdhury *et al.*, 2008; Perlot *et al.*, 2008; Ronai *et al.*, 2007). Although these findings have been called into question (Zhao *et al.*, 2009), the identification of ~11 nt single-strand DNA patches on both the strands of DNA in Ramos cells supports the presence of transient transcription bubbles moving in opposite directions (Ronai *et al.*, 2007). However, the finding that cytosines are also mutated on both strands in the S regions ((Xue *et al.*, 2006) is particularly perplexing, since the transcribed strand contains an RNA–DNA hybrid in the R-loop structures. Two other theories have been proposed to make this strand available for attack by AID. (1) The DNA upstream of an elongating RNA polymerase II may be supercoiled and unwound, which allows AID access to both the strands (Shen and Storb, 2004). (2) The DNA in R-loops may be collapsed by endogenous RNase H digestion, which would expose single-strand regions on the transcribed strand (Huang *et al.*, 2007). Taken together, this data suggest that the models for AID activity within the *Ig* loci are still in a state of flux and require further experimentation to fully define AID targeting.

3.5. Protein cofactors

In addition to the mechanisms discussed earlier, extensive work has been performed in an attempt to identify AID protein partners. It has been hypothesized that targeting will be tightly regulated by protein cofactors which coordinate the recruitment and activity of AID. With recent advances in AID protein biochemistry, the intricate network of AID interactions is just beginning to emerge.

As discussed in Section 2.4, AID is phosphorylated by PKA and PKC (Basu *et al.*, 2005; McBride *et al.*, 2008; Pasqualucci *et al.*, 2006). The phosphorylation by PKA is required for interaction with RPA and disruption of this interaction inhibits AID activity (Basu *et al.*, 2005; Cheng *et al.*, 2009; McBride *et al.*, 2006; Vuong *et al.*, 2009). While these interactions are well characterized, the precise mechanism by which RPA assists AID is unclear. Recently Chaudhuri and colleagues found that neither PKA or RPA is required to physically recruit AID to DNA (Vuong *et al.*, 2009). *In vitro* analysis suggests that RPA stabilizes the transcription bubble to allow AID activity on single-strand DNA (Chaudhuri *et al.*, 2004). Yet it remains unclear whether the interaction between the proteins promotes a coordinated handoff of the DNA between the two proteins and whether the RPA–AID interaction exists during the deamination step.

While a genetic interaction between transcription and SHM has been well documented, very little is known about the role of the transcription complex in physically recruiting AID to the DNA. AID has been shown to physically interact with RNA polymerase II; however, no further analysis was done to examine which subunit is responsible for this interaction (Nambu *et al.*, 2003). Recently, Neuberger and colleagues identified an interaction between AID and CTNNBL1. Deletion of CTNNBL1 or mutation of AID residues 39–42 abolished AID activity (Conticello *et al.*, 2008). Interestingly, CTNNBL1 physically interacts with proteins associated with the RNA polymerase II spliceosome, suggesting that AID may travel with the transcription complex. This interaction also highlights the potential role for splice sites in initiating AID activity on DNA. In a critical experiment, Radbruch and colleagues (Hein *et al.*, 1998) reported that switching was abrogated when the splice donor site for Iγ1 was deleted, even though Iγ1 transcripts were made. Another study showed that mice lacking the splice donor site for the Iμ exon had switching, but splicing still occurred in transcripts using pseudo-splice donor sites (Kuzin *et al.*, 2000). Thus, AID could be brought to the *Ig* loci through interaction with *cis*-acting elements (potentially using E2A family members; Michael *et al.*, 2003; Schoetz *et al.*, 2006; Tanaka *et al.*, 2010), bind to the RNA polymerase II spliceosome complex through CTNNBL1, and load onto DNA at donor splice sites to interact with RPA and deaminate cytosine residues.

This may explain why mutation is highest in V genes after donor splice sites in the leader and V exons, and in S regions after donor splice sites in intronic exons preceding Sμ, Sγ, and Sα (Fig. 6.2).

4. ROGUE URACILS

4.1. Deoxyuracil in DNA

Since the first identification of AID, extensive work has been performed in an attempt to elucidate the mechanism of how it promotes genomic mutation. Initial identification of the sequence similarity between AID and APOBEC1 suggested that AID may function as an RNA deaminase (Muramatsu *et al.*, 1999). Honjo *et al.* (2005) proposed an RNA editing model in which AID binds to an unidentified mRNA partner in the cytoplasm and deaminates C to U. The edited mRNA would then produce a protein, perhaps an endonuclease, that cleaves DNA during the immune response. Alternatively, identification of a mutational hotspot in SHM (WRC, discussed earlier) suggested a mechanism in which alterations occur directly at dC:dG basepairs (Rada *et al.*, 1998). Neuberger and colleagues proposed a DNA deamination model in which AID deaminates dC bases to dU, which initiates error-prone processing by some proteins in the base excision repair (BER) and mismatch repair (MMR) pathways. Support for direct deamination of DNA came from the finding that UNG is required for CSR and GC, and alters SHM frequency and mutational spectra (Di Noia and Neuberger, 2002, 2004; Petersen-Mahrt *et al.*, 2002; Rada *et al.*, 2002b; Saribasak *et al.*, 2006). The importance of UNG to the mechanism of CSR is further confirmed by genetic mutations in the human *UNG* gene that block CSR and cause hyper-IgM syndrome (Imai *et al.*, 2003; Kavli *et al.*, 2005). During classical BER, UNG binds to dU:dG mispairs in DNA, and the uracil base is cleaved to form an abasic site. Abasic sites are then cleaved by apurinic/apryimidinic endonuclease (APE1) to remove the abasic nucleotide, and DNA polymerase (Pol) β resynthesizes the DNA strand. However, during SHM and CSR, the canonical mechanism of BER is impaired by altering the resynthesis step with low-fidelity polymerases, which introduce mutations and single-strand DNA breaks. Consistent with this model shown in Fig. 6.3, deletion or inhibition of UNG and APE1 results in decreased CSR (Guikema *et al.*, 2007; Rada *et al.*, 2002b; Schrader *et al.*, 2005). Alternatively, deletion of Polβ supports increased CSR and double-strand breaks by inhibiting the faithful resynthesis step of canonical BER (Wu and Stavnezer, 2007).

In opposition to the existence of uracil in DNA, several reports from Honjo and colleagues have suggested that UNG is important for CSR through an alternative mechanism not requiring DNA glycosylase

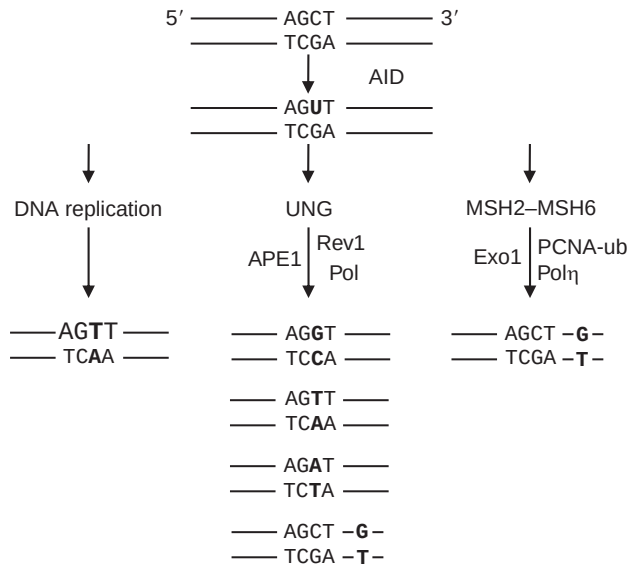

FIGURE 6.3 *Three error-prone pathways for processing U:G mismatches.* AID deaminates dC to dU in a hotspot, WGCW. Mutations are shown in bold. (1) DNA replication. U mimics T, and replication from the 5′ strand will incorporate C:G to T:A transitions. (2) UNG recognition. UNG removes the U, and several possibilities could occur. Rev1 may bypass the abasic site with a C:G to G:C transversion. APE1 may nick the abasic site, and the 5′ strand would be extended by a low-fidelity polymerase to insert other transversions. Polη may extend the synthesis to insert G opposite a downstream template T, to cause A:T to G:C transitions. (3) MSH2–MSH6 recognition. The two proteins bind to the U:G mismatch and recruit Exo1 to form a gap. It is not known what triggers a nick for Exo1 to access the DNA. Monoubiquitinated PCNA brings in Polη to synthesize predominantly A:T mutations downstream of the initial deamination event.

activity (Begum *et al.*, 2004). Mutants of active site residues in UNG had no detectable glycosylase activity *in vitro*, but they were proficient for CSR when complementing *Ung*$^{-/-}$ cells (Begum *et al.*, 2004, 2009). Additionally, the identification of normal γH2AX foci formation and strand break junctions in the absence of UNG supports a model by which UNG is involved in resolving the double-strand breaks, perhaps as a scaffold for other proteins, but not in the direct formation of the breaks (Begum *et al.*, 2007). However, these results have been called into question because of a possible dissociation between *in vitro* UNG glycosylase activity and *in vivo* CSR (Di Noia *et al.*, 2007; Kavli *et al.*, 2005; Stivers, 2004). It has been shown that several UNG active-site mutants with severally diminished *in vitro* activity still retain enough glycosylase activity *in vivo* to promote CSR. Honjo and colleagues also report that deletion of either

APE1 or APE2 had no effect on CSR (Sabouri *et al.*, 2009), in contrast to an earlier finding by Stavnezer and colleagues (Guikema *et al.*, 2007). Furthermore, the results looking at γH2AX and strand breaks did not take into account the interplay between BER and MMR in causing double-strand breaks and CSR in the S region, since it is possible that breaks were still being formed because of cleavage during MMR (Di Noia *et al.*, 2007; Rada *et al.*, 2004; Shen *et al.*, 2006).

Additional evidence for AID acting as a DNA deaminase comes from direct analysis of the purified protein *in vitro*. Using specific single-strand DNA substrates, recombinant AID is able to convert a single dC residue to dU, creating a UNG/APE1-sensitive substrate (Bransteitter *et al.*, 2003; Dickerson *et al.*, 2003). Additionally, looking at whole cellular extracts from either splenic B cells or HEK293T cells expressing recombinant AID, single-strand DNA oligomers containing multiple hotspot motifs become susceptible to treatment with UNG and APE1 (Chaudhuri *et al.*, 2003). Furthermore, double-strand DNA substrates were protected from AID deamination except in the presence of transcription, which explains the requirement for a single-strand DNA substrate (Chaudhuri *et al.*, 2003; Ramiro *et al.*, 2003). However, in these experiments, AID was able to bind to RNA molecules, albeit with lower affinity than the single-strand DNA template, allowing the slight possibility that AID may also function on RNA (Dickerson *et al.*, 2003). Additionally, the known RNA editing enzyme APOBEC1 has residual activity on single-strand DNA in similar assays, suggesting that these enzymes might act promiscuously (Harris *et al.*, 2002). Taken together with the genetic data, most evidence suggests that AID functions as a DNA deaminase. However, the true test of AID activity would be to directly detect the accumulation of dU residues in genomic DNA during an immune response, which has yet to be characterized at this time.

4.2. Mismatch repair and DNA polymerases

In addition to being processed by UNG, deoxyuracil can be recognized by some proteins in the MMR pathway (Fig. 6.3). The canonical role of MMR is to remove DNA mismatches and repair DNA in an accurate manner. The MSH2–MSH6 or MSH2–MSH3 heterodimer binds to a mismatch and recruits MLH1–PMS2 to the site. This nicks the DNA downstream of the mismatch and attracts exonuclease 1 (Exo1) to remove the strand containing the mismatch. The gap is then filled in by high-fidelity Polδ to restore the original sequence. However, during the immune response, deficiency in some MMR proteins resulted in decreased, not increased, mutation frequencies, suggesting that these proteins actually generate mutations. Extensive analyses have examined the roles of these proteins in processing mismatches in the V and S regions (Bardwell *et al.*, 2004; Ehrenstein

and Neuberger, 1999; Ehrenstein *et al.*, 2001; Frey *et al.*, 1998; Jacobs *et al.*, 1998; Kim *et al.*, 1999; Kong and Maizels, 1999; Li *et al.*, 2004b, 2006; Martin *et al.*, 2003; Martomo *et al.*, 2004; Phung *et al.*, 1998, 1999; Rada *et al.*, 1998, 2004; Schrader *et al.*, 2003; Shen *et al.*, 2006; Wiesendanger *et al.*, 2000; Winter *et al.*, 1998). The prevailing model is that MSH2–MSH6 binds to a U:G mismatch and recruits DNA Polη (Wilson *et al.*, 2005), a low-fidelity polymerase that preferentially synthesizes mispairs when copying T nucleotides (Matsuda *et al.*, 2001). This explains why the frequency of mutations at A:T bp drops dramatically in the absence of MSH2, MSH6, and Exo1, which interact with Polη. In contrast, mice deficient for the other MMR proteins had no alteration in the SHM spectra.

Throughout evolution, specialized DNA polymerases have evolved to copy DNA with low fidelity to bypass DNA damaging lesions. To see whether these polymerases are recruited to the *Ig* loci to increase sequence diversity, SHM was examined in mice deficient for eight polymerases. Pols β, μ, λ, and ι are not involved in SHM (Bertocci *et al.*, 2002; Esposito *et al.*, 2000; Martomo *et al.*, 2006; McDonald *et al.*, 2003), while the role of Polθ is currently unclear because of conflicting results (Martomo *et al.*, 2008; Masuda *et al.*, 2005, 2006, 2007; Zan *et al.*, 2005). However, there is well-defined evidence that Polζ, Rev1, and Polη have distinct roles during SHM. Conditional inactivation of Polζ in mice resulted in ~2–3-fold decrease in mutation frequency without altering mutation spectra, consistent with a role for Polζ in extending DNA mismatches (Diaz *et al.*, 2001; Schenten *et al.*, 2009; Zan *et al.*, 2001). However, due to the embryonic lethality and genomic instability seen in Polζ-deficient mice and B cells, the full extent for a role of Polζ has yet to be defined. Rev1 is a cytidyl transferase which causes G:C to C:G transversions in SHM (Arakawa *et al.*, 2006; Jansen *et al.*, 2006; Masuda *et al.*, 2009; Ross and Sale, 2006). More recently, a catalytically inactive Rev1 mutant has been examined and suggests a minor role for mouse Rev1 contributing to transition mutations as well (Masuda *et al.*, 2009); however, this was not seen in DT40 cells (Ross and Sale, 2006).

Compared to the modest roles of Polζ and Rev1, Polη has been shown to contribute significantly to diversity during SHM. Genetic mutation of Polη in humans with xeroderma pigmentosum variant disease or deletion of the gene in mice resulted in a dramatic decrease in mutations at A:T residues with a modest decrease in the overall mutation frequency (Delbos *et al.*, 2005; Faili *et al.*, 2004; Zeng *et al.*, 2001, 2004). Significantly, the decrease in A:T mutations is similar to the effects seen in mice with deficiencies in MSH2, MSH6, and Exo1, suggesting that they all act in the same pathway. Additionally, the MSH2–MSH6 heterodimer interacts physically and functionally with Polη, suggesting a shared role in producing A:T mutations during SHM (Wilson *et al.*, 2005). However, close examination of the spectra from *Msh6*$^{-/-}$ mice or double deletion of both

MSH2 and Polη indicates that the effects are not completely overlapping (Delbos *et al.*, 2007; Martomo *et al.*, 2005). Individually, *Polh* $^{-/-}$ (which encodes Polη) or *Msh2* $^{-/-}$ mice display ~15% residual A:T mutations, while the double knockout shows only ~1% A:T mutations. This suggests that while Polη and MSH2 function together, they are also able to contribute to SHM independently of each another. Interestingly, a recent paper by Reynaud and colleagues analyzed a *Polk* $^{-/-}$ *Polh* $^{-/-}$ mouse strain that shows ~7% mutations at A:T, suggesting that Polκ may function during SHM and contribute a modest amount of mutations to the overall spectra (Faili *et al.*, 2009). The identification of Polκ-dependent mutations is significant as previous reports failed to identify such a role (Schenten *et al.*, 2002; Shimizu *et al.*, 2003, 2005). However, it is difficult to differentiate between the possibility that Polκ function is obscured by the high mutation rate of Polη, or whether the normal presence of Polη blocks Polκ access in wildtype mice. The residual A:T mutations seen in the absence of both Polη and Polκ also suggest that a third polymerase can function in SHM. The total lack of A:T mutations in the *Polh* $^{-/-}$ *Msh2* $^{-/-}$ mice indicates that MSH2 is responsible for recruiting Polη, Polκ, and perhaps other polymerases.

One key aspect that promotes error-prone replication is the role of proliferating cell nuclear antigen (PCNA) monoubiquitination. PCNA is a replication accessory factor which functions in recruiting, tethering, and switching DNA polymerases at the primer-template junction. To coordinate these events, PCNA is posttranslationally modified at residue K164 to initiate either error-free or error-prone repair (Ulrich, 2009). Mutation of the K164 residue, or deletion of PCNA ubiquitin ligase Rad18, resulted in a dramatic decrease in A:T mutations during SHM (Arakawa *et al.*, 2006; Bachl *et al.*, 2006; Langerak *et al.*, 2007; Roa *et al.*, 2008). This suggests that PCNA modification is regulated to cause DNA synthesis by Polη in activated B cells. Additionally, loss of PCNA ubiquitination in DT40 cell lines, but not mice, showed a decrease in overall mutation frequency, indicating an increased utilization of PCNA-Ub in DT40 (Arakawa *et al.*, 2006; Bachl *et al.*, 2006). Interestingly, in DT40 cells, the combination of a PCNA mutant and deletion of Rev1 showed almost complete loss of SHM, suggesting a potential inability of the canonical DNA replication machinery to bypass dU (Arakawa *et al.*, 2006). Therefore, it would be interesting to further understand the role of PCNA modification in SHM, as the K164 mutation also effects other modifications such as sumoylation and polyubiquitination.

Finally, do the UNG and MSH2–MSH6 pathways shown in Fig. 6.3 operate at the same time and compete for the same dU? Some models suggest that they do (Rada *et al.*, 2004; Schanz *et al.*, 2009), whereas others propose that the pathways are temporally separated during the G1 and S phases of the cell cycle (Krijger *et al.*, 2009; Weill and Reynaud, 2008).

A noncompetitive model is appealing in that MSH2–MSH6 could recognize U:G in double-strand DNA during G1 and UNG would be most active on single-strand DNA in replication during S phase. The latter model is based on the recent finding that UNG is weakly expressed during G1 and is upregulated during early S phase (Hagen *et al.*, 2008). However, Stavnezer and colleagues show that UNG is fully active during the G1 phase and that double-strand DNA breaks are produced during G1 (Schrader *et al.*, 2007). This process of breaks is dependent upon both UNG and MSH2–MSH6, suggesting that both pathways function in G1. Taken together, it is currently unclear when and by what nature UNG and MSH2–MSH6 function in relation to each other when processing AID-generated uracils.

5. CONCLUSION

Even with the shear girth of information on AID biology, it is still unclear how the cell fully coordinates deamination events with mutagenic repair. As mentioned earlier, the mechanisms of AM, GC, and CSR start with a single protein, yet require extensive cellular coordination to produce the initiating deamination. It has been established that AID is tightly regulated at the levels of transcription, translation, phosphorylation, ubiquitination, cellular localization, protein stability, and protein–protein interaction. While much has been discovered, many components are still unknown. It is clear that *cis*-regulatory elements and transcription are involved, yet no true recruiting factor has been identified for AID. AID specifically interacts with RPA, yet it is unclear how or whether this interaction assists in AID localizing to single-strand DNA. Does AID travel with the transcription machinery in association with CTNNBL1 alone or is it more complicated? These questions and many more require further studies to understand how AID is targeted to the *Ig* loci to cause SHM.

In addition to regulation of AID deamination, the processing of dU also plays a significant role in achieving efficient antibody diversity. Of specific interest is how a B cell can manipulate DNA repair to function either less efficiently or less faithfully at the *Ig* loci, while still maintaining overall genomic integrity. Does the frequency and/or proximity of deamination events overwhelm the faithful capacities of BER and MMR? Is DNA repair specifically inhibited at the *Ig* loci during an immune response, or does repair become error-prone throughout the cell? It has been reported that DNA repair can be more or less efficient in different regions of the genome (Alrefai *et al.*, 2007; Liu *et al.*, 2008); however, it is not known what mechanisms coordinate this phenotype. Therefore, it remains to be fully understood what role DNA repair plays in transforming AID-dependent uracils into mutations.

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CHAPTER 7

BCL6: Master Regulator of the Germinal Center Reaction and Key Oncogene in B Cell Lymphomagenesis

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Contents

Abstract

BCL6 is a transcriptional repressor which has emerged as a critical regulator of germinal centers (GC), the sites where B cells are selected based on the production of antibodies with high affinity for the antigen. BCL6 is also a frequently activated oncogene in the pathogenesis of human B cell lymphomas, most of which derive from the GC B cells. A thorough understanding of the biological

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role of BCL6 in normal B cell development and lymphomagenesis depends upon the identification of the full set of genes that are targets of its transcriptional regulatory function. Recently, the identification of BCL6 targets has been implemented with the use of genome-wide chromatin immunoprecipitation and gene expression profiling approaches. A large set of promoters have been shown to be physically bound by BCL6, but only a fraction of them appears to be subjected to transcriptional repression in GC B cells. This set of BCL6 targets points to a number of cellular functions which are likely to be directly controlled by BCL6 during GC development, including activation, survival, DNA-damage response, cell cycle arrest, cytokine-, toll-like receptor-, TGFβ-, WNT-signaling, and differentiation. Overall, BCL6 is revealing its dual role of "safe-keeper" in preventing centroblasts from responding to signals leading to a premature exit from the GC and of contributor to lymphomagenesis by allowing the instauration of conditions favorable to malignant transformation.

1. GERMINAL CENTER: THE SITE OF BCL6 EXPRESSION IN B CELLS

The immune system is dedicated to the recognition of foreign antigens and to the defense from invading microorganisms. B lymphocytes are critical players of the humoral immune responses being involved in the production of antibodies. The differentiation process leading to the generation of effector B cells starts when mature naïve B cells migrate from the bone marrow to the secondary lymphoid organs where, upon encountering the antigen, they are stimulated to proliferate and further differentiate into centroblasts (CB). The high rate of cell division occurring at this stage of development leads to the formation of characteristic histological structures called germinal centers (GCs). The GCs provide the environment where B cells undergo genetic modifications of their immunoglobulin (Ig) genes and are then selected according to their newly acquired ability to recognize with high-affinity the antigen. At the CB stage, B cells are subjected to the somatic hypermutation (SHM) of the Ig variable region locus, a key mechanism for the generation of high-affinity antibodies. Highly proliferating CB further differentiate into centrocytes that undergo class switch recombination (CSR), a somatic recombination mechanism that allows the expression of different Ig classes associated to distinct effector functions. At the end of the GC reaction, B cells that acquired the ability of expressing high-affinity Ig receptors are positively selected and further differentiate into memory B cells or plasma cells. Plasma cells are the effector cells dedicated to the production of a high amount of antibodies, while the immunological memory is maintained by memory B cells that are the effectors of rapid immunological response upon

later exposure to the same antigen (Klein and Dalla-Favera, 2008). The inability of inducing GC reactions in response to an antigenic challenge is associated with severe immune deficiency syndromes consistent with the critical role of this stage of B cell differentiation for humoral immune response (Durandy *et al.*, 2007). Moreover, each stage of B cell development can be associated with malignant transformation giving rise to different types of lymphoma and leukemia. However, most mature B cells malignancies, including Burkitt lymphoma (BL), diffuse large B cell lymphoma (DLBCL), and follicular lymphoma (FL) originate from malignant transformation of GC B cells. The physiologic genetic modifications occurring during the GC reaction appear to have a role in the process of malignant transformation, suggesting that a tight regulation of B cell differentiation is critical. Thus, considerable effort has been made to investigate the role of GC in physiology and malignancy.

2. BCL6 AND ITS FUNCTION

A major player of the GC reaction is represented by BCL6, a transcriptional repressor identified in 1993 as the target of chromosomal translocations affecting band 3q27 in DLBCL (Baron *et al.* 1993; Ye *et al.*, 1993a). Then, it was shown that BCL6 is a key regulator of the GC reaction since BCL6-null mice are characterized by lack of GC formation and the inability to produce high-affinity antibodies (Dent *et al.* 1997; Ye *et al.* 1997).

The BCL6 gene encodes a 95-kD nuclear phosphoprotein belonging to the BTB/POZ/ZincFinger (ZF) family of transcription factors (Ye *et al.*, 1993b). The N-terminal BTB/POZ domain is linked to a central region, including three PEST motifs and to six C-terminal ZF DNA-binding motifs. BTB domain dimerization is required for the activities of the BTB-ZF proteins, implying that BCL6 works as dimer. BCL6 functions as a transcriptional repressor via its C-terminal ZF domain that binds to specific DNA sequences in the promoter region of target genes, and two transcriptional repression domains (Chang *et al.*, 1996) that interact with distinct corepressor complexes (Dhordain *et al.*, 1997, 1998; Fujita *et al.*, 2004; Huynh and Bardwell, 1998) (Fig. 7.1A).

The BCL6 repression activity involves the recruitment of class I and II histone deacetylase complexes (HDAC) directly or through corepressors (Lemercier *et al.* 2002; Wong and Privalsky, 1998). Several corepressors have been reported to interact with BCL6, including NCOR2 (SMRT) (Dhordain *et al.*, 1997; Huynh and Bardwell, 1998; Wong and Privalsky, 1998), NCOR1 (Huynh and Bardwell, 1998), BCOR (Huynh *et al.*, 2000), MTA3 (Fujita *et al.*, 2004), and CTBP1 (Mendez *et al.*, 2008). At least three of BCL6 corepressors (NCOR1, NCOR2, and BCOR) bind in a mutually exclusive way to the BTB domain of BCL6 (Ahmad *et al.*, 2003; Huynh

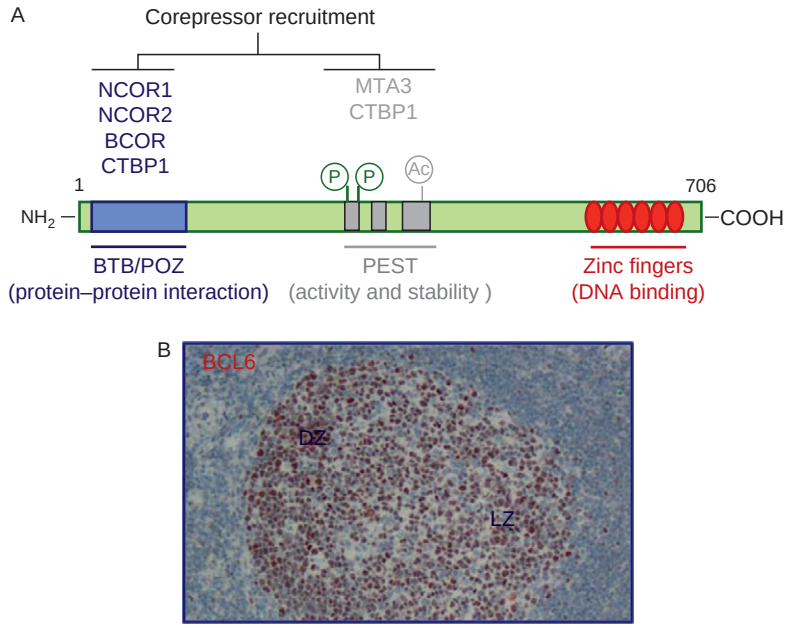

FIGURE 7.1 *BCL6 protein and its expression pattern.* (A) Schematic representation of BCL6 protein. The main protein domains are displayed, including critical protein modifications (P, phosphorylation; Ac, acetylation). Different sets of corepressors are recruited through the BTB domain or the BCL6 middle portion. (B) BCL6 detection by immunoistochemistry is restricted to GC B cells. BCL6 expression is high in B cells located in the dark zone (DZ) and becomes dim in a subset of light zone (LZ) B cells.

et al., 2000), MTA3 binds in the middle portion and CTBP1 appears to interact with both the BTB and the middle domains of BCL6 (Fig. 7.1A). The recruitment of corepressors through the BTB domain or through the BCL6 middle portion appears to be associated with the regulation of different subset of targets (Parekh *et al.*, 2007).

The C-terminal portion of BCL6 binds to DNA and recognizes specific DNA motifs. BCL6-binding motifs were initially identified based on the binding of recombinant BCL6 protein to synthetic oligonucleotides *in vitro* (Chang *et al.* 1996; Kawamata *et al.* 1994). More recently, the use of Chromatin Immunoprecipitation (ChIP)-on-chip technology led to the identification of promoters bound by BCL6 *in vivo* and allowed the redefinition of its consensus-binding motif in normal B cells under physiologic conditions (Basso *et al.* 2010; Ci *et al.* 2009). In particular, an extensive analysis of BCL6 ChIP-on-chip data generated from GC B cells showed that motif combinations (modules) had a stronger predictive value for BCL6 binding compared to single motifs. The most significantly reported module included: (i) the M00424 motif, a consensus sequence for the

NKX-homeobox family of transcription factors; (ii) the novel motif M2, which mimics an M00424 half site; and (iii) the M0 motif, which is similar but not entirely compatible with previously *in vitro* identified BCL6-binding motifs. Further experimental validation demonstrated that BCL6-mediated transcriptional repression is dependent on the M0 site, but not on M00424 and M2, which may represent sites for the binding of other transcription factors complementing the biological activity of BCL6 or facilitating its transcriptional function by appropriately modifying chromatin (Basso *et al.*, 2010).

Though direct binding to DNA is generally required for BCL6 transcriptional repression, at least two targets (CDKN1A and BCL2) have been shown to recruit BCL6 to their promoters through ZBTB17 (miz1) binding to Inr elements (Phan *et al.*, 2005; Saito *et al.*, 2009). The presence of Inr elements in BCL6-bound regions, as detected by ChIP-on-chip, in the absence of other BCL6-binding motifs, suggests that the repression of a fraction of BCL6 targets occurs via ZBTB17 (Basso *et al.*, 2010).

In conclusion, BCL6 is a transcriptional repressor which recruits the repression machinery directly or through several corepressors into the regulatory regions of its targets by binding to specific DNA motifs or through interaction with ZBTB17.

3. REGULATION OF BCL6

BCL6 expression is restricted to the GC B cells in the B cell lineage, suggesting the presence of a tight regulation (Fig. 7.1B). Indeed, several signaling pathways, known to have a role during GC reaction, have been shown to modulate BCL6 expression both at the transcriptional and protein level (Fig. 7.2).

Activation of B cell receptor (BCR) by the antigen induces MAP kinase-mediated phosphorylation of BCL6 protein which leads to BCL6 degradation by the ubiquitin–proteasome pathway (Niu *et al.*, 1998). Stimulation of the CD40 receptor by CD40 ligands expressed on T cells leads to transcriptional downregulation of BCL6 (Allman *et al.*, 1996; Basso *et al.*, 2004; Niu *et al.*, 2003). Recently, the downregulation of BCL6 by CD40 stimulation has been shown to be linked to a signaling pathway that involves NF-κB-mediated transcriptional activation of IRF4, which directly represses BCL6 transcription (Saito *et al.*, 2007). Both BCR and CD40 signaling pathways are involved in the selection of cells which acquired the ability to express high-affinity Ig receptors occurring at the late stages of the GC reaction. At this stage of B cell differentiation, BCL6 needs to be downregulated to enable the cells to further differentiate.

Another layer of BCL6 regulation is represented by the DNA damage which induces BCL6 protein degradation through a pathway which is

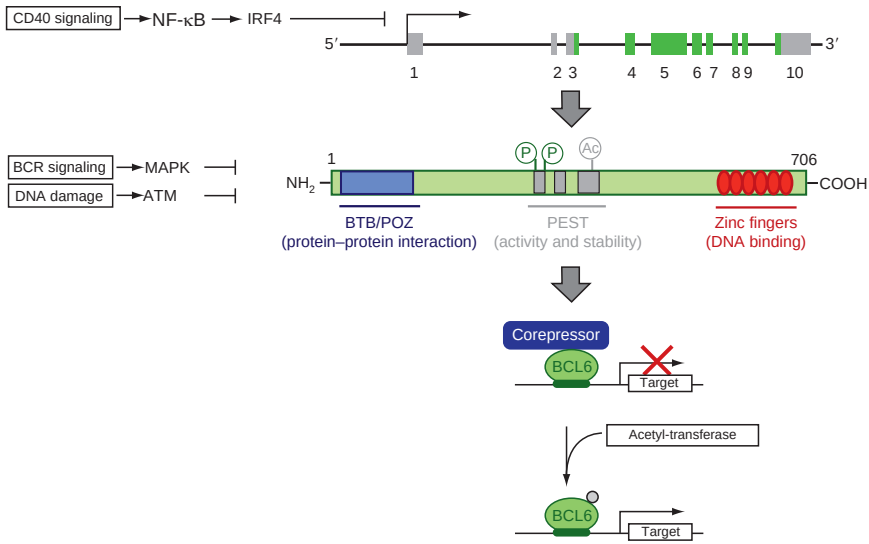

FIGURE 7.2 *Regulation of BCL6*. Schematic representation of the main signaling pathways associated to transcriptional and posttranscriptional regulation of BCL6.

independent from the one induced by BCR signaling. DNA-damage accumulation leads to ATM-promoted BCL6 phosphorylation, followed by its interaction with the isomerase Pin1 and BCL6 degradation by the ubiquitin–proteasome system (Phan *et al.*, 2007). This regulatory pathway is likely to represent a failsafe mechanism in order to grant BCL6 degradation in the event of massive DNA damage and assure that the damaged cells undergo apoptosis.

BCL6 function is also impaired by acetylation, which triggers BCL6 dissociation from corepressor complexes (Bereshchenko *et al.*, 2002). Interestingly, acetylation has a negative effect on BCL6 activity while it positively affects the function of TP53, one of the well-characterized BCL6 targets, suggesting a coordinated action between functional silencing of BCL6 and activation of its targets.

BCL6 expression is also modulated during the GC reaction through an autoregulatory circuit. Indeed, BCL6 binds to its promoter and likely acts to monitor its own expression levels. This autoregulatory circuit is disrupted in a subset of DLBCL by the SHM mechanism which introduces mutations which impair BCL6 binding to its promoter (Pasqualucci *et al.*, 2003).

Overall, different layers of BCL6 regulation acts coordinately to assure a safe level of expression in CB and to grant an efficient switch-off in the late stages of the GC reaction, an essential requirement to move further into the differentiation process.

4. DISCOVERY OF BCL6 TARGETS

A full understanding of BCL6 role in normal B cell development and in lymphomagenesis relies on the identification of the complete set of genes which are targets of its transcriptional regulatory function.

Initial target discovery was based on educated guesses (Niu *et al.*, 2003) and on the use of gene expression profiling (GEP) to identify on a large scale the genes whose expression is affected by the variations of BCL6 expression (Shaffer *et al.*, 2000). The latest approach has the advantage of producing a relevant number of candidates; however, it cannot distinguish between direct and the presumably very large set of secondary targets whose expression is indirectly affected by BCL6. Using these approaches, BCL6 has been shown to modulate the expression of genes involved in B cell activation, differentiation, cell cycle arrest, and apoptosis (Niu *et al.*, 2003; Polo *et al.*, 2004; Shaffer *et al.*, 2000; Tunyaplin *et al.*, 2004). Since normal GC B cells undergo apoptosis within hours of *ex vivo* culture and therefore cannot be experimentally manipulated *in vitro*, the discovery of BCL6 targets has been performed mainly on cell lines established from GC-derived tumors, leaving open the possibility that BCL6 function may be altered in lymphoma cells. A small set of genes has also been fully validated as functionally and physiologically relevant targets in normal cells, including the gene encoding the coactivator molecule CD80 (Niu *et al.*, 2003), genes involved in the sensing and response to DNA damage (TP53, ATR, and CHEK1) (Phan and Dalla-Favera, 2004; Ranuncolo *et al.*, 2007, 2008), the cell cycle arrest gene CDKN1A/p21 (Phan *et al.*, 2005), and the plasma cell differentiation master gene PRDM1 (Tunyaplin *et al.*, 2004).

More recently, a few studies based on genome-wide ChIP-on-chip have identified a large set of genes whose promoter regions are bound by BCL6 *in vivo* using cell lines (Polo *et al.*, 2007) or purified GC B cells (Basso *et al.*, 2010; Ci *et al.*, 2009). ChIP-on-chip analysis is critical to identify which promoters are occupied by a transcription factor *in vivo*; however, the physical binding does not necessarily imply functional activity, as shown for other transcription factors (Fernandez *et al.*, 2003). Therefore, the combination of GEP and ChIP-on-chip technologies has been instrumental toward the identification of the complete set of BCL6 targets. Two studies have been performed using this approach on normal GC B cells. The first one aiming at the comparison of the BCL6 transcriptional program in normal and malignant B cells reported that BCL6 binds to the promoter of almost 2000 genes and approximately 180 of them (out of 900 for which GEP were available) also showed downregulation in GC B cells (Ci *et al.*, 2009). The second study relies on a different platform and analysis method of ChIP-on-chip data as well as on a more recent whole genome GEP array and reports over 4000 promoters being targeted by

BCL6, a quarter of which display lower expression in GC B cells representing the largest set of BCL6 physiologic targets identified (Basso *et al.*, 2010). Based on these studies, the BCL6 transcriptional program is unfolding and becoming available to be fully interpreted.

5. CELLULAR PATHWAYS REGULATED BY BCL6

The discovery of a large number of BCL6 targets in normal GC B cells led to a broader and more complete picture of BCL6 physiologic function and opened the path to unveil its role in GC-derived malignancies.

The over 1200 gene targets found to be bound in their promoter by BCL6 and downregulated in normal GC B cells are clearly enriched in genes belonging to multiple functional pathways (Basso *et al.*, 2010). The current large set of novel targets confirmed the activity of BCL6 in modulating B cell activation, differentiation, and apoptosis and significantly extended the role of BCL6 in controlling DNA-damage sensing and response via the identification of a number of additional genes regulated by BCL6 in the same pathway. Moreover, BCL6 function appeared to be relevant on a number of not previously considered cellular pathways by modulating signaling through toll-like receptors, INF-R, a variety of cytokines, TGF-R, and WNT signaling. An intriguing feature of BCL6 function appears to be the broad control of several targets along the same pathway, often involving the simultaneous modulation of expression of cell surface receptors, signaling molecules, and nuclear effectors.

The criteria used in the target identification include not only binding of BCL6 to the promoter target but also evidences of transcriptional downregulation of the targets and mutually exclusive expression *in vivo*. Nonetheless, BCL6 has been shown to be part of an autoregulatory circuit by which it modulates its own expression binding to its promoter. Interestingly, unbiased genome-wide promoter binding studies revealed that BCL6 binds to the promoters of several genes which are highly expressed and play essential roles in GC B cells, including AICDA, MYB, CD38, and PAX5. Moreover, BCL6 binds to the promoters of its own corepressors (NCOR1, MTA3, CTBP1) (Basso *et al.*, 2010). Taken together, these observations suggest that BCL6 may act not only as a strong repressor impairing expression of its targets but also as a general modulator of transcription that allows a controlled expression of molecules critical for GC functions.

5.1. BCL6 modulates GC B cell activation and differentiation

B cell activation occurring in the late stages of the GC reaction is essential for the selection of B cells based on their Ig receptor affinity and to stimulate the positively selected cells toward further steps of

differentiation. The engagement of the BCR by the antigen in combination with costimulatory signals is required to deliver survival signals rescuing from apoptosis B cells which display high-affinity Ig receptors on their surface. In this rescuing process, a critical role is also played by the B–T cell interaction which contributes to B cell activation through the engagement of receptors by T cell surface-bound ligands. A well-characterized interaction occurs between the CD40 receptor on B cells and its ligand (CD154) expressed mainly on activated CD4+ T cells (van Kooten and Banchereau, 2000). CD40 signaling in B cells is required for a proper immune response as shown by patients affected by hyper-IgM (HIGM) syndrome in which mutations in CD154 lead to a severe immunodeficiency characterized by elevated levels of IgM, low levels of other Ig classes, absence of GC, and inability to mount a T-cell dependent humoral response (Aruffo *et al.*, 1993; DiSanto *et al.*, 1993; Korthauer *et al.*, 1993). The characteristics of HIGM syndrome have been recapitulated in CD40- or CD154-deficient mice (Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994). These observations suggest that CD40 signaling is required for T-cell dependent Ig class switch and GC formation. Evidences of CD40 signaling are not traceable in the bulk of the GC cells, but only in a small subset of centrocytes which indeed downregulate BCL6 expression (Basso *et al.*, 2004). CD40 signaling activates different mediators and pathways whose outcome is the activation of multiple transcription factors, including NF-κB, NF-AT, and AP-1 (Berberich *et al.*, 1994; Francis *et al.*, 1995). BCL6 acts on modulating a number of molecules involved in both the BCR and CD40 signal transduction from the surface to the nucleus, including Ca^{2+}-mediated signaling, MAPK, and NF-κB pathways, assuring that none of these pathways is prematurely activated (Fig. 7.3).

The B–T cell interaction is also dependent on the presence of costimulatory molecules such as those belonging to the B7 family. CD80 (B7-1) and its related molecule CD86 (B7-2) are expressed on antigen-presenting cells, including B cells, and their interaction with CD28 and CD152 molecules is required for T-cell activation, GC formation, and Ig class switching (Borriello *et al.*, 1997). The discovery of more members of the B7:CD28 family has revealed additional costimulatory pathways that have broadened the concept of costimulation (Dong *et al.*, 1999). CD274 (B7-H1, PDL1) has been shown to bind CD80, and to regulate the balance of activation and inhibition of the T cell response (Keir *et al.*, 2008). Both CD80 and CD274 are reported to be actively repressed by BCL6 in GC B cells (Basso *et al.*, 2010; Niu *et al.*, 2003), suggesting that BCL6 intervenes in modulating the presence of costimulatory molecules involved in the B–T cell interaction (Fig. 7.3).

B cells producing high-affinity antibodies are destined to differentiate into memory B cells and plasma cells. Although the molecular mechanisms leading to these final steps of differentiation are mostly unknown,

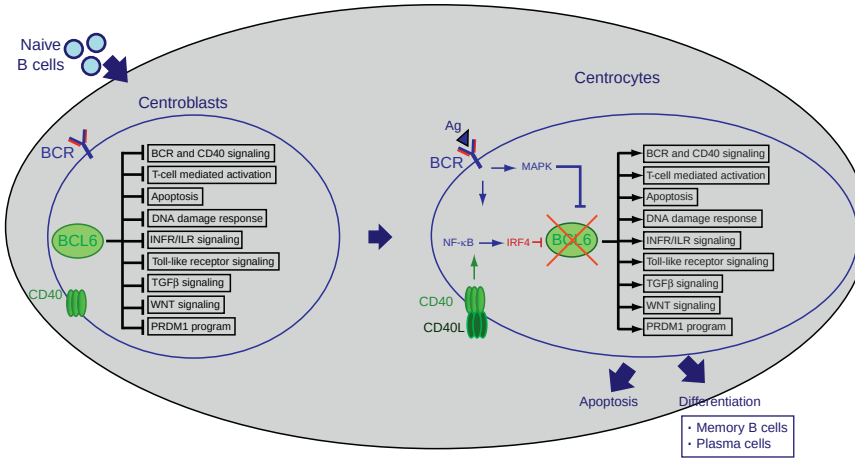

FIGURE 7.3 *Cellular pathways regulated by BCL6*. Schematic representation of BCL6 regulatory functions in GC B cells. BCL6 transcriptional repression is released in centrocytes upon the activation of signaling pathways that lead to BCL6 downregulation and protein degradation.

a few transcription factors, including PRDM1, XBP1, and IRF4, have been identified, the regulation of which is crucial for plasma cell commitment. PRDM1 (BLIMP1) is expressed in a subset of centrocytes and in plasma cells (Angelin-Duclos *et al.*, 2000) and it is required for the formation and maintenance of Ig-secreting B cells (Shapiro-Shelef *et al.*, 2003). PRDM1 has been shown to act upstream of XBP1, a transcription factor that is required for the secretory phenotype of plasma cells (Shaffer *et al.*, 2004). IRF4, a transcription factor expressed in a subset of centrocytes in the GC and in plasma cells (Falini *et al.*, 2000), is required for the generation of plasma cells and plays a critical function in CSR (Klein *et al.*, 2006). IRF4 has been suggested to act upstream of (Sciammas *et al.*, 2006) or in parallel to (Klein *et al.*, 2006) PRDM1 for the generation of plasma cells. Consistent with their pattern of expression restricted in GC to BCL6-negative centrocytes, PRDM1 and IRF4 have been reported to be transcriptionally repressed by BCL6 (Basso *et al.*, 2010; Shaffer *et al.*, 2000; Tunyaplin *et al.*, 2004), supporting a role for BCL6 in blocking the differentiation of GC B cells (Fig. 7.3).

BCL6 direct targets include an increasing number of genes pointing to several signaling pathways which may have a role in GC B cell activation and differentiation. Multiple interferon-type and interleukin receptors that lead to the activation of JAK/STAT are broadly represented among BCL6 targets. Furthermore, STAT family members were also found to be directly repressed by BCL6 (Basso *et al.*, 2010; Ding *et al.*, 2008).

Interestingly, a deficiency of STAT3 expression has been associated with a defect in the generation of plasma cells that produce IgG subclasses (Fornek *et al.*, 2006). A modulatory activity on the toll-like-receptor pathway has been also suggested by the presence among BCL6 target genes of those encoding both toll-like receptors and transducers of the toll-derived signals. Since this pathway has been reported to have a role in T-dependent immune response and the development of memory B cells (Meyer-Bahlburg *et al.*, 2007; Pasare and Medzhitov, 2005), these findings suggest that its silencing by BCL6 may also be necessary to avoid activating stimuli during the proliferative stage of GC reaction. BCL6 appear to have a modulator action on the ability of TGFβ to regulate post-GC differentiation targeting genes encoding TGFβ-type receptors, a ligand (BMP2), and nuclear effectors. Previous studies in TGFβ$^{-/-}$ mice showed the role of TGFβ in promoting the differentiation of IgA-secreting plasma cells and in attenuating B cell response to low-affinity antigens (Cazac and Roes, 2000). The WNT-signaling pathway also appears to be affected by BCL6 through the control of genes encoding its receptors, signal transducers, and downstream transcription factors. In conjunction with the report that mice bearing B cell-specific deletion of β-catenin show defective plasma cell formation *in vitro* (Yu *et al.*, 2008), these results suggest a role of WNT signaling in the late stage of B cell differentiation and support the silencing of this pathway by BCL6 in the early stage of the GC reaction (Fig. 7.3). Together, these findings point to a broad function of BCL6 in modulating a variety of incoming signals that may prematurely activate CB in the GC and indicate that while BCL6 is required for GC formation, its downregulation may be critical for B cell exiting from the GC and differentiation toward memory and plasma cells.

5.2. BCL6 controls the DNA damage and apoptotic responses in GC B cells

The role of BCL6 in protecting cells against DNA-damage-induced apoptosis has been unveiled by the initial discovery of TP53 being a direct BCL6 target (Phan and Dalla-Favera, 2004). Following this discovery, several more genes (CDKN1A, ATR, and CHEK1) involved in the DNA-damage response were accounted among BCL6 targets (Phan *et al.* 2005; Ranuncolo *et al.* 2007, 2008). The application of genome-wide ChIP-on-chip technology in association with GEP has further consolidated the role of BCL6 in modulating the sensing and execution of responses to DNA damage by the identification of a large set of BCL6 targets involved in this pathway (Basso *et al.*, 2010) (Fig. 7.3). The modulation of the DNA-damage sensing and response pathway also appears to be functionally correlated with the broad control that BCL6 has on the apoptotic machinery in which it affects multiple genes, encoding both pro- and antiapoptotic proteins.

The transcriptional profile of GC B cells is characterized by a downregulation of multiple antiapoptotic genes and upregulation of proapoptotic genes (Klein *et al.*, 2003), resulting in the well-known high susceptibility to apoptosis displayed by CB. This represents a critical phenotype ensuring that GC B cells will be eliminated by apoptosis if not rescued by efficient BCR engagement and other signaling pathways. Of note, among BCL6 direct targets involved in apoptosis was recently identified BCL2, a key antiapoptotic molecule with oncogenic functions in DLBCL and FL (Ci *et al.*, 2009; Saito *et al.*, 2009).

These findings collectively suggest that BCL6 may prevent normal cell cycle arrest and apoptotic responses in GC B cells to allow the execution of DNA-remodeling processes (SHM and CSR) without eliciting responses to DNA damage.

6. BCL6 AND LYMPHOMAGENESIS

BCL6 was identified in virtue of its involvement in chromosomal translocations affecting band 3q27 in DLBCL (Baron *et al.*, 1993; Ye *et al.*, 1993a,b). Further studies confirmed that rearrangements of BCL6 were detectable in ~40% of DLBCL and 5–10% of FL (Butler *et al.*, 2002; Kerckaert *et al.*, 1993; Lo Coco *et al.*, 1994; Ye *et al.*, 1993a). The translocations involving the 3q27 locus affect the 5′ regulatory region of BCL6 juxtaposing its coding sequence to heterologous promoters derived from other chromosomes (Ye *et al.*, 1995) (Fig. 7.4A). The common denominator of these promoters is their constitutive activity in the B cell lineage, and in particular, their persistent activity in post-GC cells, such as immunoblasts and plasma cells, contrasting with the GC-specific activity of the BCL6 promoter (Chen *et al.*, 1998). Indeed, the GC-restricted expression of BCL6 in the B cell lineage is altered by the promoter substitution. A different mechanism leading to BCL6 deregulation was later found to be associated with the SHM occurring on BCL6 5′ regulatory region. BCL6 was reported to be the first non-Ig target affected by SHM, a mechanism thought to be restricted to Ig genes (Pasqualucci *et al.*, 1998; Shen *et al.*, 1998). Further investigations identified a subset of DLBCL (~14% of the cases) carrying in BCL6 5′ regulatory region-specific mutations which lead to its deregulated expression by disrupting an autoregulatory circuit (Pasqualucci *et al.*, 2003; Wang *et al.*, 2002) or impairing IRF4-mediated repression (Saito *et al.*, 2007) (Fig. 7.4B). The link between BCL6 deregulation and its oncogenic function was conclusively demonstrated in mouse models in which BCL6 deregulated expression leads to the development of lymphomas (Cattoretti *et al.*, 2005).

The large number of BCL6 targets involved in the DNA damage responses suggests that an important function of BCL6 is to allow

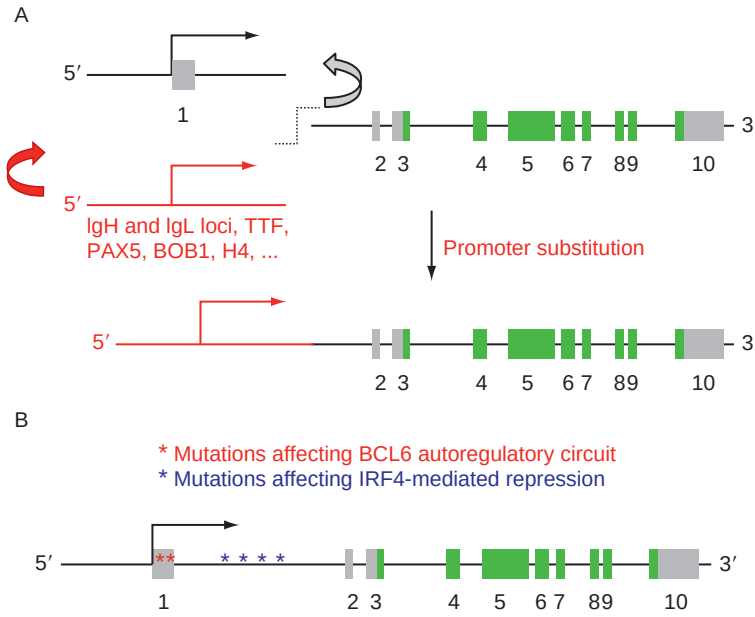

FIGURE 7.4 BCL6 gene is targeted by genetic aberrations associated with lymphomagenesis. (A) BCL6 gene is affected by chromosomal translocations substituting its 5′ regulatory region with heterologous promoters. (B) BCL6 autoregulatory circuit and IRF4-mediated repression are found to be impaired by somatic mutations in a subset of DLBCL.

GC B cells to tolerate the physiologic DNA breaks required for SHM and CSR. However, this state of tolerance for genomic instability is likely to play a role in lymphomagenesis. A direct link between physiologic DNA-remodeling events and GC lymphoma development has been shown in mice lacking AICDA, the enzyme required both for CSR and SHM (Muramatsu *et al.*, 2000). In AICDA-deficient mice, the development of BCL6-driven GC-derived lymphomas is impaired, but no effect is observed on pre-GC lymphomas (Pasqualucci *et al.*, 2008). The BCL6-driven unresponsiveness to DNA-damage checkpoints may allow the instauration of genetic aberrations introduced by AICDA errors, including those affecting BCL6 itself and leading to its deregulated expression.

The dissection of the role of BCL6 in DLBCL pathogenesis is complicated by the characteristic heterogeneity of this disease. Indeed, GEP-based class discovery was able to identify novel molecular subtypes in DLBCL suggesting that this disease is unlikely to be a single entity. A GEP-based classification (Cell Of Origin, COO, classification) led to distinguish at least two distinct groups of DLBCL, including GCB and ABC types (Alizadeh *et al.*, 2000). The first group has been associated with

cells in the GC stage and display high expression of BCL6, CD10, and other GC markers. The ABC subgroup resembles cells in a later stage of differentiation when BCL6 is downregulated and displays a transcriptional signature mimicking the activation signature obtained in B cells upon *in vitro* stimulation. Although the GCB type of DLBCL is usually associated with BCL6 activity, translocations affecting the BCL6 locus occur more frequently in the ABC subtype, suggesting that BCL6 deregulation may also play a role at the stage of post-GC differentiation, impairing BCL6 downregulation (Iqbal *et al.*, 2007). The COO classification does not fully recapitulate the heterogeneity of DLBCL which appear to hide a larger number of molecular entities. Indeed, a study using multiple data analysis methods to recognize robust gene clusters in GEP datasets identified three DLBCL biological subtypes not related with the COO, but associated with tumor microenvironment and host inflammatory response as defining features of DLBCL (Monti *et al.*, 2005). The first cluster (oxidative phosphorylation, OxPhos type) was enriched in genes involved in mitochondrial functions, the second cluster was marked by genes involved in the BCR signaling and proliferation (BCR type), the third cluster was defined mainly by an associated host response gene signature. The COO classification applied at the same dataset was able to discriminate between GCB and ABC subtypes, suggesting that the two classification systems appear to capture different aspects of DLBCL biology. The BCR-type DLBCL showed an enrichment for BCL6 targets among downregulated genes compared to the OxPhos-type of DLBCL (Ci *et al.*, 2009). Inhibition of BCL6 activity by BPI, a peptide which inhibits the interaction between BCL6 and its corepressors, was shown to be effective in a subset of DLBCL cell lines belonging to the BCR-type, suggesting that the inhibition of BCL6 may be more effective in this subset of DLBCL (Polo *et al.*, 2007). These data suggest that BCL6 may play distinct roles in the pathogenesis of different molecular subtypes of DLBCL.

BCL6 targets the transcription of at least two critical oncogenes for GC-derived lymphomas, MYC and BCL2, which are affected by translocations representing the hall mark of BL and FL, respectively. The t(8;14) or its variants t(8;2) and t(8;22) translocations are found in all cases of BL and cause the substitution of the 5′ regulatory region of MYC with different regions of the Ig genes, leading to MYC deregulated expression. As consequence of the translocation, the BCL6-driven downregulation of MYC is impaired. In FL, different mechanisms have been shown to be involved in the ZBTB17-mediated BCL6 repression of BCL2, including chromosomal translocations of the BCL2 gene, somatic mutations in the BCL2 promoter region, and deregulated expression of ZBTB17 (Saito *et al.*, 2009).

Overall, BCL6 is genetically targeted and plays a role in the pathogenesis of DLBCL and in a fraction of FL, however, it is likely to have a function in all GC-derived tumors. BCL6 expression grants to the tumors

a certain resistance to genomic damage and apoptosis and impairs the differentiation processes. Moreover, as observed in FL and BL, it appears preferable to impair BCL6 repression on a subset of its targets (i.e., MYC and BCL2) than lose the advantages provided by BCL6 expression in GC-derived tumor cells.

7. CONCLUSIONS

The essential role of BCL6 in GC development and its involvement in GC lymphomagenesis have been widely explored using a number of complementary approaches and recently in a genome-wide fashion. The discovery of the large set of its targets suggests that BCL6 controls B cell activation, differentiation, susceptibility to DNA damage, and apoptosis during the proliferative phase of the GC reaction. BCL6 is expressed in all GC-derived malignancies, including BL, FL, DLBCL, and a subset of Hodgkin lymphoma. A large fraction of the BCL6 physiologic functions is likely to be maintained in the malignant cells, suggesting that all lymphoma subtypes expressing BCL6 may be relatively insensitive to a variety of activation and differentiation stimuli. Therefore, direct targeting of BCL6 (Cerchietti *et al.*, 2009) may represent a strategy to complement other therapeutic approaches aiming to the induction of apoptosis, activation, and/or differentiation.

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