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CHAPTER 1

T Cell Activation and the Cytoskeleton: You Can't Have One Without the Other

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Contents

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Abstract More than a quarter of a century has passed since the observation that T cells rapidly polarize their actin and microtubule cytoskeletal systems toward antigen-presenting cells during activation. Since this initial discovery, several receptors on T cells (e.g., T cell receptor [TCR], co-receptors, integrins, and chemokine receptors) have been identified to regulate these two cytoskeletal networks through complex signaling pathways, which are still being elucidated. There is now an undeniable body of biochemical, pharmacological, and genetic evidence indicating that regulators of actin and microtubule dynamics are crucial for T cell activation and effector functions. In fact, the actin cytoskeleton participates in the initial clustering of TCR–major histocompatability complex or peptide complexes, formation and stabilization of the immune synapse, integrin-mediated adhesion, and receptor sequestration, whereas both the actin and microtubule cytoskeletons regulate the establishment of cell polarity, cell migration, and directed secretion of cytokines and cytolytic granules. Over the past several years, we have begun to more thoroughly understand the contributions of specific actin-regulatory and actin-nucleating proteins that govern these processes. Herein, we discuss our current understanding of how activating receptors on T lymphocytes regulate the actin and microtubule cytoskeletons, and how in turn, these distinct but integrated cytoskeletal networks coordinate T cell immune responses.

1. INTRODUCTION

T lymphocytes develop in the thymus and perform their immune-surveillance activities by migrating between the blood and lymphatics in response to chemokines, which promote the deformation of T cells from ~8–10-μm wide rigid spheres covered in microvilli, into elongated, polarized cells that can bind to and extravasate through vascular endothelium into surrounding parenchyma. This migration is essential for T cells to locate and interact with antigen-presenting cells (APCs) in stimulating an immune response. $CD4^+$ T cells represent a key lymphocyte subset required for the development of humoral and cell-mediated responses, through the production of cytokines that support antibody maturation (class-switching and somatic hypermutation) and B cell growth as well as the cytolytic activity of $CD8^+$ T cells. For $CD4^+$ or $CD8^+$

T cells to carry out their effector functions, they must interact with peptide major histocompatability complex (pMHC) on an APC or target cell, respectively. During APC recognition, initial signals through the T cell receptor (TCR) drive the production of an actin-dependent lamellipodial structure over the APC interface, which facilitates further engagement of TCR–pMHC complexes, integrin-mediated adhesion, and formation of the immune synapse (IS), a structure involved in signaling and establishing cell polarity (Bromley *et al.*, 2001). As the IS forms, several receptor–ligand interactions activate signaling cascades, which further impact actin reorganization and lead to polarization of the microtubule network to the IS.

It has been more than 25 years since the discovery that polarization of filamentous (F)-actin and the microtubule-organizing center (MTOC, or centrosome) toward the APC was a mark of productive lymphocyte activation (Geiger *et al.*, 1982; Ryser *et al.*, 1982). Over the ensuing decades, the signaling pathways that regulate these cytoskeletal systems have begun to be elucidated. These studies indicate that the actin cytoskeleton regulates numerous T cell processes including the formation and stabilization of the IS, maintenance of macromolecular signaling assemblies, integrin-mediated adhesion, cell motility, and receptor sequestration and internalization (Billadeau *et al.*, 2007). Additionally, microtubules and F-actin participate in the establishment of cell polarity, a process required for directed cellular migration and delivery of cytokines or cytolytic granules toward the APC or target cell, respectively (Billadeau *et al.*, 2007). Thus, TCR-, integrin-, and chemokine-driven signaling pathways coordinate the regulation of these two cytoskeletal networks, which in turn, fundamentally contribute to the activation of T cells and the generation productive immune responses.

During the past several years, our understanding of the molecular mechanisms regulating the actin and microtubule networks in T cells has expanded exponentially. This is in part due to the functional characterization of cytoskeletal regulators in other cell types, which have been extrapolated into T cells. Additionally, the use of RNAi and gene-targeted animal models as well as the characterization of human diseases in which key regulators of the cytoskeleton are defective have helped us identify new cytoskeletal regulators and have provided insight into the physiological roles of these molecules in T cells. Lastly, the use of new microscopy techniques and tools that permit the spatiotemporal resolution of signaling molecules during T cell activation have had a profound impact on our understanding of cytoskeletal regulation. Altogether, these studies have yielded new, exciting ideas, which have been incorporated into our existing conceptual framework, and have contributed to a deeper understanding of the role of the cytoskeleton in T cell activation.

2. TCR SIGNALING LEADING TO ACTIN CYTOSKELETAL REMODELING

2.1. Proximal signals from the TCR to actin nucleation

TCR engagement during APC recognition leads to activation of the SRC-related proximal tyrosine kinase LCK, which then phosphorylates the TCR-associated ζ chain, resulting in the recruitment and activation of the tyrosine kinase ζ-associated protein of 70 kDa (ZAP70). These two proximal kinases subsequently phosphorylate numerous cellular substrates (e.g., LAT [linker for activation of T cells], SLP76 [SRC homology-2 (SH2) domain-containing leukocyte protein of 76 kDa], ITK [interleukin-2-inducible T cell kinase], VAV1), which are required for the activation of signaling pathways that regulate the T cell cytoskeleton (Fig. 1.1) (Koretzky *et al.*, 2006; Saito and Yokosuka, 2006; Wilkinson *et al.*, 2005). Not surprisingly, given the proximal role of LCK in the initiation of TCR signaling, pharmacological inhibition of LCK abrogates accumulation of F-actin at the IS (Morgan *et al.*, 2001) and TCR-dependent spreading (Bunnell *et al.*, 2001). Additionally, ZAP70 controls optimal actin reorganization in T cells (Morgan *et al.*, 2001), in part through phosphorylation of the membrane-associated adaptor protein LAT at several tyrosine residues, which mediates recruitment of numerous signaling proteins, including growth-factor-receptor-bound protein 2 (GRB2), the GRB2-related adaptor protein (GADS)–SLP76 complex, and phospholipase-γ1 (PLCγ1). Although a LAT-deficient Jurkat T cell line is capable of spreading on anti-TCR coated coverslips, maximal spreading peaks rapidly and is highly irregular compared with control cells (Bunnell *et al.*, 2001). Since intracellular Ca^{2+} levels are required for TCR-stimulated actin dynamics, LAT could possibly contribute to actin reorganization by mediating the activation of PLCγ1, which generates the second messenger inositol 1,4,5-trisphosphate (IP_3) to stimulate intracellular Ca^{2+} release from the endoplasmic reticulum (ER) and subsequent extracellular influx through the calcium release-activated calcium (CRAC) channel. However, expression of a LAT mutant that can only interact with PLCγ1 was unable to support TCR-stimulated actin dynamics (Bunnell *et al.*, 2001). Additionally, a LAT mutant unable to recruit GADS–SLP76 complexes was similarly defective in spreading in response to TCR cross-linking. Thus, while there are clearly LAT-independent mechanisms downstream of the TCR that permit limited bursts of actin nucleation, LAT-mediated organization of signaling complexes seems essential for proper F-actin reorganization in T cells.

SLP76 is recruited to phosphorylated LAT through the adaptor molecule GADS, and once recruited is phosphorylated by ZAP70 on tyrosine resides within its acidic region, which mediate its interaction with three actin cytoskeletal regulatory proteins, ITK, VAV1 (a hematopoietically-expressed

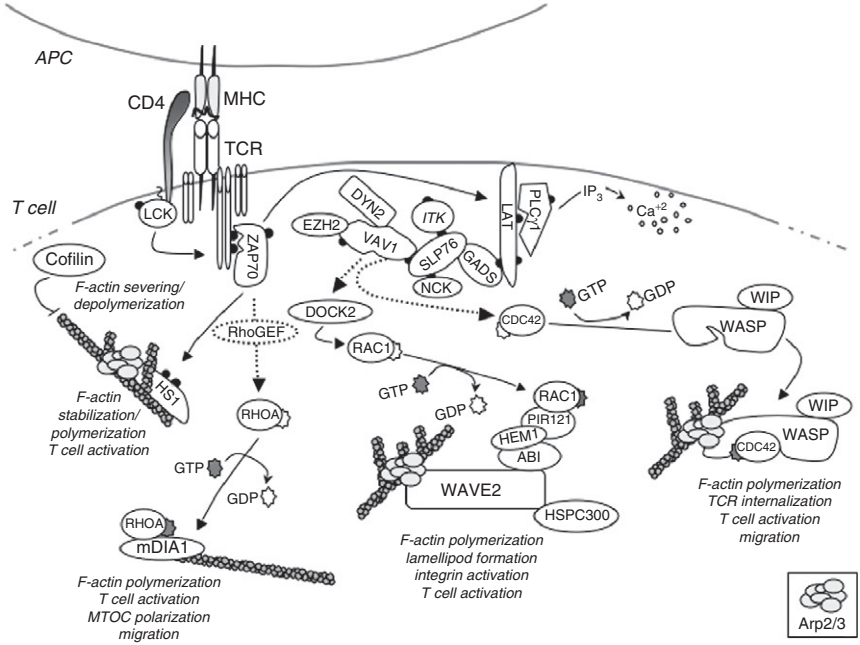

FIGURE 1.1 Regulation of F-actin polymerization during T cell–APC interaction. Following TCR ligation, LCK and ZAP70 are activated. LAT is then phosphorylated by ZAP70, and SLP76 is recruited to LAT through its association with GADS. LAT and SLP76 serve as a scaffold for actin-regulatory proteins, including PLCγ1, NCK, ITK, and VAV1. PLCγ1 activity leads to calcium mobilization, which is essential for F-actin remodeling in T cells. VAV1 is required for TCR-stimulated activation of the small GTP-binding proteins RAC1 and CDC42 (dashed lines). DOCK2 can also stimulate RAC activation following TCR cross-linking, but the mechanism regulating its activation by the TCR is unclear and might involve VAV1 (dashed line). GTP-bound CDC42 activates WASP, which is recruited by NCK and is constitutively associated with WIP. The binding of active CDC42 to WASP releases its auto-inhibition allowing WASP to polymerize F-actin through its association with the ARP2/3 complex. GTP-bound RAC1 interacts with the WAVE2 complex through its association with PIR121 or IRSp53 (not shown) and is proposed to either localize or activate WAVE2-mediated activation of the ARP2/3 complex. Moreover, through an interaction with VAV1, Dynamin2 is localized to the IS where it participates in regulating F-actin reorganization, and likely influences membrane dynamics and recruits additional actin regulators. EZH2 interacts with VAV1 and is required for TCR-stimulated F-actin dynamics through its methylation of undefined substrates. HS1 is phosphorylated by TCR proximal kinases, interacts with ARP2/3, and stabilizes newly generated actin filaments. TCR stimulation also results in the activation of RHOA, but the GEF mediating this activation is unknown (dashed line). RHOA can activate the formin mDIA1, which is involved in F-actin and microtubule dynamics in T cells. Cofilin has a role in T cell actin dynamics through its F-actin severing or depolymerizing activity.

RHO family GEF), and NCK (Koretzky *et al.*, 2006). Consistent with its role in regulating F-actin dynamics, expression of a phosphorylation-deficient SLP76 mutant in Jurkat T cells abrogates recruitment of NCK, VAV1, and the local activation of the small GTPase CDC42 at the IS (Zeng *et al.*, 2003). Localized activation of CDC42 is required to stimulate the ARP2/3-regulatory Wiskott–Aldrich syndrome protein (WASP), and although WASP can interact with numerous proteins, its localization to the IS is dependent on its association with NCK (Zeng *et al.*, 2003). Indeed, fluorescence–resonance energy transfer (FRET) experiments have verified that NCK and WASP are rapidly recruited to the ligated TCR in a LAT- and SLP76-dependent manner (Barda-Saad *et al.*, 2005). Consistent with the notion that NCK participates in TCR-stimulated actin cytoskeletal reorganization, overexpression of a phosphorylation mutant of SLP76 that cannot interact with NCK leads to dramatic defects in TCR-stimulated actin-capping (Bubeck Wardenburg *et al.*, 1998). Additionally, ITK is involved in TCR-stimulated F-actin dynamics (Gomez-Rodriguez *et al.*, 2007) as well as accumulation of VAV1 and active CDC42 at the IS (Labno *et al.*, 2003; Zeng *et al.*, 2003). Interestingly, ITK recruits VAV1 and regulates actin dynamics at the IS independent of its kinase activity domain, but requires its SH2 domain (Dombroski *et al.*, 2005). Thus, since active CDC42 directly stimulates WASP activity, SLP76 might affect WASP-mediated actin dynamics by coordinating the recruitment of NCK–WASP complexes with the localized activation of CDC42 regulated by ITK and VAV1.

Since RHO family GTPases, such as RAC1, RHOA, and CDC42, are fundamental for F-actin dynamics in other cell types (Scheele *et al.*, 2007), they are obvious candidates for regulating similar pathways in T cells. RHO proteins cycle between an inactive GDP-bound state and active GTP-bound state through interactions with GEFs, which facilitate loading of GTP, whereas GTPase activating proteins (GAPs) inactivate GTP-loaded RHO proteins by facilitating cleavage of the terminal phosphate group of GTP. Additionally, RHO GDP-dissociation inhibitors (GDIs) bind to various RHO GTPases and regulate their function by either sequestering GDP-loaded RHO proteins to the cytosol or targeting them to molecular assemblies at the plasma membrane to ensure their stabilization, activation, and interaction with downstream effector molecules (Scheele *et al.*, 2007). The recent knockout of *rhogdiα* and *rhogdiβ*, the two isoforms most highly expressed in T cells, has uncovered an important role for these proteins in regulating TCR-stimulated proliferation, migration, and maintenance of active RAC1 and RHOA in thymocytes and peripheral T cells (Ishizaki *et al.*, 2006).

The RHO family GEF VAV1 can activate RAC1 and CDC42 and is stimulated by phosphorylation of tyrosine residues within its acidic region by LCK (Turner and Billadeau, 2002) (Fig. 1.1). As indicated above, VAV1 is recruited to SLP76 through an interaction of the VAV1-SH2

domain with phosphorylated SLP76. The generation of *vav1*$^{-/-}$ mice has identified it as a critical regulator of thymocyte development, peripheral T cell activation and proliferation, formation of the mature IS, and accumulation of F-actin at the IS (Tarakhovsky *et al.*, 1995; Turner *et al.*, 1997; Wulfing *et al.*, 2000; Zhang *et al.*, 1995). Additionally, clustering of TCRs, protein kinase C-θ (PKCθ), lipid rafts, and lymphocyte-associated antigen-1 (LFA-1) to the IS is abrogated in VAV1-deficient cells (Villalba *et al.*, 2000, 2001, 2002), all of which are regulated by F-actin. Lastly, T cells from a subset of patients with a common variable immunodeficiency (CVID) syndrome associated with significantly reduced levels of VAV1 display similar defects (Paccani *et al.*, 2005). Taken together, these data show that VAV1 is a pivotal regulator of TCR-induced F-actin dynamics and T cell activation.

RAC1 is a major target of VAV1 and can link receptor signaling to the actin cytoskeleton as well as other signaling cascades in many cell types. Thus, RAC1 is likely to be essential for T cell development, activation, and actin dynamics. Previous investigations of the role of RAC1 in T cell development and activation expressed a constitutively active (CA)-RAC1 transgene in developing thymocytes using the proximal *lck* promoter. These studies revealed that CA-RAC1 controls cell fate decisions in the thymus by diverting thymocytes from positive to negative selection (Gomez *et al.*, 2001). Moreover, they found that RAC1 activation was sufficient to restore pre-T cell differentiation and thymocyte cellularity in *vav1*$^{-/-}$ mice, suggesting that some of the defects in the *vav1*$^{-/-}$ mice are due to an inability to activate RAC1 (Gomez *et al.*, 2000). To gain a better understanding of the role of RAC in thymopoesis and T cell function, conditional *rac1*$^{-/-}$ mice have been mated with the total *rac2* (a highly homologous isoform also expressed in T cells) knockout. Importantly, deletion of either *rac1* (conditionally) or *rac2* shows no discernible effect on thymic development (Dumont and Tybulewicz, personal communication). However, removal of both *rac* genes causes a severe block at the pre-TCR checkpoint. This effect is strong, since there are very few T cells in the periphery, and those that are present still have a *rac1* gene, suggesting a strong counter selection (Dumont and Tybulewicz, personal communication). A clear view of the effect of RAC protein loss on F-actin dynamics in T cells awaits the generation of TCR-transgenic animals that bypass the pre-TCR checkpoint.

Since loss of VAV1 affects TCR-stimulated RAC1 activation, it has been assumed that VAV1 is the predominant RAC-GEF downstream of the TCR. Aside from its regulation of F-actin reorganization, VAV1 can also activate pathways that regulate mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NFκB) and Ca^{2+} signaling, which impact on transcriptional activation of interleukin-2 (IL-2) (Costello *et al.*, 1999), a cytokine whose expression is tightly regulated by TCR signaling. However, it was

shown that a GEF-inactivating mutant of VAV1 still retained its ability to stimulate IL-2 promoter activity following TCR engagement (Kuhne *et al.*, 2000). Even more surprisingly, retroviral transduction of a VAV1 GEF mutant into bone marrow from *vav*-null mice (mice lacking expression of all three *vav* genes) reconstitutes TCR-stimulated RAC1 activation, thymocyte development, peripheral T cell activation, TCR microcluster formation and actin dynamics to levels seen in VAV1 wild-type expressing cells (Swat, personal communication). This is not to say that VAV1 GEF activity is not important for signaling in hematopoietic cells, since bone marrow-derived macrophages from *vav*-null mice expressing the same VAV1 GEF-inactivating mutant fail to generate reactive oxygen species following LPS stimulation (Miletic *et al.*, 2007). These data call into question the role of VAV1 GEF activity during T cell activation, and may point to a primary role for VAV1 as an adaptor molecule involved in stabilizing signaling assemblies through protein–protein interactions, as well as the recruitment of other GEFS for the activation of RAC1.

Dedicator of cytokinesis-2 (DOCK2), a member of the CDM (CED-5, DOCK180, Myoblast City) family of GEFs, regulates TCR-mediated RAC1 activation (Sanui *et al.*, 2003) (Fig. 1.1). Importantly, *dock2*$^{-/-}$ T cells do not localize TCRs and lipid rafts to the IS, but do polarize PKCθ and LFA-1. This suggests that DOCK2-dependent RAC1 activation is responsible for TCR and lipid raft clustering, whereas PKCθ and LFA-1 clustering are RAC1-independent, but VAV1-dependent. Thus, the effects on F-actin reorganization regulated by DOCK2-RAC1 may be inherently different from those mediated through VAV1 to drive LFA-1 and PKCθ clustering to the IS. Unfortunately, the role of DOCK2 in regulating RAC1-mediated actin dynamics at the T cell–APC contact site has not been investigated. Lastly, many other signaling pathways that are defective in VAV1-deficient T cells, including the activation of phosphatidylinositol 3-kinase (PI3K), proline-rich tyrosine kinase-2 (PYK2), PLCγ1, NFκB, and Ca^{2+} mobilization, are intact in *dock2*$^{-/-}$ T cells (Sanui *et al.*, 2003), again implying that these are VAV1-regulated events that are RAC1-independent. Taken together, these data suggest the possibility that DOCK2 acts downstream of VAV1 to regulate TCR-stimulated RAC1 activation. However, whether a VAV1-established signaling platform serves to recruit DOCK2 to the IS remains to be determined.

RHOA regulates F-actin dynamics through interaction with effector proteins, such as the cytoskeletal-related p160-RHO-associated coiled-coil containing kinase (ROCK) and the F-actin-nucleating formin diaphanous-1 (mDIA1) (Scheele *et al.*, 2007). Although TCR stimulation results in RHOA activation (Gomez and Billadeau, unpublished observation), the GEF responsible is unknown (Fig. 1.1). In an attempt to understand the role of RHOA in thymopoesis and peripheral T cell activation, investigators have either pharmacologically inhibited RHOA, through the expression of

a C3-exoenzyme (which ADP-ribosylates RHOA, RHOB, and RHOC inhibiting their activity) or a CA-RHOA transgene in thymocytes. These studies indicate that RHOA function is essential for thymocyte survival and TCR-stimulated responses (Corre *et al.*, 2001; Galandrini *et al.*, 1997; Henning *et al.*, 1997). Moreover, integrin-mediated adhesion to fibronectin is enhanced in CA-RHOA-expressing cells (Corre *et al.*, 2001), consistent with the previously identified role of RHOA in regulating chemokine-induced adhesion in T cells (Laudanna *et al.*, 1996). Additionally, C3-exoenzyme inhibited TCR-mediated spreading in a T lymphoma cell line (Verschueren *et al.*, 1997). Altogether, these studies suggest that RHOA is a critical regulator of thymopoesis, T cell activation, and F-actin dynamics.

CDC42 has been considered to be a prominent regulator of actin dynamics because of its ability to directly stimulate WASP-mediated actin polymerization in T cells (Fig. 1.1). In fact, active CDC42 rapidly accumulates at the center of the T cell–APC interface in a TCR- and CD28-dependent manner (Cannon *et al.*, 2001; Tskvitaria-Fuller *et al.*, 2006). Moreover, protein transduction of a dominant-negative (DN)-CDC42 TAT fusion protein into TCR-transgenic T cells revealed a critical role for CDC42 in the regulation of sustained F-actin accumulation at the IS and cell proliferation (Tskvitaria-Fuller *et al.*, 2006). In contrast, while a DN-RAC1 TAT fusion protein also exhibited decreased antigen-induced F-actin polymerization at the IS, a DN-RHOA TAT fusion protein had no effect, thus highlighting differential roles of these three GTPases (Tskvitaria-Fuller *et al.*, 2006). DN-CDC42 and DN-RAC1 also decreased the localization of TCR–pMHC complexes to the center of the IS, suggesting that these two GTPases are involved in TCR clustering (Tskvitaria-Fuller *et al.*, 2006). Although these studies have begun to illuminate the where and when of CDC42 activation, the generation of a conditional *cdc42*$^{-/-}$ mouse will certainly be necessary to establish its role in thymocyte development, peripheral T cell activation, and actin cytoskeletal regulation. While we can appreciate the importance of RAC1, RHOA, and CDC42 in controlling TCR-mediated cytoskeletal remodeling, we still do not have a complete picture of how each of these GTPases are regulated, their individual contributions to TCR-induced cytoskeletal changes, nor their cytoskeletal-independent effects during thymocyte development and activation.

2.2. Generation of F-actin in T cells by ARP2/3 regulators

TCR-stimulated F-actin remodeling occurs as a result of actin depolymerization and repolymerization through the synchronized activation of a diverse array of actin-regulatory proteins (Billadeau *et al.*, 2007). Actin filaments are two-stranded helical polymers built from individual

monomeric subunits of globular (G)-actin, a 43-kDa GTPase (Goley and Welch, 2006). These polymers are partially stabilized by contacts between adjacent G-actin subunits. Thus, two actin monomers bind relatively weakly to each other, but the addition of a third monomer, leading to formation of a trimer, makes it a more stable complex. This actin trimer can then act as a nucleation point for further actin subunit addition, generating an elongating actin filament. Actin filaments are asymmetric with a fast growing ''barbed end'' and a more slowly growing ''pointed end,'' and can arrange into different structural conformations, such as branched F-actin networks or linear F-actin bundles. These conformations can lead to the formation of higher-order structures, such as flat lamellipodia or long stress fibers, which perform specific cellular functions (Chhabra and Higgs, 2007). The spontaneous formation of an actin trimer within cells is thermodynamically unlikely, but various actin-nucleating proteins (or complexes) can overcome this barrier to actin nucleation and are essential for the efficient generation of these cellular F-actin-based structures.

The first actin-nucleating complex to be identified was the actin-related protein-2/3 (ARP2/3) complex, which consists of seven distinct polypeptides, including ARP2, ARP3, and ARP complex proteins 1–5 (ARPC1–5) (Goley and Welch, 2006). The ARP2/3 complex is integrated into the F-actin network during ARP2/3-mediated nucleation, and has been visualized at the branch points of F-actin. For this reason, its nucleation activity is suggested to produce branched F-actin networks by generating new actin filaments off of the sides of preexisting filaments. This type of ''dendritic'' actin meshwork is commonly associated with the lamellipodium of migrating fibroblasts and epithelial cells. Even though filopodia are also predicted to arise from the ARP2/3 complex-dependent dendritic network through selective bundling of ARP2/3 complex-generated filaments, this idea remains controversial (Gomez *et al.*, 2007). ARP2 and ARP3 themselves are structurally similar to actin, and are proposed to substitute for two actin monomers during F-actin nucleation. Thus, ARP2/3-nucleation promoting proteins (or protein complexes), which are essential for bringing ARP2, ARP3, and an additional actin monomer together for F-actin nucleation and subsequent filament assembly, are essential for regulating the activity of the ARP2/3 complex (Goley and Welch, 2006).

During T cell–APC interaction, the lamellipodial structure that is produced over the APC surface is ARP2/3-dependent, as ARP2- or ARP3-depleted T cells cannot form an F-actin-rich lamellipodia, and instead form polarized filopodial protrusions in response to anti-TCR-coated coverslips or APCs (Gomez *et al.*, 2007). This observation underscores the importance of cytoskeletal regulatory proteins that impact ARP2/3-mediated nucleation in T cells, but also indicate that other

ARP2/3-independent mechanisms of actin nucleation are activated by the TCR. Several ARP2/3-nucleation promoting factors, which regulate the activity of the ARP2/3 complex downstream of the TCR, have been identified. The most thoroughly characterized ARP2/3-interacting regulator in T cells is WASP, the hematopoietic-cell-specific form of N-WASP (Ochs and Thrasher, 2006). However, recent studies in T cells have also identified the ARP2/3-regulator WASP-family verprolin-homologous protein-2 (WAVE2) and the ARP2/3- and actin-binding protein hematopoietic lineage cell-specific protein-1 (HS1) as effectors of actin reorganization T cells (Gomez *et al*., 2006; Nolz *et al*., 2006; Zipfel *et al*., 2006). There are several excellent reviews that discuss the mechanisms by which these proteins directly regulate ARP2/3-mediated F-actin nucleation in T cells (Billadeau and Burkhardt, 2006; Billadeau *et al*., 2007; Huang and Burkhardt, 2007). Therefore, we will briefly describe our current understanding of the function of these distinct ARP2/3-interacting proteins with regard to T cell activation, and then will more thoroughly focus on emerging F-actin regulators.

WASP is an effector of CDC42 and constitutively interacts with WASP-interacting protein (WIP), an actin-binding adaptor that is important for F-actin reorganization, proliferation, homing, and IS formation in T cells (forming the WIP–WASP complex) (Fig. 1.1). WIP also interacts with the G-actin-binding protein profilin, providing a pool of monomeric G-actin for WASP-mediated polymerization. Additionally, several recent reports indicate that WIP is important for stabilizing WASP (de la Fuente *et al*., 2007; Dong *et al*., 2007). Although TCR signaling, actin reorganization, TCR internalization, and migration are defective in T cells from WAS patients and *wasp*$^{-/-}$ mice, T cell development is intact (Ochs and Thrasher, 2006). Thus, unlike other actin-regulatory proteins (VAV1, RAC1/RAC2), which were shown to be required for thympoesis, WASP initially appeared to be dispensable for thymocyte development, but was required for peripheral T cell activation. Recently, using either the RAG2-deficient blastocyst complementation system or conditionally targeted alleles of *n-wasp*, *wasp*$^{-/-}$/*n-wasp*$^{-/-}$ animals were produced. While *n-wasp*$^{-/-}$ did not affect thymopoesis or T cell activation, transition from the DN3-to-DN4 stage of thymocyte development was blocked in *wasp*$^{-/-}$/*n-wasp*$^{-/-}$ mice, with an overall decrease in thymocyte numbers (Cotta-de-Almeida *et al*., 2007). These data parallel the thymocyte developmental block observed in transgenic mice expressing a mutant WASP, which cannot interact with ARP2/3 or actin (Zhang *et al*., 2002), and in *vav1*$^{-/-}$ mice (Turner *et al*., 1997). Interestingly, signaling in *wasp*$^{-/-}$/*n-wasp*$^{-/-}$ DP thymocytes appears intact, whereas TCR/CD28-induced proliferation is defective in SP thymocytes (Cotta-de-Almeida *et al*., 2007). Additionally, defects in receptor-mediated spreading and chemokine-induced migration were more pronounced in *wasp*$^{-/-}$/*n-wasp*$^{-/-}$ compared

with *wasp*$^{-/-}$ SP thymocytes. However, peripheral T cells from these two mice displayed a comparable spreading defect, suggesting that in peripheral T cells, WASP has a more prominent role in TCR-mediated F-actin dynamics than N-WASP. Lastly, compared with *wasp*$^{-/-}$ mice, which have decreased numbers and activation of T_{reg} cells that is associated with early-onset colitis (Maillard *et al.*, 2007), *wasp*$^{-/-}$/*n-wasp*$^{-/-}$ mice had even fewer T_{reg} cells and more severe early-onset colitis (Cotta-de-Almeida *et al.*, 2007).

Although it is generally accepted that WASP regulates T cell activation (i.e., IL-2 production and proliferation), questions have recently emerged regarding its role in F-actin accumulation at the IS and TCR-stimulated integrin activation (Cannon and Burkhardt, 2004; Krawczyk *et al.*, 2002; Nolz *et al.*, 2006), suggesting that other ARP2/3 regulators must be involved in regulating these ARP2/3-dependent processes. In fact, WAVE2 was identified as a central regulator of F-actin polymerization downstream of the TCR (Nolz *et al.*, 2006; Zipfel *et al.*, 2006). In contrast to WASP, which exists in an autoinhibited conformation that is relieved by CDC42 binding, WAVE2 is thought to be constitutively active and is regulated by RAC1-mediated recruitment to areas of cellular activation (Fig. 1.1). In fact, mouse embryonic fibroblasts from WAVE2$^{-/-}$ mice, which die during gestation from developmental defects, show impaired RAC1-mediated migration, lamellipodia formation, and dorsal ruffling (Yamazaki *et al.*, 2003; Yan *et al.*, 2003), which firmly places the WAVE2 complex downstream of RAC1 signaling. WAVE2 exists in an interdependent multimolecular complex with several other proteins including the abelson tyrosine kinase (ABL)-interacting adaptor proteins (ABI-1 and -2), hematopoietic protein-1 (HEM1) (or its homologue NCK-associated protein-1, NAP1), p53-inducible messenger RNA-121 (PIR121) (or its homologue specifically-RAC1 associated protein-1, SRA-1), and hematopoietic stem progenitor cell-300 (HSPC300) (Takenawa and Suetsugu, 2007).

Recent studies have shown that the WAVE2 complex is recruited to the T cell–APC contact site, and that RNAi-mediated depletion of WAVE2, HEM1, or ABI inhibits TCR-induced spreading and F-actin polymerization at the IS (Nolz *et al.*, 2006; Zipfel *et al.*, 2006). While *wave2*$^{-/-}$ mice have not been generated to study the role of this protein in thymocyte development and peripheral T cell function, these early observations establish the WAVE2 complex as an integral component of TCR-initiated signaling leading to T cell activation. In addition to cytoskeletal defects, T cells from *abi1*$^{+/-}$/*abi2*$^{-/-}$ mice have decreased proliferation and produce diminished IL-2 in response to TCR stimulation (Zipfel *et al.*, 2006), and RNAi suppression of WAVE2 in Jurkat T cells abrogates TCR-mediated activation of nuclear factor of activated T cells (NFAT) and CD28REAP elements within the IL-2 promoter (Nolz *et al.*, 2006, 2007a). NFAT activity is highly dependent on the levels of intracellular Ca^{2+}, and these transcriptional defects in WAVE2-depleted cells stem

from an inability to couple TCR-stimulated intracellular Ca^{2+} store release from the ER to the activation of the CRAC channel (Nolz *et al.*, 2006). Similar defects in Ca^{2+} signaling were observed in cells treated with actin disrupting agents (Holsinger *et al.*, 1998; Rivas *et al.*, 2004), suggesting a causal link between the actin cytoskeleton and regulation of CRAC channels. However, reexpression of a WAVE2 mutant unable to bind ARP2/3 fully rescues the CRAC channel defect in WAVE2-depleted Jurkat T cells, but was unable to promote F-actin accumulation at the IS (Nolz *et al.*, 2007b). This suggests that WAVE2 (or another component of the WAVE2 complex) may be involved in regulating the CRAC channel in an actin-independent manner. In this regard, HEM1 is predicted to have four transmembrane-spanning regions (Hromas *et al.*, 1991) and might be integrated into the plasma membrane to participate in CRAC channel regulation. With the recent identification of the CRAC channel component ORAI1 and the ER-resident CRAC channel regulator stromal interaction molecule-1 (STIM1) (Feske, 2007), this possibility can be formally tested.

In addition, the hematopoietic-specific cortactin homologue, HS1, regulates the ARP2/3 complex in T cells. TCR ligation results in LCK-dependent tyrosine phosphorylation of HS1, providing docking sites for several SH2 domain-containing proteins, including VAV1, LCK, and PLCγ1 (Gomez *et al.*, 2006). Importantly, phosphorylation of HS1 correlates with its localization to the IS and provides a mechanism for VAV1 stabilization at areas of F-actin polymerization (Gomez *et al.*, 2006). Once recruited to the IS, HS1 has the capacity to stimulate ARP2/3-induced actin assembly as well as stabilize filament branch-points (Uruno *et al.*, 2003). Consistent with this notion, RNAi-mediated silencing of HS1 in Jurkat T cells results in disorganized and erratic TCR-stimulated F-actin structures and decreased stability of F-actin at the IS *in vivo* (Gomez *et al.*, 2006). Additionally, T cells from $hs1^{-/-}$ mice also have defects in F-actin accumulation at the IS as well as diminished TCR-induced proliferation, perhaps due to defects in Ca^{2+} signaling and NFAT and NFκB transactivation of the IL-2 promoter (Gomez *et al.*, 2006; Taniuchi *et al.*, 1995).

2.3. Formin-mediated F-actin nucleation in T cells

As mentioned, studies of ARP2/3-depleted T cells indicate the existence of ARP2/3-independent F-actin reorganization events downstream of the TCR, suggesting that other actin nucleation promoting factors are required for T cell activation and function. Formins represent a large family of proteins (15 genes in humans) that are activated by RHO GTPases to nucleate actin filaments in an ARP2/3 complex-independent manner (Faix and Grosse, 2006). Unlike the ARP2/3 complex, formins nucleate linear actin filaments and remain associated with the barbed end

during filament elongation where they might also function as actin-capping proteins (Chhabra and Higgs, 2007). Formins have been implicated in generating unbranched structures such as actin cables, filopodia, stress fibers, and are also involved in the generation of actin at the contractile ring during cytokinesis (Chhabra and Higgs, 2007). Formins are conserved modular proteins sharing characteristic formin homology (FH) domains and function as homo- or heterodimers to promote actin filament growth through their highly conserved FH2 and tandem FH1 domain (Higgs, 2005). The crystal structure indicates that formins oligomerize during nucleation, forming a ring-like FH2 dimer, with each FH2 domain binding two actin monomers, oriented, as they would be in a filament (Higgs, 2005). The proline-rich FH1 domain interacts with the G-actin-binding protein profilin, providing a pool of monomeric G-actin for filament assembly, while the adjacent FH2 domain nucleates F-actin and mediates barbed-end association (Higgs, 2005). This allows formins to rapidly promote processive actin addition at the barbed end. In addition to regulating actin dynamics, formin proteins have also been shown to participate in positioning of the mitotic spindle during mitosis, as well MTOC polarity in migrating cells (Sagot *et al*., 2002). Thus, these proteins can coordinate both the actin and microtubule cytoskeletons during cellular activation.

Initial studies indicate that the RHOA effector protein mDIA1 (encoded by the *drf1* gene) is upregulated upon T cell activation and that expression of an activated form of mDIA1 results in increased F-actin content in T cells (Vicente-Manzanares *et al*., 2003) (Fig. 1.1). However, although formin-like-1 (FMNL1; a formin overexpressed in T non-Hodgkin's lymphoma) and mDIA1 localize with F-actin at the IS and into the lamellipod of TCR-stimulated spreading T cells (Eisenmann *et al*., 2007; Gomez *et al*., 2007), suppression of either protein alone or in combination did not affect the generation of ARP2/3-independent filopodial structures (Gomez *et al*., 2007). Thus, other formins might be involved in the generation of these structures and in fact, at least 8 of the 15 formin genes are expressed in T cells (Gomez and Billadeau, unpublished observation). Although Jurkat T cells suppressed for mDIA1 or FMNL1 did not show defects in the accumulation of F-actin at the IS, or spreading in response to anti-TCR stimulation, spreading of $drf1^{-/-}$ T cells is compromised (Eisenmann *et al*., 2007). This could be due to incomplete suppression of mDIA1 using RNAi compared with the complete removal in the knockout mouse, although MTOC polarization is still affected in the RNAi-treated Jurkat T cells (Gomez *et al*., 2007). In addition to regulating actin dynamics in T cells, many actin-regulatory proteins also participate in antigen-induced proliferation and T cell activation. It is of interest that $drf1^{-/-}$ T cells show defective antigen-receptor stimulated proliferation (Eisenmann *et al*., 2007; Sakata *et al*., 2007), which might be a result of

diminished TCR-stimulated ERK activation. How exactly mDIA1 couples TCR signaling to the activation of ERK remains to be established.

It is likely that formins collaborate with ARP2/3-dependent events regulated by proteins such as WASP–WIP, WAVE, and HS1 in T lymphocytes. In fact, many of the defects seen in the *drf1*$^{-/-}$ T cells are remarkably similar to those seen in T cells lacking WASP. Interestingly, it was found that *drf1*-deficient cells had an ~75% reduction in the amount of WASP protein, however reexpression of WASP in *drf1*$^{-/-}$ T cells could not rescue defects in cell migration or actin accumulation (Sakata *et al.*, 2007). WASP underwent proteasomal degradation more rapidly in *drf1*$^{-/-}$ cells suggesting that mDIA1 functions to prevent this rapid turnover of WASP by either regulating a degradation-promoting posttranslational modification on WASP or through multimolecular complex stabilization, as is seen with the WAVE complex. Interestingly, SRC-mediated phosphorylation was found to target N-WASP for ubiquitination and proteasomal degradation (Suetsugu *et al.*, 2002) and mDIA1 can interact with SRC kinase to regulate its localization (Tominaga *et al.*, 2000; Yamana *et al.*, 2006). Moreover, the SH3 domain-containing mDIA1-interacting protein (DIP/WISH) can interact with both mDIA1 and WASP (Fukuoka *et al.*, 2001; Meng *et al.*, 2004), and perhaps functions to stabilize an mDIA1–DIP–WASP–WIP complex. It is interesting that DIP regulates RHOA and RAC1 signaling in an SRC-dependent manner (Meng *et al.*, 2004) and can activate N-WASP-stimulated ARP2/3-mediated F-actin nucleation independent of CDC42 (Fukuoka *et al.*, 2001). Additionally, another formin family member, mDIA2, was recently shown to localize to the lamellipodial edge through interactions with ABI in mouse melanoma cells (Yang *et al.*, 2007), and formin proteins were found in complex with HEM1 (Weiner *et al.*, 2006). This raises the intriguing possibility for cooperative regulation of actin nucleation by the WAVE2 complex and formins, similar to mDIA1 and WASP. In fact, while WAVE2-depleted cells completely fail to spread in response to anti-TCR (Nolz *et al.*, 2006), ARP2/3-depleted cells form filopodia, which are enriched with WAVE2 (Gomez *et al.*, 2007). This suggests that WAVE2 may also be involved in the generation of these filopodial structures independent of its role in regulating ARP2/3 F-actin nucleation. Taken together, these observations suggest that interactions occurring between these actin-nucleating systems functionally cooperate to achieve optimal F-actin dynamics.

2.4. Up-and-coming regulators of F-actin in T cells

The dynamin family of large GTPases consists of three conventional isoforms (DYN1–3) that share at least 70% homology and show discrete patterns of gene expression (Orth and McNiven, 2003). Dynamins are multidomain proteins that include an N-terminal GTPase domain, middle

region, phosphatidylinositol-4,5-bisphosphate (PIP_2)-binding pleckstrin homology (PH) domain, GTPase effector domain, and C-terminal PR domain (PRD). These proteins are postulated to function as mechanoenzymes, performing ''work'' through oligomerization (tetramer), protein–lipid or protein–protein interactions, and GTPase activation, culminating in the tubulation and severing of membranes (Orth and McNiven, 2003). They have been implicated in internalization of multiple receptors through studies using DN mutants (such as the K44A mutant of DYN2) (Damke *et al.*, 1994). It has been proposed that DYN2, the dynamin isoform expressed in T cells, is involved in the internalization of the pre-TCR complex in developing thymocytes (Panigada *et al.*, 2002). However, suppression of DYN2 in Jurkat T cells does not affect TCR-stimulated internalization of the receptor (Gomez *et al.*, 2005). More recently, dynamins were proposed to reshape the cortical actin meshwork at sites of receptor activation through interactions with multiple actin-regulating proteins, and link the plasma membrane to F-actin (Krueger *et al.*, 2003; McNiven *et al.*, 2000).

In the case of DYN2, these interactions with actin-regulatory proteins occur primarily through the PRD, although the exact mechanism by which it regulates F-actin reorganization is unclear. DYN2 has, however, been predicted to associate with WIP–WASP through the adaptor NCK, and with cortactin, which is responsible for DYN2 recruitment to sites of actin reorganization (McNiven *et al.*, 2000). Moreover, it was suggested that dynamins may also localize active RAC1 and interact with ABI1/2, thereby possibly linking them to WAVE2-mediated actin reorganization (Schlunck *et al.*, 2004; So *et al.*, 2000), and hence, ARP2/3-mediated F-actin nucleation to specialized membrane domains. Indeed, DYN2 accumulates at the IS and depletion of DYN2 by RNAi in Jurkat T cells results in diminished F-actin accumulation at the IS. DYN2 was also found to couple TCR signaling to the activation of PLCγ1, ERK, and c-Jun N-terminal kinase (JNK) as well as Ca^{2+} mobilization and CD69 upregulation (Gomez *et al.*, 2005). It is known that the SH3 domain of PLCγ1 interacts with the PRD of DYN2 (Seedorf *et al.*, 1994) providing one possible link to PLCγ1 activation in T cells. Additionally, the VAV1 C-terminal SH3 domain was found to interact with the DYN2 PRD and this interaction is required for VAV1-mediated transcriptional activation, as well as the stable recruitment of DYN2 to the IS (Gomez *et al.*, 2005).

The regulation of the F-actin cytoskeletal network requires posttranslational modification of numerous proteins. Until recently, much of our attention has focused on phosphorylation and dephosphorylation events that regulate this complex process. However, a recent report has identified an important role for the enhancer of zeste homologue-2 (EZH2) in the regulation of F-actin dynamics in T cells and other cell types (Su *et al.*, 2005). EZH2 is a histone methyltransferase, which participates in the

epigenetic silencing of genes through phosphorylation of histone H3 at lysine 27 (Nolz *et al.*, 2005). However, it is now clear that EZH2 is also located in the cytoplasm, where it regulates TCR-stimulated, as well as growth factor-stimulated actin polymerization in a methyltransferase-dependent manner (Su *et al.*, 2005). Thus, the methylation of certain actin-regulatory proteins must be required for their function. Interestingly, EZH2 interacts with VAV1 providing a mechanism to localize EZH2 to areas of receptor activation (Hobert *et al.*, 1996) and conditional deletion of *ezh2* in T cells results in a block at the double negative stage of thymocyte development, similar to that seen in $vav1^{-/-}$ mice (Su *et al.*, 2005). However, although CDC42 activity is impaired in $ezh2^{-/-}$ cells, tyrosine phosphorylation of VAV1 was not affected and methylation of VAV1 has not been detected. Therefore, VAV1 does not appear to be the direct target of EZH2. Clearly, the identification of cytosolic targets of EZH2 will aid in our understanding of how methylation of proteins can affect F-actin dynamics. Moreover, since EZH2 is overexpressed in several human malignancies, the role of EZH2 in cancer cell migration should not be overlooked.

BCL10 is an adaptor protein, which contains an N-terminal caspase recruitment domain (CARD) and a C-terminal serine/threonine-rich region. BCL10 was originally identified as a translocation partner in mucous-associated lymphoid tissue (MALT) lymphomas, but has subsequently been demonstrated to be part of a large complex involving MALT1 and CARD-membrane-associated guanylate kinase-1 (CARMA1) (Rawlings *et al.*, 2006), which participates in the activation of NFκB following antigen-receptor engagement. T cell development, TCR-induced proliferation, NFκB activation, IL-2 production, and upregulation of activation markers are severely impaired in $bcl10^{-/-}$ mice (Ruland *et al.*, 2001). However, BCL10 has been unexpectedly shown to be involved in TCR-stimulated F-actin dynamics and FcγR-mediated phagocytosis and actin cup formation (Rueda *et al.*, 2007). In fact, $bcl10^{-/-}$ T cells show diminished TCR-induced F-actin, TCR-mediated spreading, and integrin-mediated adhesion. This effect on F-actin required Ser138 phosphorylation of BCL10, but DID does not involve the entire BCL10–MALT1–CARMA1 complex, since suppression of CARMA1 did not affect TCR-induced F-actin accumulation. It is currently unclear how BCL10 affects actin reorganization in T cells, but expression of CA-RAC1 and CA-CDC42 induce actin polymerization in the absence of BCL10, suggesting that it might organize signaling leading to actin polymerization, or stabilize F-actin structures (Rueda *et al.*, 2007). Indeed, BCL10 associates with the actin-binding or cross-linking protein α-actinin (Guiet and Vito, 2000), which can also link actin microfilaments to the cytoplasmic tails of transmembrane receptors, including integrins (Brakebusch and Fassler, 2003). The further identification of other BCL10-binding partners in T cells, as well as determining

if the CARD is required for TCR-stimulated F-actin nucleation will help define the mechanism by which BCL10 regulates this process.

3. CYTOSKELETAL CONTROL OF IS FORMATION

3.1. The immunological synapse

TCR clustering was first described as global redistribution of the TCR to the T cell–APC contact site in an antigen-specific manner (Kupfer *et al.*, 1987). This TCR enrichment toward stimulating APCs not only overlapped with the already discovered cytoskeletal polarization of T cells (Geiger *et al.*, 1982; Ryser *et al.*, 1982), but was later found to coincide with spatial segregation and clustering of additional receptors as well as intracellular proteins into distinct domains at the T cell–APC junction, collectively known as the IS (Grakoui *et al.*, 1999; Monks *et al.*, 1998). The IS was characterized to consist of a central domain enriched in TCR–pMHC complexes and signaling components such as PKCθ and LCK, which were surrounded by a ring of adhesion-associated molecules, including LFA-1 and talin. These concentric zones within the IS were termed the central (TCR-rich) and peripheral (integrin-rich) supramolecular activation clusters (cSMAC and pSMAC, respectively) (Monks *et al.*, 1998) (Fig. 1.2).

Through biochemical studies, it was already known that TCR ligation during IS formation led to rapid initiation of tyrosine kinase activation and signaling complex assembly that was sufficient to trigger immediate events like cytoskeletal reorganization, but that more complex transcriptional-dependent functions, such as proliferation and cytokine production, were predicted to require prolonged TCR engagement. Although a matter of debate, it is generally accepted that 4–10 h of continuous stimulation are required for T cells to commit to an activation program (Mempel *et al.*, 2004). Therefore, the molecular events of IS formation, including integrin-mediated adhesion and cytoskeletal remodeling, were suggested to stabilize transient TCR–pMHC interactions allowing T cells to integrate signal strength with T cell function (Grakoui *et al.*, 1999). Thus, the cSMAC was predicted to mediate sustained TCR signaling for the optimization of complex T cell effector responses.

Our understanding of the IS was partly altered with the discovery that immediately following APC recognition a broad central zone of adhesion molecules surrounded by TCR–pMHC complexes initially formed, which then dynamically inverted within minutes to form the originally observed ''mature'' IS, with concomitant redistribution of TCRs to the cSMAC and integrins to the pSMAC (Grakoui *et al.*, 1999). Not surprisingly, this rapid

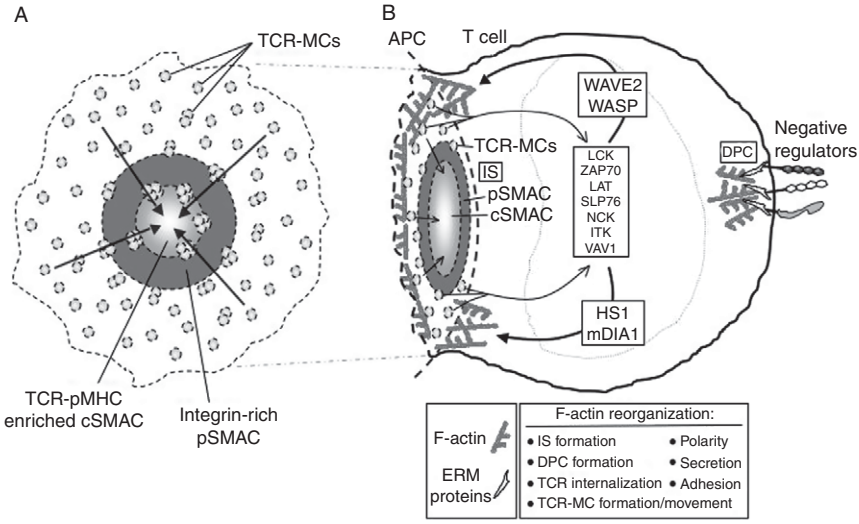

FIGURE 1.2 Regulation of IS formation and polarization by F-actin. (A) Upon APC recognition, initial signals emanating from TCR–pMHC complexes lead to F-actin-dependent TCR microcluster (TCR-MC) formation. TCR-MCs are initially immobile following formation, but then translocate inward through F-actin dynamics, to allow for formation of the TCR-enriched cSMAC. Signals generated by TCR-MCs also result in T cell spreading over the surface of the engaged APC, facilitating further TCR–pMHC engagement and TCR-MC formation. Although the size of TCR-MCs increases upon redistribution toward the cSMAC, perhaps due to F-actin-mediated TCR-MC fusion, this central flow of TCR-MCs correlates with decreased phosphorylation of TCR-MC-associated proteins. This indicates that TCR signaling might be diminished upon formation of the cSMAC. Even after mature IS formation, many TCR-MCs continuously form peripherally and migrate to the cSMAC. (B) Proximal signaling molecules, such as LCK, ZAP70, LAT, SLP76, NCK, ITK and VAV1, drive TCR-mediated F-actin reorganization. These signaling intermediates regulate the downstream activation of RHO family GTPases (not shown) and subsequent recruitment or activation of key actin-regulatory proteins, including WAVE2, WASP, HS1, and mDIA1. These F-actin regulators then cooperatively orchestrate cytoskeletal remodeling, resulting in TCR-MC formation or movement, T cell polarization, lamellipodial protrusion, and formation of the actin-rich mature IS (containing a TCR-rich cSMAC and surrounding integrin-rich pSMAC). Additionally, ERM proteins, which link plasma membrane-associated proteins to the actin cytoskeleton, are inactivated causing decreased cellular rigidity during APC recognition, and are then localized to the F-actin-rich distal pole complex (DPC), sequestering potential negative regulatory receptors and cytoplasmic proteins away from the IS via F-actin anchoring.

spatiotemporally regulated molecular segregation of TCRs and integrin activation during IS formation were highly dependent on F-actin dynamics (Billadeau *et al.*, 2007). In fact, the TCR, which associates with the detergent-insoluble ''cytoskeletal fraction'' of T cells, redistributes upon

stimulation with a greater speed than that predicted for simple diffusion, suggesting that the cytoskeleton directly generates the force needed to quickly relocate engaged TCRs to the cSMAC (Moss *et al*., 2002; Rozdzial *et al*., 1995). Thus, it seemed logical that TCR signaling could first occur peripherally leading to the cytoskeletal remodeling required for the generation of a mature IS, which would then allow sustained signaling through the cSMAC and T cell activation. However, this notion that the cSMAC was the primary site for sustained TCR signaling was quickly questioned with the observation that a significant amount of TCR-mediated tyrosine kinase activity precedes mature IS formation (Lee *et al*., 2002). This finding, along with the recent characterization of TCR microclusters in T cells has led to a reevaluation of the role that the IS plays in TCR-mediated signaling.

3.2. What are TCR microclusters?

We have learned a lot regarding the proximal TCR signaling components that are required for T cell activation, but we still do not understand the dynamic (spatial, temporal, and stoichiometric) regulation involved in the initiation and maturation of TCR signaling, or the distinct regulatory points at which the T cell cytoskeleton can impact TCR function during IS formation. It is known that many TCR-mediated signaling events, such as tyrosine kinase activation, Ca^{2+} mobilization, and F-actin polymerization precede IS formation, peeking within minutes of TCR ligation (Bunnell *et al*., 2001; Lee *et al*., 2002). The origin of these immediate responses was recently revealed with the discovery that fully functional signaling complexes could assemble with small, distinct clusters of TCRs (called TCR microclusters; TCR-MCs) forming in peripheral membrane domains of activated T cells spreading on stimulating surfaces (Bunnell *et al*., 2002; Campi *et al*., 2005; Yokosuka *et al*., 2005) (Fig. 1.2). Recruitment of TCR-associated signaling molecules, such as LCK, ZAP70, LAT, GRB2, GADS, SLP76, VAV1, and c-CBL to TCR-MCs was observed within 30 s following T cell stimulation (Bunnell *et al*., 2002; Miletic *et al*., 2006). Moreover, each of these proximal components seemed to temporally depart from TCR-MCs via distinct mechanisms (Bunnell *et al*., 2002). In fact, LAT-nucleated signaling complexes are rapidly internalized following TCR-MC formation via a mechanism that requires c-CBL-mediated ubiquitylation of LAT (Balagopalan *et al*., 2007), and movement of TCR-stimulated SLP76-containing clusters corresponds with microtubule-dependent endocytosis (Barr *et al*., 2006; Bunnell *et al*., 2002). This reveals that TCR-MCs are highly dynamic because simultaneous with the assembly of signaling proteins, disassembly factors are recruited to immediately limit signal duration through cytoskeletal-dependent mechanisms.

In addition, this dynamic nature of TCR-MCs was further illuminated when T cells were activated on antigen-presenting lipid bilayers, which permits the movement of engaged receptor–ligand pairs. TCR-MCs were initially immobile following formation, but then translocated inward to form the cSMAC as the T cell reached maximal spreading. This central flow of TCR-MCs correlated with decreased phosphorylation of both LCK and ZAP70 as well as decreased SLP76 association, indicating that TCR-MC signaling was diminished upon cSMAC formation (Campi *et al.*, 2005; Yokosuka *et al.*, 2005). In fact, even after mature IS formation, many TCR-MCs continuously formed peripherally and migrated to the cSMAC, suggesting that the primary site of sustained TCR signaling might actually be newly generated TCR-MCs. This is supported by the fact that once the cSMAC is formed its stability does not seem to be actin-dependent, while the pSMAC disorganizes and formation of new TCR-MCs rapidly ceases upon perturbation of actin dynamics (Varma *et al.*, 2006). Interestingly, inhibition of SRC kinases prevents recruitment of proximal molecules to TCR-MCs altogether but does not affect F-actin-dependent formation of TCR-MCs, showing that actin-dependent movement of TCRs into microclusters is SRC-independent (Bunnell *et al.*, 2002; Campi *et al.*, 2005; Yokosuka *et al.*, 2005). Moreover, size and intensity of TCR-MCs increases upon redistribution toward the cSMAC, raising the possibility of TCR-MC fusion, an event that would likely be linked to cytoskeletal dynamics (Campi *et al.*, 2005; Yokosuka *et al.*, 2005). However, the duration of phospho-Tyr signaling by TCR-MCs seems to correlate primarily with radial position rather than cluster size (Mossman *et al.*, 2005). Remarkably, one study showed that TCR-MC movement and fusion, although altered, occurred when SRC activity was blocked (Yokosuka *et al.*, 2005). Thus, F-actin-dependent formation and movement of TCR-MCs is the earliest known regulatory point at which F-actin dynamics are requisite to T cell activation. Of note, WASP and NCK were shown to rapidly localize to TCR-MCs and then redistribute to the actin-rich cell perimeter in spreading T cells. However, as was shown for other TCR-associated molecules, SRC inhibition abrogated NCK–WASP localization to TCR microclusters (Barda-Saad *et al.*, 2005). Thus, WASP might not regulate the SRC-independent, actin-dependent clustering and movement of TCRs.

Our current understanding of TCR signaling raises the question of the primary function of the IS. While it is clear that disruption of IS formation does negatively impact T cell activation (Billadeau *et al.*, 2007), the generation of the cSMAC might not propagate sustained TCR signaling as once thought. However, it is possible that the cSMAC serves as a site for stabilization of the few specific TCR–pMHC interactions during T cell–APC recognition, supports late activation signals, or acts as an anchor providing a ''stop signal'' for T cells interacting with APCs

(Yokosuka *et al.*, 2005). In addition, the IS may be essential for regulating directed exocytosis of cytokines or granules and receptor down-regulation via endocytosis, both cytoskeletal-dependent events (Kupfer *et al.*, 1991; Stinchcombe *et al.*, 2001; Varma *et al.*, 2006). Regardless of the exact role of the IS in T cell activation, these recent studies of TCR-MC formation confirm that F-actin is fundamental for the earliest identified TCR signaling events and likely underlies the sum of TCR signaling from TCR-MC formation to termination via TCR endocytosis.

3.3. Potential links between the T cell cytoskeleton and TCR-MCs

Since we now understand that the formation and then radial translocation of TCR-MCs might be a means to adjust signal strength (Campi *et al.*, 2005; Mossman *et al.*, 2005; Yokosuka *et al.*, 2005), cytoskeletal-dependent mechanisms directly controlling TCR clustering and movement to the cSMAC are potentially critical regulatory points for modulating TCR signaling. Therefore, the resounding question in the field still relates to the direct mechanisms that spatially and temporally link the cytoskeleton to the TCR. One recently explored mechanism that could connect the TCR to the F-actin cytoskeleton involves the CD3ε subunit of the TCR. It was proposed that TCR ligation induces a conformational change in CD3ε that exposes a proline-rich sequence in its cytoplasmic tail, permitting interaction with the cytoskeletal adaptor NCK (Gil *et al.*, 2002). Interestingly, it was reported that this ligand-induced conformational change occurs earlier than TCR-mediated SRC kinase activation (Gil *et al.*, 2002), and that phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) within CD3ε might abrogate this CD3ε–NCK interaction (Kesti *et al.*, 2007). In light of our current understanding of TCR-MC formation, this very early structural change in CD3ε is an appealing mechanism for linking F-actin to SRC-independent formation of TCR-MCs. Even though this idea was challenged in a study showing that NCK localization to TCR-MCs was abrogated upon treatment with SRC inhibitor (Barda-Saad *et al.*, 2005), another recent report has identified that epidermal growth factor receptor kinase substrate 8 (EPS8), a protein that binds actin, interacts with CD3ε (Kesti *et al.*, 2007). This CD3ε–EPS8 interaction, which occurs via the same motif bound by NCK and is similarly blocked by CD3ε phosphorylation, might also link the antigen-engaged TCR to actin.

EPS8 has been linked to F-actin remodeling in non-immune cells where it was shown to enter into a complex with ABI1, son of sevenless-1 (SOS-1) and PI3K, which regulates RAC1 activation as well as F-actin downstream of receptor tyrosine kinases (Innocenti *et al.*, 2003). Additionally, an IRSp53–EPS8 complex is activated downstream of CDC42 resulting in F-actin bundling and filopodia formation (Disanza *et al.*, 2006).

Furthermore, EPS8 can negatively influence F-actin dynamics via its barbed-end capping activity, which is modulated by its interaction with ABI1 (Disanza *et al.*, 2004). Interestingly, this EPS8 interaction with ABI1 could link it to the WAVE2 complex, providing a mechanism for immediate *de novo* actin polymerization upon TCR ligation. Moreover, EPS8 associates with receptors during endocytosis and could also play a later role in TCR internalization upon CD3ε dephosphorylation (Burke *et al.*, 2001). While this early ligand-induced conformational change of CD3ε is exciting in the context of F-actin-dependent TCR clustering, it must be noted that the importance of the NCK–EPS8-interacting motif within CD3ε was recently disputed (Szymczak *et al.*, 2005). Therefore, it will be important to determine whether the cytoskeleton can directly link to the TCR in this manner to control TCR-MC formation in the absence of SRC kinase activity.

Another attractive model whereby cytoskeletal dynamics could influence TCR clustering during T cell activation involves the concept of ''lipid rafts,'' an idea that has been around for some time but has become increasingly controversial (Manes and Viola, 2006; Shaw, 2006). Rafts are lipid subdomains that are hypothesized to arise within the plasma membrane because of phase separation and the inherent tendency for certain lipids to coalesce based on their physical properties. It is thought that these distinct lipid microdomains can then serve as stable platforms for membrane-associated protein organization through selective protein–lipid affinities and clustering, which brings signaling molecules together for activation. Thus, lipid rafts are enriched in specific lipids such as cholesterol and sphingomyelin, are selectively associated with a subset of membrane-anchored proteins such as glycosylphosphatidylinositol (GPI)-linked proteins, and are biochemically-defined by insolubility in nonionic detergents. Shortly after inception of the raft hypothesis, biochemical studies revealed that the TCR along with TCR signaling components, such as LCK and LAT, resided in lipid rafts in stimulated T cells and that direct cross-linking of glycosphingolipid (GM1) or GPI-linked proteins induced raft clustering and T cell activation (Shaw, 2006). LFA-1 was also shown to mobilize into lipid rafts following GM1 cross-linking (Leitinger and Hogg, 2002), and early imaging studies indicated polarized accumulation of lipid rafts toward the cSMAC during T cell activation (Burack *et al.*, 2002). Thus, the recruitment of TCRs into lipid microdomains and the subsequent TCR-mediated polarization of these rafts appeared to be a key element in the assembly of higher-order signaling complexes, the exclusion of negative regulators, and the accumulation of second messenger molecules during lymphocyte activation.

Recently, the theory that lipid raft formation occurs passively through phase separation based on lipid structure has been challenged, and this is supported by the fact that raft clustering requires F-actin dynamics

(Villalba *et al.*, 2001). In fact, studies utilizing mass spectrometry have identified numerous cytoskeletal-associated proteins within T cell lipid rafts, further suggesting a strong link between actin and lipid raft dynamics (Kobayashi *et al.*, 2007; von Haller *et al.*, 2001). So far, we do not mechanistically understand how the cytoskeleton can influence raft polarization. However, a recent study of gelsolin, an F-actin capping and severing protein, indicated that F-actin not only regulates lipid raft aggregation upon TCR stimulation but also actively maintains the spatial segregation of raft-associated signaling molecules in resting cells (Morley *et al.*, 2007). In addition, the ability for WASP and WAVE2 to be activated by inositol phospholipids (Higgs and Pollard, 2000; Oikawa *et al.*, 2004), and other actin-binding proteins, including filamin A and ezrin–radixin–moesin (ERM) family members, to form cytoskeletal linkages with membrane resident proteins might directly connect cortical F-actin remodeling to lipid raft dynamics allowing TCR clustering (Charrin and Alcover, 2006; Tavano *et al.*, 2006). Indeed, T cells from WAS patients were reported to have impaired lipid raft accumulation (Ochs and Thrasher, 2006), and ERM proteins influence TCR clustering at the IS (Roumier *et al.*, 2001). Actin-binding flotillin proteins, which are lipid-modified and associate with membrane rafts in T cells, may also have a role in raft clustering (Neumann-Giesen *et al.*, 2004). In addition, many GEFs are associated with lipid rafts, including VAV1, SWAP70, and β-PAK-interacting exchange factor (β-PIX) (Kobayashi *et al.*, 2007; Villalba *et al.*, 2001), suggesting that activation of small GTPases is important for raft clustering via cytoskeletal remodeling. Indeed, VAV1- or DOCK2-deficient T cells are unable to polarize TCR or lipid rafts upon stimulation (Sanui *et al.*, 2003; Villalba *et al.*, 2001).

An intriguing hypothesis to mechanistically explain how cytoskeletal remodeling could actively control lipid and TCR distribution has recently emerged (Kusumi *et al.*, 2005). Based on single-molecule imaging at the cell surface, a model was proposed in which F-actin forms a lattice that underlies the plasma membrane, called a ''membrane-skeleton fence'', and that this ''actin fence'' creates physical barriers to diffusion, leading to membrane compartmentalization. Thus, lateral diffusion is limited with molecules (proteins and lipids) having to undergo hop diffusion to enter into an adjacent actin-lined compartment. As part of this model, transmembrane proteins corralled within the ''actin fence'' can be anchored to the cytoskeleton, acting as a row of ''pickets'' along the F-actin fence to limit diffusion of lipids within the outer leaflet of the membrane. In the case of the TCR, TCR-MC formation in stimulated T cells is proposed to increase the density of F-actin near the cluster site, which as a result decreases local diffusion of signaling components and corrals proteins and lipids together into smaller ''fences'' to promote signaling. Although this model nicely suggests

how compartmentalization can occur within the T cell membrane to bring the TCR into close proximity with membrane-associated signaling molecules, it does not yet adequately explain how TCR-MCs can rapidly form in such a compartmentalized membrane, how specific lipids (lipid rafts) and proteins are locally concentrated during TCR-MC signaling, or assimilate the rapid, dynamic translocation of TCR-MCs from the periphery to the cSMAC.

One recent study might extend this ''picket-fence'' hypothesis. It was proposed that small carbohydrate-binding proteins, called galectins, induce multivalent cross-linking of glycoproteins on the cell surface, leading to the formation of a galectin-mediated protein lattice (Chen *et al*., 2007). Since these galectin-bound protein complexes are larger, they have restricted movement within the plasma membrane (potentially because they cannot undergo hop diffusion) and can be more easily compartmentalized through cytoskeletal interactions (perhaps mediated by the actin ''picket-fence''). During T cell activation, it was suggested that the distribution of glycosylated proteins, such as CD45 and TCR/CD4, were dependent on a competition between the degree of extracellular galectin cross-linking versus cytoplasmic cytoskeletal restriction. Thus, disruption of galectin binding to N-glycan chains on the TCR following stimulation could release TCRs from larger protein lattices, permitting movement into lipid rafts and signaling. Conversely, loss of galectin binding to CD45 phosphatase leads to its exclusion from lipid rafts through cytoskeletal interactions. This finding adds yet another facet to our understanding of how the cytoskeleton can regulate IS formation. Therefore, the further integration of emerging hypotheses, such as the ''membrane-skeleton fence'' and ''galectin-lattice'', with the ''lipid raft hypothesis'' may be getting us closer to the truth regarding the notion of cytoskeletal-dependent membrane compartmentalization.

Although the idea of lipid rafts is still widely accepted, it must be noted that the actual existence of lipid rafts and their potential association with TCR signaling complexes during T cell activation is currently under scrutiny (Manes and Viola, 2006; Shaw, 2006). Consequently, the lipid raft paradigm is in a state of evolution. Although many studies have supported TCR-mediated lipid raft clustering (Manes and Viola, 2006), it was recently shown that raft-anchored proteins do not co-localize with TCR-MCs, suggesting that lipid microdomains might not direct TCR clustering (Bunnell *et al*., 2002; Saito and Yokosuka, 2006). Other studies have agreed with these findings and indicate that there is no detectable global enrichment of raft-associated proteins and GM1 upon TCR stimulation, and instead support a model of diffusional trapping via protein–protein interaction networks for TCR-MC formation and TCR signalosome assembly (Bunnell *et al*., 2006; Douglass and Vale, 2005; Glebov and Nichols, 2004). These studies, along with others, have led to a reevaluation of the concept

of lipid rafts in T cell biology, which suggests that instead of being large, stable, and homogeneous, rafts may actually be extremely small, highly unstable, and heterogeneous entities dependent on protein–protein interactions and F-actin for stability (Kusumi *et al.*, 2005; Shaw, 2006). Thus, while it is clear that the cytoskeleton enables TCR and lipid clustering at the IS, it remains enigmatic whether plasma membrane microdomains play a role in organizing TCR-MC formation and signaling.

3.4. New perspectives: VAV1, WASP, and IS formation

Although we do not firmly understand how the T cell cytoskeleton controls TCR-MC formation and subsequent IS formation, we have recently gained additional insight into this process through the study of key cytoskeletal regulatory proteins. The central F-actin regulator VAV1 is essential for IS formation, and controls the accumulation of TCR, PKCθ, and phospho-tyrosine-containing proteins to the cSMAC, as well as influences integrin activation at the pSMAC (Tybulewicz, 2005). VAV1 is now known to dynamically assemble into stable microclusters within seconds after TCR stimulation (Miletic *et al.*, 2006). However, by reexpressing VAV1 mutants in a $VAV1^{null}$ T cell line, it was shown that a mutant $VAV1^{Y174F}$ with constitutive GEF activity forms clusters that are transient, disappearing immediately upon formation (Miletic *et al.*, 2006). Intriguingly, if this same $VAV1^{Y174F}$ is mutated further to abrogate its GEF activity ($VAV1^{Y174F/GEF-}$), then this combined $VAV1^{Y174F/GEF-}$ mutant regains the ability to form stable microclusters similar to wild-type VAV1. This suggests that TCR-MC formation and stability are independent of VAV1 GEF activity, although TCR clustering itself was not directly analyzed in this study. These findings may also indicate a role for VAV1 GEF activity in negatively regulating VAV1 clustering at the TCR, or perhaps in TCR-MC down-regulation altogether, since $VAV1^{Y174F}$ clusters quickly disassemble. Thus, it is clear, that the regulation of IS formation by VAV1 will require more in depth study to determine which of its effects are driven by cytoskeletal dynamics versus its role as an adaptor in T cell signaling.

Additionally, in a recent, compelling study of naïve T cells it was demonstrated that during the early stages of antigen recognition (the first 2 h after an initial IS is formed) the cells alternated between repeated cycles of stable IS formation and migration, which relied on a fine balance between PKCθ- and WASP-regulated signals (Sims *et al.*, 2007). Interestingly, PKCθ was seen primarily in the pSMAC rather than the cSMAC of these naïve T cells. In the study, $pkcq^{-/-}$ T cells formed hyperstable synapses with pSMACs that did not break symmetry to allow for subsequent migration, whereas $wasp^{-/-}$ T cells displayed normal IS formation, as previously demonstrated (Cannon and Burkhardt, 2004), but

were then incapable of reforming synapses following entry into the migration phase. However, these *wasp*$^{-/-}$ T cells, which were stuck in a state of continuous migration on stimulating bilayers, could reform synapses upon PKCθ inhibition. Thus, it seems that under normal circumstances WASP negatively regulates PKCθ activity to permit pSMAC stabilization during IS formation, and that PKCθ must then be reactivated to allow the pSMAC to break apart for subsequent migration. This observation might explain conflicting results obtained with regard to *wasp*$^{-/-}$ T cells and IS formation.

A mechanism for these opposing effects mediated by PKCθ and WASP was suggested based on a previous observation (Krzewski *et al.*, 2006). TCR-mediated PKCθ phosphorylation of WIP might result in a WIP–myosinIIA complex, which could break symmetry of the pSMAC via tensional changes in the F-actin network at the IS. It was speculated that WASP, which is also a part of this WIP–myosinIIA complex, could then inhibit the activity of myosinIIA for stable IS formation. As a functional consequence of this balance between PKCθ and WASP, those cells that cycled periodically between synaptic and migratory states during this T cell priming phase, ultimately produced more IL-2 than those that formed one stable IS (Sims *et al.*, 2007). Thus, it seems that another crucial role for the T cell cytoskeleton is to balance the antigen-sensing process and IS formation with cellular migration during early antigen recognition.

3.5. ERM family proteins and the distal pole complex

The ERM family of membrane-cytoskeleton cross-linkers are also important regulators of IS formation. These proteins possess an N-terminal FERM domain that interacts with the cytoplasmic tails of various cell-surface receptors, including CD43, CD44, L-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), and intercellular adhesion molecule-1 (ICAM1) and ICAM3, anchoring them directly to cortical F-actin through a C-terminal actin-binding region (Charrin and Alcover, 2006). Additionally, ERM proteins may interact with various cytoskeletal-related cytoplasmic proteins, such as RHO-GDI, DBL, CRK, p21-activated kinase (PAK), and discs large-1 (DLG1) (Charrin and Alcover, 2006; Huang and Burkhardt, 2007). ERM proteins exist in an autoinhibited conformation, via direct interaction between FERM and C-terminal domains, unless they are phosphorylated or bound by certain phosphoinositides (Charrin and Alcover, 2006). Importantly, this folded conformation cannot bind F-actin or cargo molecules. Upon TCR ligation, ERM family proteins are inactivated by dephosphorylation through a pathway involving VAV1-regulated RAC1 (Faure *et al.*, 2004). As a result, ERM-mediated plasma membrane and cortical F-actin linkage is severed, leading to reduced cellular rigidity, increased cell-surface protein mobility, and

more efficient T cell–APC interaction (Faure *et al.*, 2004). In this regard, ERM proteins influence lipid raft dynamics and TCR clustering at the IS (Ilani *et al.*, 2007; Roumier *et al.*, 2001). Following antigenic stimulation, ERM proteins are rapidly reactivated, localizing to and regulating the formation of the actin-rich distal pole complex (DPC), a posterior cap-like structure forming opposite the IS (Fig. 1.2). As a result, several ERM-interacting molecules are actively carried rearward to the DPC through F-actin anchoring, sequestering negative regulators (such as CD43 and DLG1) away from the IS (Huang and Burkhardt, 2007; Xavier *et al.*, 2004). Indeed, DLG1 has been shown to negatively influence NFAT transcriptional activity and $dlg1^{-/-}$ T lymphocytes are hyperproliferative (Stephenson *et al.*, 2007; Xavier *et al.*, 2004). Although ezrin and moesin, the two lymphocyte-expressed ERM family proteins, have largely been thought to act redundantly during T cell activation, recent evidence indicates that these proteins may be distinctly activated or inactivated by the TCR with ezrin organizing IS formation and moesin regulating DPC formation (Ilani *et al.*, 2007).

4. REGULATION OF TCR-MEDIATED MTOC POLARIZATION

Upon APC recognition, the MTOC, along with the microtubule cytoskeleton and Golgi apparatus also reorient toward the T cell–APC contact site, a widely recognized hallmark of T cell polarization and activation (Geiger *et al.*, 1982; Kuhn and Poenie, 2002). Until recently, MTOC (or centrosome) repositioning in T cells was shown to rely on only a few signaling molecules, including LCK, FYN, ZAP70, LAT, SLP76, VAV1, CDC42, and intracellular Ca^{2+} (Ardouin *et al.*, 2003; Kuhne *et al.*, 2003; Lowin-Kropf *et al.*, 1998; Martin-Cofreces *et al.*, 2006; Stowers *et al.*, 1995) (Fig. 1.3). However, although DN-CDC42 expression studies have indicated its importance in MTOC positioning during T cell activation (Stowers *et al.*, 1995), it was recently found that depletion of RAC1, but not CDC42 abrogates MTOC polarity in T cells (Gomez *et al.*, 2007). Nevertheless, for the most part, these molecules are TCR proximal components, which affect many pathways downstream of the TCR. Therefore, the molecular signaling pathways, which are directly involved in the physical movement of the MTOC following TCR engagement, have not been defined.

Functionally, MTOC polarization governs directed secretion of lymphokines or cytotoxins, which travel along microtubules using minus-end directed movement to the centrosome (Stinchcombe *et al.*, 2006), and are then released toward the engaged APC or target cell (Fig. 1.3). In fact, stores of IL-2, IL-4, and IL-5 in T helper cells are directed toward the APC (Kupfer *et al.*, 1991), and cytolytic T cells form a specialized synapse, where lytic granules polarize along with the MTOC toward the target

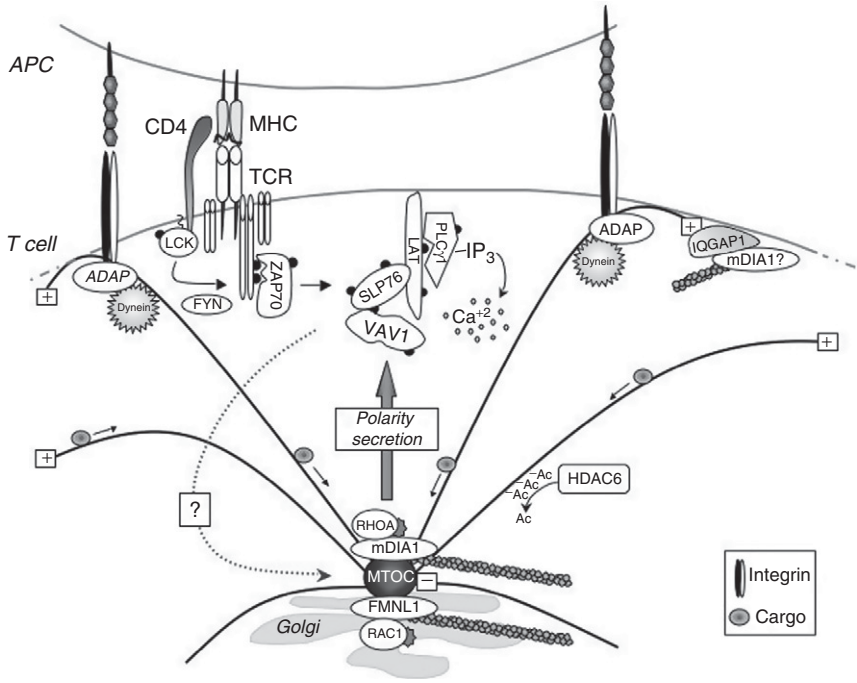

FIGURE 1.3 Regulation of TCR-mediated MTOC polarization. Upon APC recognition, the MTOC reorients to a region just beneath the T cell–APC contact site. This MTOC polarization relies on several TCR proximal events, including activation of various kinases, SLP76 and VAV1 recruitment, as well as Ca^{2+} mobilization. These events lead to downstream activation of the RHO family GTPases, RHOA and RAC1, through an unknown mechanism (dashed line), which in turn activate the actin-nucleating formins, mDIA1 and FMNL1, respectively. Most likely through F-actin polymerization, mDIA1 and FMNL1 each distinctly regulate MTOC polarization to the IS. Moreover, a complex consisting of ADAP and the microtubule motor, Dynein, has been suggested to "winch in" the MTOC toward the bound APC with a distinctive sliding motion. Microtubules have been observed to anchor to the integrin-rich pSMAC during APC recognition, perhaps through this ADAP interaction. Moreover, microtubule plus-end complexes, which contain IQGAP1 and mDIA1, are proposed to link the microtubule cytoskeleton to the cortical T cell actin cytoskeleton, providing additional force for MTOC movement. HDAC6 is also required for TCR-mediated MTOC polarization. TCR ligation leads to a transient deacetylation of microtubules and then enhanced microtubule acetylation at the IS, which is important for microtubule stability. Once the MTOC is polarized, cargo (e.g., lytic granules and cytokines) travels along microtubules using minus-end-directed movement to the centrosome, which directs secretion toward the APC surface through specialized domains within the IS.

cell and are secreted through a unique area in the cSMAC (Stinchcombe *et al.*, 2001). Interestingly, contact between the centrosome and the plasma membrane in T cells has recently been shown to be necessary for directed secretion of lytic granules and to be F-actin-dependent (Stinchcombe *et al.*, 2006). Moreover, links established between microtubules and the actin cytoskeleton are likely crucial in endocytic processes occurring during IS formation. Unfortunately, the putative actin regulators controlling MTOC polarization are largely unknown. However, a connection between F-actin dynamics and MTOC polarity was recently uncovered through the analysis of formin proteins, which not only stimulate F-actin nucleation, but also bind microtubule plus-end complexes, thus physically linking them to microtubules (Gomez *et al.*, 2007) (Fig. 1.3). Actin-nucleating FMNL1 and mDIA1 co-localized near the centrosome and controlled MTOC polarity and cell-mediated killing, whereas TCR-mediated MTOC repositioning was unexpectedly ARP2/3-independent. Moreover, FMNL1 and mDIA1 display strikingly different patterns of MTOC co-localization and loss of either FMNL1 or mDIA1 individually affects MTOC reorientation, suggesting that they are not functionally redundant in this regard. These findings indicate that formin proteins control distinct actin polymerization processes that are required for MTOC positioning.

Additional insight into how the microtubule cytoskeleton reorients during T cell activation was gained through a recent study, which indicated that the microtubule motor protein, dynein, participates in MTOC polarization through an interaction with the integrin-associated adhesion- and degranulation-adaptor protein (ADAP) (Combs *et al.*, 2006). This is interesting considering that microtubules were already observed to anchor to the pSMAC during APC recognition (Kuhn and Poenie, 2002). Also, it provides a link between microtubules, microtubule motor proteins, and the actin cytoskeleton (through ADAP/vasodilator-stimulated phosphoprotein (VASP)-mediated actin binding), which could mechanistically connect formin-mediated F-actin dynamics to MTOC positioning (Fig. 1.3). Also, microtubules conversely affect the formation of the lamellipod during T cell activation, as T cells with disrupted microtubules show early retraction of actin-based protrusions during activation (Bunnell *et al.*, 2001). Altogether, these data highlight the integrated nature of these two cytoskeletal systems in defining T cell polarity.

Other molecules that link microtubule plus-ends to the cortical actin cytoskeleton in non-hematopoietic cells, such as IQ-motif-containing GTPase activating protein-1 (IQGAP1), are also emerging as potential regulators of MTOC polarity in T cells (Gomez *et al.*, 2007; Stinchcombe *et al.*, 2006). Indeed, we have found that RNAi-mediated depletion of IQGAP1 abrogates TCR-mediated MTOC polarization (Gomez *et al.*, 2007).

Interestingly, a recent study in macrophages has identified a functional interaction between IQGAP1 and mDIA1 that is required for phagocytosis and phagocytic cup formation (Brandt *et al.*, 2007). Whether these two proteins also interact in T cells remains to be determined, but it could provide another mechanism for simultaneously stabilizing microtubules through IQGAP1 and coordinately regulating localized F-actin dynamics through mDIA1. Lastly, microtubule acetylation might be important for microtubule stability, since TCR ligation leads to a transient deacetylation of microtubules by histone deacetylase-6 (HDAC6) followed by enhanced microtubule acetylation at later time points (Serrador *et al.*, 2004). Interestingly, HDAC6 is required for TCR-mediated MTOC polarization and overexpression of HDAC6 results in disorganized TCR and integrin localization at the IS, abrogated MTOC polarization and impaired IL-2 production (Serrador *et al.*, 2004). Taken together, these data indicate a potential role for microtubules in establishing cell polarity and IS organization, as well as in regulating endocytosis and exocytosis.

5. F-ACTIN-MEDIATED REGULATION OF INTEGRINS

5.1. Integrin overview

To generate a functional adaptive immune response, T cells must be able to migrate between blood and secondary lymphoid organs (SLOs) and respond to APCs (Burbach *et al.*, 2007; Kinashi, 2007). Integrins are a large family of heterodimeric proteins that consist of an α- and β-chain, which facilitate T cell trafficking and activation by promoting interactions with extracellular matrix (ECM) components and tight contact with APCs. LFA-1 (αLβ2 integrin) and very late antigen-4 (VLA-4; α4β1 integrin) promote T cell adhesion to endothelium and facilitate extravasation into inflammatory tissue (Burbach *et al.*, 2007). LFA-1 is also an important mediator of T cell–APC conjugate formation through its interaction with ICAM on the APC surface (Dustin and Springer, 1989). LFA-1 blocking antibodies abrogate T cell–APC adhesion and $CD8^{+}$-mediated cytotoxicity (Lancki *et al.*, 1987; Rothlein and Springer, 1986). Thus, integrins represent a key family of cell-surface proteins that regulate T cell activation by affecting cell–cell interactions and the migration and retention of T cells during inflammation.

On resting naïve T cells, integrins are present in a low-affinity conformation, which facilitates the rapid migration of T cells through SLOs (Kinashi, 2007). However, in response to chemokines or TCR stimulation, a process known as 'inside-out' signaling, low affinity integrins will cluster, increasing adhesive avidity. In addition, 'inside-out' signaling also results in a conformational change from a low affinity to a high affinity

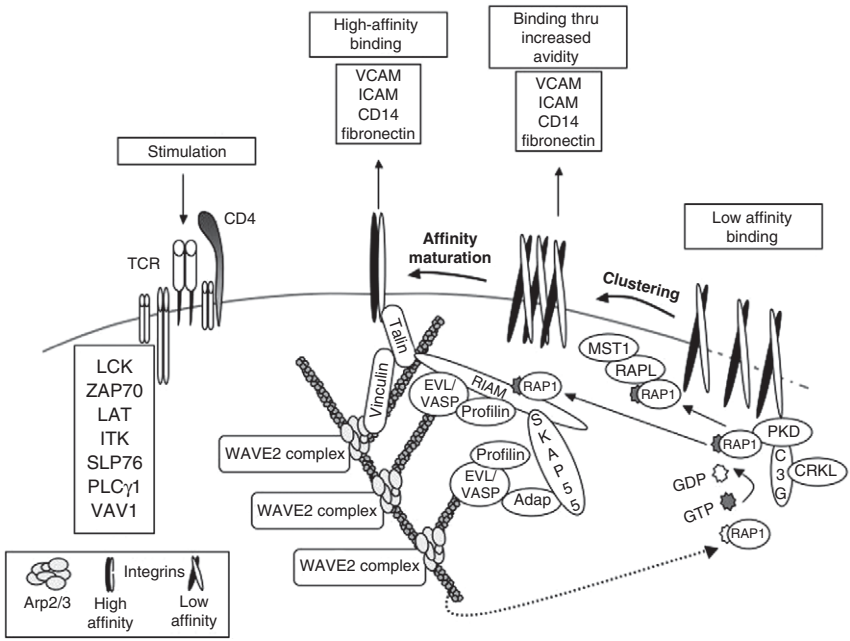

FIGURE 1.4 Pathways that contribute to TCR-mediated integrin activation in T cells. Stimulation of the TCR by pMHC complexes presented by APCs leads to the activation of both β_1- and β_2-integrins on the T cell surface through "inside-out" signaling. These integrins bind fibronectin and intercellular adhesion molecule-1 (ICAM1), respectively. "Inside-out" signaling from the TCR is initiated by the proximal kinases (LCK and ZAP70) and the LAT signalosome (shown in box; see Fig. 1.1 for details), which directs WAVE2-mediated cytoskeletal reorganization and subsequently integrin-mediated adhesion. WAVE2-stimulated *de novo* actin polymerization results in the association of vinculin with the ARP2/3 complex, leading to the stable recruitment of talin to the IS and β-integrin tails. This local accumulation of talin is required for high-affinity binding by integrins. In addition to regulating high-affinity maturation of integrins, the WAVE2 complex and ARP2/3 affect integrin clustering and might participate in the activation of RAP1 (dashed line). PKD binding to β_1-integrin tails helps recruit and activate RAP1. PKD can also interact with the RAP1 GEF C3G, which is constitutively associated with the adaptor protein CRKL. RAP1-GTP can interact with two known effector molecules, RAPL and RIAM. RAP1–RAPL–MST1 complexes are involved in integrin clustering. RAPL also interacts with RIAM, which can bind to F-actin through associations with EVL and VASP proteins, as well as the G-actin binding protein profilin. RIAM also has the potential to interact with talin and the ADAP–SKAP55 complex. These interactions most likely help stabilize F-actin and regulate the local accumulation of RAP1 for optimal integrin activation (see text for details).

state increasing integrin binding (Dustin and Springer, 1989; Shimizu *et al.*, 1990) (Fig. 1.4). These events slow motility and induce strong adhesion to ECM components and counterreceptors on other cells (Dustin and

Springer, 1989). As mentioned, LFA-1 localizes to the T cell–APC contact site to participate in the formation or stabilization of the IS (Monks *et al.*, 1998). Importantly, many regulators of TCR-stimulated integrin clustering and affinity also affect TCR-stimulated F-actin dynamics, suggesting an important role for the actin cytoskeleton in this process (Burbach *et al.*, 2007). Indeed, disruption of F-actin inhibits conjugate formation and binding of T cells to ECM components (Koopman *et al.*, 1992), thus providing compelling evidence that the actin cytoskeleton regulates ''inside-out'' signaling leading to integrin activation in T cells. Proximal signaling pathways leading to integrin activation through chemokine receptors and the TCR are relatively well understood and several excellent reviews are available (Burbach *et al.*, 2007; Kinashi, 2007). However, new information regarding the mechanisms by which cytoskeletal regulatory proteins participate in integrin activation in T cells has recently emerged and will be discussed in the following sections.

5.2. RAP1 regulation of integrins

The importance of the GTPase RAP1 in regulating integrin activation is highlighted by the fact that RAP1A-deficiency abrogates T cell adhesion and TCR-stimulated polarization (Duchniewicz *et al.*, 2006). RAP1 is also involved in chemokine-induced affinity maturation (Parmo-Cabanas *et al.*, 2007) and patients with a rare form of leukocyte adhesion deficiency fail to activate RAP1 in response to chemokines (Kinashi *et al.*, 2004). Although the mechanism by which RAP1 is activated remains undefined in T cells, RAP1 is localized to the tail of the β1 integrin by PKD (Medeiros *et al.*, 2005). PKD localization of RAP1 promotes an interaction with a RAP1 GEF called CRK-SH3 domain binding GEF (C3G) (Medeiros *et al.*, 2005). However, whether C3G is required for TCR-stimulated RAP1 activation is unclear. In fact, although C3G constitutively associates with the adaptor protein CRKL, which is involved in localizing C3G, homotypic adhesion is not impaired in *crkl*$^{-/-}$ mice (Peterson *et al.*, 2003). While other CRK family adaptors might compensate in this model, it is also possible that other RAP1 GEFS activate RAP1 in the absence of C3G. Indeed, PLCγ1 is required for RAP1 activation in T cells, but this can be bypassed by treating the cells with PMA (Katagiri *et al.*, 2004b), a potent PKC activator that is capable of promoting integrin clustering and affinity maturation independent of the TCR. T cells also express the RAP1 GEF, calcium- and DAG-regulated GEF (CALDAG-GEF), and overexpression of CALDAG-GEF in PLCγ1-deficient Jurkat T cells increased RAP1 activation and adhesion to ICAM (Katagiri *et al.*, 2004b). Thus it remains possible that the combination of both C3G and CALDAG-GEF regulates TCR-stimulated activation of RAP1.

The exact mechanism by which active RAP1 promotes integrin clustering is unclear. Similar to other GTPases, RAP1-GTP can interact with effector proteins, stimulating their diverse functions. Two RAP1 effectors have been identified, including RAP1 ligand (RAPL) (Katagiri *et al.*, 2003) and RAP1-interacting adaptor protein (RIAM) (Lafuente *et al.*, 2004) (Fig. 1.4). Importantly, RAPL overexpression enhances integrin clustering (Katagiri *et al.*, 2003) and chemokine-induced integrin-mediated adhesion (Parmo-Cabanas *et al.*, 2007). Also, T cells from *rapl*$^{-/-}$ mice have profound defects in cell migration and adhesion (Katagiri *et al.*, 2004a). Recently, mammalian sterile twenty-like-1 (MST1) kinase was found to interact with RAPL in response to RAP1 activation, which localized MST1 to the IS and stimulated its kinase activity (Katagiri *et al.*, 2006). Additionally, RAPL and MST1 localized to intracellular vesicles containing LFA-1, suggesting that this complex mediates the polarized transport of LFA-1 during migration or IS formation. Importantly, RNAi-mediated knockdown of MST1 impairs IS formation, chemokine-mediated T cell polarization, as well as TCR- and chemokine-stimulated LFA-1 clustering and adhesion.

Similar to RAPL, RIAM overexpression increases integrin-mediated adhesion and RNAi toward RIAM abrogates RAP1-induced adhesion and membrane localization of active RAP1 (Han *et al.*, 2006; Lafuente *et al.*, 2004). Additionally, RAP1 increases the association of RIAM with talin (Han *et al.*, 2006). RIAM is a multidomain protein that contains a RAS-association domain for RAP1 binding, a PH domain and an N- and C-terminal PR region. These PR regions contain several profilin- and EVH1-binding sequences that provide a link between active RAP1 and the actin cytoskeleton (through profilin and mammalian enabled [ENA]-VASP proteins). In fact, RIAM localizes to the edge of the lamellipodium of spreading T cells similar to ENA-VASP-like (EVL), VASP, and WAVE2 (Gomez *et al.*, 2007; Lafuente *et al.*, 2004) (Fig. 1.4). Profilin, VASP, and EVL constitutively associate with RIAM in T cells (Lafuente *et al.*, 2004), and CA-RAP1 co-immunoprecipitation with profilin was RIAM-dependent. Lastly, ADAP also interacts with EVL/VASP proteins, which might facilitate integrin linkage to F-actin. Interestingly, the ADAP-binding partner SRC kinase-associated phosphoprotein of 55 kDa (SKAP55) binds constitutively to RIAM and stabilizes RAP1 membrane localization (Kliche *et al.*, 2006; Menasche *et al.*, 2007), thus providing a direct link with the ADAP–SKAP55 complex and active RAP1.

LFA-1 aggregation independent of TCR ligation induces the formation of a ring-shaped ''actin-cloud'' at the center of the T cell–APC contact site that is enriched in phospo-tyrosine proteins and whose formation is dependent on an interaction of ADAP with SLP76 and LFA-1 as well as JNK activity (Suzuki *et al.*, 2007). Interestingly, *adap*$^{-/-}$ T cells retain the

ability to polymerize F-actin at the IS, indicating that ADAP is required for the generation of specific F-actin structures during T cell activation. HIP55, an actin-binding protein, which can activate JNK through its association with HPK, also interacts with ADAP (Yuan *et al.*, 2005), but since *hip55*$^{-/-}$ T cells home to SLOs, it is unlikely that HIP55 is involved in ''inside-out'' integrin activation during migration (Han *et al.*, 2005). However, HIP55 may play a role in the generation of the ''actin-cloud'' through HPK1-mediated JNK activation. The ''actin-cloud'' was also found to lower the overall signaling threshold through the TCR, and thus may be a mechanism by which chemokine signaling leading to integrin activation could facilitate the activation of T cells by regulating their sensitivity to low-affinity TCR ligands, or to APCs displaying limited numbers of reactive pMHCs.

5.3. WAVE2 as a key regulator of integrins

T cells from *vav1*$^{-/-}$ MICE demonstrate defective conjugate formation, integrin clustering, and IS formation (Ardouin *et al.*, 2003; Krawczyk *et al.*, 2002; Wulfing *et al.*, 2000). Additionally, suppression of VAV1 and RAC1 affect chemokine-induced integrin-mediated adhesion (Garcia-Bernal *et al.*, 2005; Parmo-Cabanas *et al.*, 2007). Moreover, ARP2/3-generated F-actin is essential for TCR-stimulated integrin clustering and activation (Gomez *et al.*, 2007; Nolz *et al.*, 2007b), implicating ARP2/3-dependent F-actin nucleation as a critical event regulating TCR-stimulated integrin activation. Although *wasp*$^{-/-}$ T cells have defects in activation and actin dynamics, these T cells adhere to integrin ligands normally (Cannon and Burkhardt, 2004; Krawczyk *et al.*, 2002; Nolz *et al.*, 2006). In contrast, WAVE2-suppressed Jurkat T cells and primary human T cells have defective TCR-stimulated adhesion (Nolz *et al.*, 2006, 2007b). Interestingly, CD4^{+} T cells isolated from patients with multiple sclerosis show increased expression of PIR121 (a WAVE2 complex component) and knockdown of RAC1 or PIR121 resulted in diminished T cell adhesion (Mayne *et al.*, 2004), thereby implicating RAC1 and its target effector, the WAVE2 complex, as a key ARP2/3 regulator involved integrin-mediated adhesion in T cells.

Several F-actin-interacting proteins are components of focal adhesions and regulate integrin activation. Talin is an actin-binding protein, which localizes to the IS (Monks *et al.*, 1998), binds the cytoplasmic tail of β integrins and is required for integrin affinity maturation (Simonson *et al.*, 2006). Vinculin, a multidoman protein that interacts with talin, F-actin, and ARP/23, localizes to the IS (Nolz *et al.*, 2007b) and is involved in integrin function in many cell types (Ziegler *et al.*, 2006). Interestingly, the interaction between vinculin with ARP2/3 is enhanced in the

presence of a VCA domain (DeMali *et al.*, 2002), and TCR/CD28-stimulated Jurkat and primary T cells form a complex containing WAVE2–ARP2/3, vinculin, and talin (Nolz *et al.*, 2007b) (Fig. 1.4). The interaction of vinculin with ARP2/3 requires the presence of WAVE2 bound to ARP2/3, since a WAVE2 protein lacking the VCA domain does not lead to the formation of the TCR-induced complex. Interestingly, WASP is unable to function in mediating this TCR-stimulated association of ARP2/3 and vinculin, despite harboring a VCA domain. Although vinculin suppression in Jurkat T cells does not affect F-actin or integrin clustering at the IS, it does abrogate conjugate formation and adhesion to fibronectin. Importantly, a vinculin mutant that cannot interact with ARP2/3 is unable to regulate integrins. Lastly, suppression of either WAVE2 or vinculin results in decreased talin localization to the IS and, as a result, diminished high-affinity integrin binding. Taken together, these data delineate a role for WAVE2–ARP2/3 in both the affinity maturation of integrins (through interaction with vinculin and talin) as wells as integrin avidity (integrin clustering) at the IS. Although it is currently unclear how WAVE2–ARP2/3 and actin regulate integrin clustering at the IS, recent studies indicate that T cells suppressed for WAVE2, ARP2/3 or treated with F-actin disrupting agents show diminished RAP1 activation (Nolz and Billadeau, unpublished observation). Thus, it is likely that F-actin not only provides the framework for integrin clustering and affinity, but also for localized RAP1 accumulation and activation.

6. CYTOSKELETAL DYNAMICS AND CELL MIGRATION

6.1. Polarity during T cell migration

Naïve T cells continuously survey the body's lymphoid compartments for antigen or specifically home to sites of inflammation following activation. This directed motility, which is coordinately regulated through intricate recognition of chemoattractants and adhesion to cellular ligands or ECM components, is highly dependent on cytoskeletal-mediated shape changes (Vicente-Manzanares and Sanchez-Madrid, 2004). T cells responding to chemoattractants rapidly polarize, forming a leading lamellipodium and filopodia containing structure protruding toward the source of stimulus, and a trailing uropod projection (Fig. 1.5). Continuous F-actin polymerization at the leading edge drives forward movement and adhesion, while the uropod undergoes repeated cycles of substrate attachment, release, and retraction. Thus, this coordinated adhesion and detachment, along with actin-mediated propulsion, allows T cells to ''crawl'' or ''chemotax'' along blood vessel walls and to transmigrate through vascular endothelium into surrounding tissues.

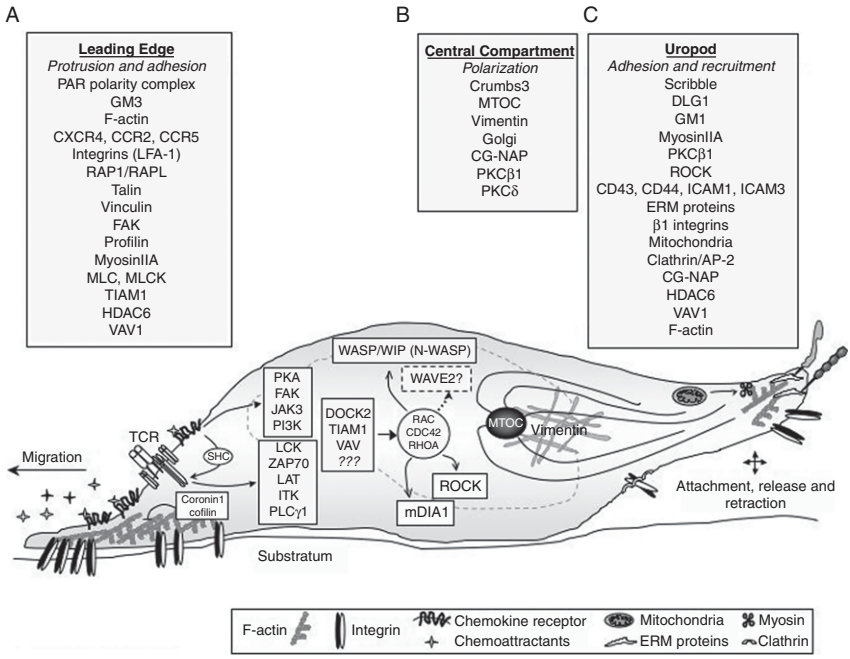

FIGURE 1.5 The role of the cytoskeleton in T cell migration. Chemokine-stimulated T cells rapidly polarize, forming a leading F-actin-rich edge directed toward the source of stimulus, and a trailing uropod projection. This leading edge and uropod, along with a central polarizing region, serve as subcellular compartments for the polar distribution of various organelles, lipid rafts components, receptors, and signaling molecules during migration (Box A, leading edge components; Box B, central polarizing compartment; Box C, uropodal components). Chemokine receptors and integrins, which localize to the leading edge of migrating T cells, sense chemoattractant gradients and allow adherence to the substratum, respectively. Together these receptors stimulate the activity of various kinases, adaptor proteins, and GEFs, which ultimately regulate the activation of RHO GTPases and their F-actin regulating effectors, including WASP, WAVE2, ROCK, and mDIA1. This results in F-actin polymerization at the leading edge to drive forward movement and adhesion, and causes the uropod to undergo cycles of substrate attachment, release, and retraction. Interestingly, chemokine receptors might regulate this F-actin-dependent locomotion through transactivation of the TCR via the adaptor SHC. Also, cofilin and Coronin1 promote F-actin turnover to allow dynamic assembly or disassembly of F-actin-based structures during migration. In addition to F-actin reorganization, the MTOC and vimentin (intermediate filaments) also rapidly relocate to the base of the uropod, with microtubules radiating into the uropod, perhaps increasing T cell deformability during chemotaxis. Mitochondria may regulate myosin activity for contraction within the uropod, whereas clathrin-mediated endocytosis occurring in the uropod could control receptor recycling during migration. Lastly, adhesion molecules are localized to the uropod via ERM-dependent anchoring to the cortical actin cytoskeleton and might allow responding T cells to recruit bystander lymphocytes to sites of inflammation or periodically anchor the cell to the substratum during locomotion.

In addition to F-actin, intermediate filaments (IFs) and microtubules may also participate in T cell migration. In circulating spherical T cells, the IF, vimentin, forms a cage-like structure, which provides structural support. However, during chemotaxis this sturdy vimentin architecture rapidly collapses and redistributes toward the uropod, a change that is predicted to facilitate reduced cellular rigidity for migration through constricted spaces (Brown *et al.*, 2001). However, analysis of *vimentin*$^{-/-}$ T cells indicate only minor defects in homing to SLOs (Nieminen *et al.*, 2006), and normal polarization, indicating that vimentin does not provide structural support to the uropod (Brown *et al.*, 2001). The MTOC also rapidly relocates to the base of the uropod, with microtubules radiating into the slender uropodal appendage (Ratner *et al.*, 1997; Serrador *et al.*, 1997; Volkov *et al.*, 2001). In many adherent cell types the MTOC is instead positioned toward the leading edge, suggesting that polarity in T cells is governed by a distinct set of rules (Rodriguez *et al.*, 2003). Similar to vimentin, this microtubule retraction is not required for uropod formation, but is instead thought to increase T cell deformability (Ratner *et al.*, 1997). In contrast to disruption of F-actin reorganization, which abrogates leading edge and uropod formation (Lee *et al.*, 2004; Verschueren *et al.*, 1995), microtubule destabilization has little impact on T cell polarization and motility (Ratner *et al.*, 1997). Therefore, the restructuring of these IF and microtubule systems does not produce motive force such as F-actin, but instead seems to be a requisite step in establishing polarity and plasticity in migrating T cells.

In addition to polarization of the cytoskeletal systems, the leading edge and uropod also serve as subcellular compartments for the polar distribution of various receptors and signaling molecules, which not only act as molecular markers to define polarity in T cells, but also give mechanistic insight into T cell migration (Fig. 1.5). Additionally, as with IS formation, lipid rafts may participate in this polar segregation of membrane-associated molecules during chemotaxis, as GM3- and GM1-enriched domains are selectively targeted in an actin-dependent manner to the leading edge and uropod, respectively (Gomez-Mouton *et al.*, 2001). In this regard, the leading edge is enriched in chemokine receptors, including CXCR4, CCR2, and CCR5, (Gomez-Mouton *et al.*, 2001; Nieto *et al.*, 1997), which are used for sensing the complex chemoattractant gradients that induce directed migration. Also, profilin at this leading edge promotes actin monomer addition to rapidly growing filaments, which orchestrate actin-based protrusion and integrin anchoring (Vicente-Manzanares *et al.*, 2003). Accordingly, integrins (including LFA-1) are enriched at the front migrating T cells (Campanero *et al.*, 1994; del Pozo *et al.*, 1995; Friedl and Brocker, 2000), along with talin, vinculin, and focal adhesion kinase (FAK) (Campanero *et al.*, 1994; Entschladen *et al.*, 1997; Gomez-Mouton *et al.*, 2001), where they function

to dynamically link F-actin to the substratum via cyclic clustering and recirculation during lymphocyte movement (Burbach *et al.*, 2007; Volkov *et al.*, 1998). Interestingly, LFA-1 cross-linking can fully induce T cell polarization and migration (Volkov *et al.*, 1998). Moreover, related to integrin function, myosinIIA has been shown to associate with activated chemokine receptors, providing an important link to the mechanosignaling required for traction during locomotion and to chemokine receptor endocytosis (Rey *et al.*, 2002, 2007). Likewise, myosin light chain (MLC) and MLC-kinase (MLCK) are enriched at this leading edge where they are crucial for integrin activation and lamellar extension (Smith *et al.*, 2003; Vicente-Manzanares *et al.*, 2002). Thus, based on this molecular polarity, the F-actin-dependent formation of an extended leading edge is critical for recognizing gradients through specialized chemosensory receptors, promoting integrin-mediated adhesion and providing contractile potential at F-actin anchored adhesion sites, which allow migrating cells to pull themselves forward (Fig. 1.5).

Migrating T cells also form a posterior protrusion called a uropod. Although the exact role of this appendage remains elusive, and is less well defined than the leading edge, the finding that various molecules selectively compartmentalize into the uropod has begun to shed light on mechanisms underlying its formation and function. It is known that several transmembrane adhesion molecules, including CD43, CD44, ICAM1, and ICAM3, are concentrated into the uropod (Sanchez-Madrid and del Pozo, 1999). Moreover, the cross-linking of these adhesion molecules directly results in T cell polarization (Campanero *et al.*, 1994; Fanning *et al.*, 2005; Serrador *et al.*, 1998; Volkov *et al.*, 1998). Interestingly, the intracellular tails of CD43, CD44, and ICAM3 can bind to activated ERM proteins, which are also found co-localized in the uropod, suggesting that these molecules are localized to the uropod through F-actin-mediated anchoring (Lee *et al.*, 2004; Serrador *et al.*, 1997, 1998). Additionally, some β1 integrins are sequestered to the uropod during migration, indicating that the uropod might function as a point of attachment to the substratum while the cell body pulls forward through leading edge dynamics (Friedl and Brocker, 2000). However, in some cases the uropod was reported to be a rigid structure, which is lifted off from, rather than attached to the substratum in lymphocytes (del Pozo *et al.*, 1997; Samaniego *et al.*, 2007). Thus, although the uropod could serve as an anchor during cell body translocation, it lifts up during retraction for net cell movement. Alternatively, it is possible that the lifted posterior sequestration of these various adhesion molecules along with ERM proteins, which have the potential to increase cellular rigidity via membrane-cytoskeletal cross-linking, might simply favor high-speed migration of T cells by imparting low adhesive potential and plasticity. Interestingly, it was shown that polarized T cells are able to form ICAM-mediated

contacts via their uropod with LFA-1 expressed on bystander T cells (del Pozo *et al.*, 1997). In this way, the uropods of polarized lymphocytes were suggested to capture neighboring T cells during transendothelial migration, leading to the cooperative transport and recruitment of bystander T cells to sites of inflammation.

In addition to accumulating at the leading edge (Rey *et al.*, 2002), myosinIIA also localizes to the uropod during chemotaxis (Jacobelli *et al.*, 2004), and either RNAi-depletion or pharmacologic inhibition of myosinIIA abrogates uropod formation. However, pseudopods, which are morphologically similar to the leading edge, still form upon myosinIIA inhibition, although these uropod-deficient T cells are nonmotile (Jacobelli *et al.*, 2004). Myosin inhibition eliminates CD43 and ICAM3 accumulation at the uropod (Sanchez-Mateos *et al.*, 1995; Serrador *et al.*, 1997), and blocking MLCK abrogates polarization altogether (Vicente-Manzanares *et al.*, 2002). A contradictory study has indicated that myosin inhibition does not ablate uropod formation, but results in loss of uropod rigidity instead (Samaniego *et al.*, 2007). In addition to regulating uropod structure, it is plausible that myosin-generated tensional strain on the cytoskeleton is important for breaking aged adhesive contacts at the rear of the cell during locomotion, allowing the uropod to retract. Also related to myosin function, mitochondria accumulate in the uropod via a microtubule-dependent mechanism, and are suggested to provide the local ATP production required for MLC phosphorylation, which is needed for uropodal F-actin contraction during migration (Campello *et al.*, 2006).

Clathrin and the endocytic adapter complex, AP-2, co-localize to the T cell uropod in a microtubule-independent manner (Samaniego *et al.*, 2007). These clathrin–AP-2 structures are capable of actin-dependent endocytosis, indicating that specialized posterior endocytic platforms function during T cell migration. Importantly, while blocking clathrin-mediated endocytosis does not affect polarization it does result in diminished chemotaxis, suggesting that uropodal vesicular transport may aid in uropod retraction, function to spatially resolve signaling, or maintain local polarization of essential proteins via recycling during migration. For instance, these endocytic platforms may be crucial for recycling integrins to new sites of adhesion, since attached receptors move backwards as the cell advances (Fig. 1.5). Altogether, this suggests that the T cell uropod is not simply a trailing appendage created during polarization, but rather an essential structure that modulates cellular adhesion, recruits bystander lymphocytes, generates contraction via actin-myosin fiber formation, and contains active machinery for endocytosis.

So far, we have only a limited understanding of how these polar subdomains are established within migrating T cells. However, it was suggested that T cell polarization is governed by a network of

PDZ-containing protein complexes including Scribble, DLG family members, Crumbs3, and partitioning defective-3 (PAR-3), which regulate epithelial cell polarity. These molecules are distributed asymmetrically in polarized T cells with Scribble and DLG localizing to the uropod. In fact, Scribble depletion leads to defects in uropod formation, CD44 and ERM localization, as well as migration (Ludford-Menting *et al.*, 2005). Additionally, PAR-3 localizes to the leading edge (Gerard *et al.*, 2007), whereas Crumbs3 concentrates at the base of the uropod (Ludford-Menting *et al.*, 2005). This suggests that these complexes may dictate the cytoskeletal-based morphological changes that are required for chemotaxis. Taken together, chemoattractant-induced asymmetric cytoskeletal polarization and compartmentalization of cellular components into a front-rear axis is a highly regulated process, which modulates the plasticity and adhesive potential of T cells for directed and rapid T cell motility.

6.2. Chemokine signaling to F-actin remodeling and cell motility

The pathways required for converting chemotactic signals into complex cytoskeletal changes during T cell migration have begun to emerge. Critically important are the chemoattractant cytokines, or chemokines (such as CXCL12, also known as stromal cell-derived factor 1α, SDF1α), which specifically interact with 7 transmembrane-spanning heterotrimeric G-protein-coupled receptors (GPCRs) (such as CXCR4, the receptor for SDF1α). Chemokines transmit signals leading to integrin activation, polarization, and F-actin remodeling, which coordinately regulate lymphocyte chemotaxis. Once activated by ''inside-out'' chemokine signals, integrins can also signal ''outside-in'' to maintain physical connections between the substratum and the inner cortical cytoskeleton during migration, thus facilitating complex crosstalk between chemokine and adhesion receptors. Therefore, circulating T cells are recruited to SLOs or sites of inflammation because they recognize vascular endothelial-displayed chemoattractants, such as chemokines, which sequentially result in integrin activation, lymphocyte arrest, microvillar collapse, polarization, and migration through the endothelial lining (transendothelial migration) (Vicente-Manzanares and Sanchez-Madrid, 2004). While the mechanisms used by chemokine receptors to elicit these diverse responses are poorly understood in T cells, many signal transduction events have been suggested to act downstream of chemokine receptors and integrins for proper cytoskeletal remodeling and polarity during chemotaxis.

Several kinases, including protein kinase A (PKA), Janus kinases, FAK, LCK, and ZAP70, are required for CXCR4-mediated migration as indicated by studies utilizing pharmacologic inhibitors (Entschladen *et al.*, 1997; Patrussi *et al.*, 2007; Stein *et al.*, 2003; Ticchioni *et al.*, 2002;

Vicente-Manzanares *et al.*, 1999) (Fig. 1.5). Interestingly, although ERK is activated by chemokines and required for neutrophil migration (Gu *et al.*, 2003), it does not play a role in polarization or migration of T lymphocytes (Bardi *et al.*, 2003; Ticchioni *et al.*, 2002; Vicente-Manzanares *et al.*, 1999). Also, the actin-regulatory Tec kinases, ITK and RLK, are phosphorylated in response to SDF1α, and necessary for CXCR4-induced F-actin polymerization, adhesion, and migration *in vitro* (Takesono *et al.*, 2004). Moreover, $itk^{-/-}/rlk^{-/-}$ T lymphocytes display severely defective homing to SLOs *in vivo* (Takesono *et al.*, 2004). Additionally, protein kinase C β1 (PKCβ1) and PKCδ are activated during LFA-1-induced polarization, and specifically localize into the uropod with the MTOC and microtubules (Volkov *et al.*, 1998, 2001). However, while PKCβ1 is dispensable for adhesion, it is required for polarization and locomotion (Volkov *et al.*, 2001). Related to PKC function, it was suggested that centrosome and golgi localized protein kinase-N associated protein (CG-NAP) acts as an essential cytoskeletal scaffolding protein for PKC and LFA-1 anchoring to microtubules (El Din El Homasany *et al.*, 2005). Similarly, it was suggested that HDAC6 might act as an adaptor to mediate crosstalk between microtubules and F-actin during chemotaxis (Cabrero *et al.*, 2006).

Several studies have implicated PI3K in establishing cell polarity in neutrophil migration (Ferguson *et al.*, 2007; Hannigan *et al.*, 2002). However the role of PI3K in regulating T cell migration is less clear. While expression of dominant negative forms of p85 and PI3Kγ affect T cell migration, and active PI3K associates with activated CXCR4 (Curnock and Ward, 2003; Vicente-Manzanares *et al.*, 1999), PI3K inhibitors only partially inhibit T cell migration in most cases (Nombela-Arrieta *et al.*, 2004; Stein *et al.*, 2003; Ticchioni *et al.*, 2002; Vicente-Manzanares *et al.*, 1999). Moreover, while treatment with PI3K inhibitors profoundly inhibits the polar redistribution of ICAM3 and moesin to the T cell uropod, it has no effect on cell morphology, global F-actin polymerization, or MTOC polarization (Vicente-Manzanares *et al.*, 1999). This suggests that partial defects in T cell migration observed with PI3K inhibitors may reflect an inability to form a functional uropod or effectively reduce cellular rigidity. PI3K activity is also required for chemokine-mediated lateral clustering of integrins for increased avidity (Constantin *et al.*, 2000), suggesting that PI3K may affect migration via regulation of actin-mediated integrin polarization. Despite these observations, T cell migration may be largely PI3K-indpendent, as demonstrated recently through analysis of $pi3k\gamma^{-/-}$ lymphocytes, which suggests that only a small proportion of T cell migration is actually PI3K-dependent (Nombela-Arrieta *et al.*, 2004). Thus, although T cells require PI3K for optimal migration, their exact level of dependency on PI3K signaling is unclear.

The SH2-containing adaptor SHC, which is essential for chemokine-induced migration, is phosphorylated following SDF1α-stimulation,

forms a complex with LCK, ZAP70 and VAV1, and is required for their phosphorylation (Patrussi *et al*., 2007). Moreover, SHC is also essential for ITK and PLCg1 phosphorylation (Patrussi *et al*., 2007). Interestingly, CD3ζ is also phosphorylated and associates with phospho-SHC in SDF1α-treated T cells, and the TCR is required for CXCR4-mediated migration and phosphorylation of ZAP70, VAV1, and SHC. This suggests that transactivation of the TCR, possibly via SHC, might be an essential step for efficient chemokine signaling leading to migration (Fig. 1.5). Overexpression of DN-ITK impairs activation of both RAC1 and CDC42 in response to SDF1α (Takesono *et al*., 2004), suggesting that this TCR transactivation may also be crucial for activating these GTPases in migrating T cells. Since SHC is necessary for the activation of TCR-induced actin-regulatory proteins such as VAV1 and ITK by chemokines, chemokine-mediated TCR transactivation might be a primary means of exploiting TCR-associated F-actin remodeling machinery during migration. This is supported by the fact that both SHC- and TCR-deficient T cells cannot efficiently internalize CXCR4 in response to SDF1α (Patrussi *et al*., 2007), a cytoskeletal-dependent process that is likely important for cellular desensitization during directed movement toward chemokines.

Importantly, chemokine receptors rely heavily on the activation of small GTPases, and subsequent activation of their effector proteins to regulate integrins, cell morphology, and chemotaxis. As mentioned, RAP1 is important in this regard through its associates with RAPL at the leading edge of migrating T cells to promote integrin activation, polarization, and transendothelial migration (Katagiri *et al*., 2003; Shimonaka *et al*., 2003). Overexpression of either RAPL or CA-RAP1 induces a fully polarized T cell morphology, and the latter also enhances motility in the absence of chemokine (Katagiri *et al*., 2003; Shimonaka *et al*., 2003). Moreover, T lymphocytes from *rapl*$^{-/-}$ mice show severe defects in chemokine-induced polarization and display diminished homing to SLOs (Katagiri *et al*., 2004a). In addition to RAP1, chemokine receptors also activate RAC, CDC42, and RHOA (Haddad *et al*., 2001; Nijhara *et al*., 2004; Vicente-Manzanares *et al*., 2002). Studies expressing mutant versions of these GTPases have highlighted their crucial role in T cell polarization and migration. Active versions of RAC1, CDC42, and RHOA abrogate T cell polarization, while dominant negative versions of these GTPases trigger uropod formation (del Pozo *et al*., 1999). Moreover, expression of the activated forms of these three GTPases or DN-CDC42 leads to significant defects in CXCR4-mediated chemotaxis, whereas DN-RHOA- and DN-RAC1-expressing T cells show only slightly diminished migration (del Pozo *et al*., 1999). However, it was also shown that expression of DN-RHOA dramatically suppresses uropod formation, and CA-RHOA localizes to the T cell uropod (Lee *et al*., 2004). In contrast, inhibition of RHOA via C3-exoenzyme results instead in decreased uropod rigidity or highly

extended uropod formation (Samaniego *et al.*, 2007; Vicente-Manzanares *et al.*, 2002). In addition, expression of CA-RAC1 or CA-CDC42 in T cells mediates ERM dephosphorylation and subsequent microvillar collapse, which is requisite for efficient migration, while CA-RHOA induces ERM activation (Nijhara *et al.*, 2004). Thus, as with reports pertaining to TCR-mediated F-actin regulation by RHO GTPases, it is difficult to deduce the role of each of these GTPases in chemotaxis based on mutant overexpression studies due to conflicted findings and the possibility for pleiotropic affects. However, from these studies, it is clear that RHO GTPases are important for cytoskeletal regulation during migration, which makes the identification of the GEFs that specifically activate them of interest.

The central GEF that has emerged for chemokine-induced migration is DOCK2. While *dock2*$^{-/-}$ T cells display normal ERK phosphorylation, protein kinase B (PKB) activation and Ca^{2+} mobilization in response to SDF1α, they cannot activate RAC (Fukui *et al.*, 2001) (Fig. 1.5). Consequently, *dock2*$^{-/-}$ T cells have severe defects in chemokine-induced F-actin polymerization, microvillar collapse, polarization, and migration (Fukui *et al.*, 2001; Nombela-Arrieta *et al.*, 2004; Shulman *et al.*, 2006). These defects are highlighted by the fact that *dock2*$^{-/-}$ mice have reduced thymic emigration and dramatically decreased $CD4^+$ and $CD8^+$ T cell numbers in SLOs (Fukui *et al.*, 2001). Interestingly, one study suggests that *dock2*$^{-/-}$ T cells are capable of arrest and transendothelial migration even though they cannot locomote laterally on vascular integrin ligands in response to chemokine. However, these *dock2*$^{-/-}$ T cells do not migrate away from the site of diapedesis into surrounding tissue once on the basal side of the vascular endothelium (Shulman *et al.*, 2006). In spite of these severe defects, *dock2*$^{-/-}$ mice do have some residual SLO architecture with separated T and B cell zones (Fukui *et al.*, 2001), suggesting that *dock2*$^{-/-}$ does not completely abolish migration. Interestingly, it was shown this residual DOCK2-independent migration could be blocked by PI3K inhibition (Nombela-Arrieta *et al.*, 2004). While *pi3kγ*$^{-/-}$ T cells showed a large defect in chemokine-mediated PKB phosphorylation, they displayed only slightly reduced migration to SLOs (15–30%). However, combined DOCK2 and PI3Kγ deficiency abrogated residual migration observed in *dock2*$^{-/-}$ T cells (Nombela-Arrieta *et al.*, 2004). Thus, it seems that T cell chemotaxis might occur via two distinct pathways, a major DOCK2-dependent and PI3K-independent pathway and a minor mechanism that is PI3K-dependent. This is interesting in light of the fact that other leukocyte subsets, such as neutrophils, which do not express DOCK2 (Nishihara *et al.*, 1999), are strongly dependent on PI3K activity for migration (Ferguson *et al.*, 2007; Hannigan *et al.*, 2002). Thus, leukocyte subsets, or even T lymphocyte subsets, clearly rely on divergent mechanisms for setting up polarity and directed migration.

Another report has implicated the RAC-specific GEF, T lymphoma invasion, and metastasis-1 (TIAM1), in lymphocyte polarity during migration (Gerard *et al.*, 2007). This study supports a role for the Scribble–Crumbs–Par polarity network in T cell polarization (Ludford-Menting *et al.*, 2005), by demonstrating that the PAR polarity complex (made up of PAR-3, PAR-6, and atypical PKCζ) is activated downstream of chemokine receptors (Gerard *et al.*, 2007). Activation of RAP1, which is required for T cell polarization, leads to localized activation of CDC42, the major activator of the PAR polarity complex. Moreover, RAP1-induced T cell polarization is impaired by expression of DN-CDC42 or RNAi-depletion of PAR-3, suggesting that the PAR complex is essential for RAP1-regulated polarity. In addition, RAC1-mediated cytoskeletal remodeling is necessary for RAP1-induced polarization, and RAC1 activation requires both CDC42 and the PAR polarity complex. Importantly, TIAM1 was found to be the missing link for this RAP1-induced RAC1 activation. Activated RAP1 was also found to interact with TIAM1, which was shown previously to bind PAR-3. This suggests that TIAM1 might be pivotal not only in connecting locally activated RAP1 to the PAR complex following chemokine stimulation, but also in subsequent chemokine-induced RAC1 activation. Consequently, CXCR4-stimulated *tiam1*$^{-/-}$ T cells show reduced RAC1 activation, polarity, and chemotaxis (Gerard *et al.*, 2007). Moreover, RAP1, CDC42, PAR-3, PKCζ, and TIAM1 all localize to the leading edge, suggesting that this signaling axis is important for anterior F-actin dynamics. This compelling study gives new mechanistic insight into the establishment of chemokine-mediated polarity by linking RAP1 activation to the CDC42-PAR polarity complex and RAC1 activation via TIAM1.

In addition, another RAC-activating GEF, VAV1, might also regulate T cell migration. Upon stimulation with SDF1α, VAV1 associates with CXCR4, is phosphorylated, and then localizes to both the leading edge and the uropod of polarized T cells (Vicente-Manzanares *et al.*, 2005). Interestingly, expression of either a GEF-inactive or a CA-VAV1 mutant in primary human T cells leads to impaired CXCR4-mediated polarization and chemotaxis. Although, these results suggest a role for VAV1 in chemokine-induced polarization and migration, it must be noted that T cells from *vav1*$^{-/-}$ mice show normal increased F-actin content and display no defects in migration in response to SDF1α (Vicente-Manzanares *et al.*, 2005). This is perhaps due to compensatory effects of VAV2 and VAV3 in these cells. Thus, further studies analyzing the ability of T cells from *vav*1/2/3 knockout mice (Fujikawa *et al.*, 2003), which have a block in thymocyte development with some T cells escaping, will be required to firmly establish the role of VAV proteins in chemotaxis.

6.3. GTPase-regulated effectors and T cell migration

Downstream effector molecules regulated by the RHO GTPases following chemokine- and integrin-mediated signaling during migration are also of great interest in the field. Actin-regulatory effectors such as WASP, mDIA1, and ROCK are thought to collaboratively control the cytoskeletal architecture that allows cells to migrate. Upon stimulation with SDF1α, WASP is tyrosine phosphorylated and forms a complex with NCK, CRK-associated substrate (CAS), and FAK in T cells (Okabe *et al.*, 2002). Moreover, T lymphocytes from WAS patients display impaired migration to SDF1α (Haddad *et al.*, 2001), and T cells from *wasp*$^{-/-}$ mice were shown to be defective in homing and chemotaxis (Snapper *et al.*, 2005). However, while it was verified that *wasp*$^{-/-}$ T cells are indeed unable to migrate efficiently to SLOs *in vivo*, it seems that they are capable of migration to SDF1α *in vitro* (Gallego *et al.*, 2006), which may be supported by recent studies showing migration of *wasp*$^{-/-}$ T cells on lipid bilayers (Sims *et al.*, 2007). In fact, *wasp*$^{-/-}$ T cells display almost no defect in chemotaxis *in vitro* whereas *wip*$^{-/-}$ T cells are much more defective, and lymphocytes devoid of both proteins have severely impaired chemotactic ability (Gallego *et al.*, 2006). This suggests that WASP and WIP play separate and complementary roles in T cell homing. In addition, migration defects observed in *wip*$^{-/-}$ T cells might partially reflect the requirement of WIP for WASP protein stability. Interestingly, it was also shown that CXCR4-induced increases in F-actin content were unaffected in both *wasp*$^{-/-}$ and *wip*$^{-/-}$ T cells, but were severely compromised with *wip*$^{-/-}$/*wasp*$^{-/-}$. However, all knockout populations did not polarize normally in response to chemokines (Gallego *et al.*, 2006). Interestingly, *wasp*$^{-/-}$/*n-wasp*$^{-/-}$ T cells display a greater defect in chemotaxis to SDF1α than do *wasp*$^{-/-}$ lymphocytes, although there is still not a complete block in migration *in vitro* (Cotta-de-Almeida *et al.*, 2007), which supports a role for additional actin-nucleators in chemotaxis.

Another actin-nucleating protein that may regulate migration is the RHO-activated formin, mDIA1. A role for mDIA1 in chemotaxis was first suggested when expression of a truncated active form of mDIA1 led to increased F-actin content and inhibited CXCR4-mediated migration in T cell lines (Vicente-Manzanares *et al.*, 2003). More recently, the role of mDIA1 in chemotaxis was more firmly established through analysis of *drf1*$^{-/-}$ T cells (Eisenmann *et al.*, 2007; Sakata *et al.*, 2007). *drf1*$^{-/-}$ T cells display severe defects in chemotaxis, F-actin polymerization, and polarization in response to chemokines *in vitro*. As a result, these *drf1*$^{-/-}$ mice exhibit impaired lymphocyte trafficking *in vivo*, with significant reductions of both CD4^{+} and CD8^{+} T cell numbers in SLOs (Eisenmann *et al.*, 2007; Sakata *et al.*, 2007). However, while one study showed reduced percentages of CD4 and CD8 single positive thymocytes in *drf1*$^{-/-}$ mice

(Eisenmann *et al.*, 2007), another report indicated a tendency toward increased total numbers of these thymocyte populations compared with control mice (Sakata *et al.*, 2007). Additionally, this latter finding was associated with significant thymic retention of $CD69^{lo}CD62L^{hi}$ single positive thymocytes, suggesting diminished migratory egress from the thymus (Sakata *et al.*, 2007). Not surprisingly, these defects in chemotaxis, along with a diminished proliferation capacity of $drf1^{-/-}$ T cells, lead to impaired immune responses (Sakata *et al.*, 2007). Interestingly, as mentioned, $drf1^{-/-}$ T cells also have severely reduced WASP levels due to accelerated WASP degradation, but polarization and migration defects could not be rescued by reexpression of WASP in $drf1^{-/-}$ T cells, suggesting that loss of mDIA1 alone is sufficient (Sakata *et al.*, 2007).

Also downstream of RHOA, the serine/threonine kinase, ROCK, has been proposed to regulate actomyosin activity. While ROCK does not influence the amount of chemokine-mediated F-actin polymerization, it might play a role in spatially positioning *de novo* F-actin to the leading edge of migrating T cells (Vicente-Manzanares *et al.*, 2002). In addition, while one study has indicated that polarized T cells treated with ROCK inhibitor lose uropod formation, but maintain phospho-ERM and CD44 capping at the site were the uropod once protruded (Lee *et al.*, 2004), other studies suggest that the uropod becomes long, unretracted, and limp upon ROCK inhibition (Samaniego *et al.*, 2007; Smith *et al.*, 2003; Vicente-Manzanares *et al.*, 2002). Yet, another study indicates complete loss of polarization in primary T cells with ROCK inhibitor (Bardi *et al.*, 2003). This suggests that while ROCK is likely required for uropod maintenance through effects on myosin, it is not responsible for ERM-mediated cytoskeletal anchoring of cellular receptors. Moreover, the fact that MLCK is localized to the leading edge, influencing integrin activation, and ROCK is concentrated in the uropod, may suggest that these two kinases cooperate in the regulation of myosin to regulate forward propulsion and mediate rear detachment, respectively (Smith *et al.*, 2003). This notion is supported by the fact that ROCK promotes lymphocyte de-adhesion by reducing F-actin-dependent cellular spreading via myosin activation (Liu *et al.*, 2002). Additionally, the accumulation of endocytic clathrin complexes to the uropod also critically depends on RHOA–ROCK–myosin signaling (Samaniego *et al.*, 2007). Importantly, while the exact role of ROCK in T cell polarization remains unclear, it seems that blocking the chemokine-induced RHOA–ROCK–myosin axis does abrogate T cell migration (Bardi *et al.*, 2003; Jacobelli *et al.*, 2004; Vicente-Manzanares *et al.*, 2002).

In addition, other emerging cytoskeletal regulatory effectors are likely to regulate chemotaxis. For example, studies of the WAVE2 complex, which we know is critical for TCR-mediated integrin function and F-actin dynamics (Nolz *et al.*, 2006), have not yet focused on T cell

chemotaxis. However, recent studies of other leukocyte subsets indicate a role for this complex in migration. In response to chemokine, WAVE2–ABI1 complexes localize to F-actin-rich membrane protrusions in macrophages, and RNAi-depletion of WAVE2 or ABI1 impairs macrophage chemotaxis (Kheir *et al.*, 2005). Moreover, HEM1 was shown to regulate cell polarity, motility, and activation of PI3K and RAC1 in neutrophils (Weiner *et al.*, 2006). Taken together, this suggests that pathways activated by GTPases in T cells are pivotal for efficient cytoskeletal-based polarization and migration.

6.4. F-actin disassembly during migration

During chemotaxis, T cells must be able to rapidly reorient by dismantling existing F-actin architecture, which allows for dynamic redirection of lamellipodial protrusions and for restructuring of adhesion sites. Therefore, along with mechanisms that promote actin assembly, pathways regulating F-actin disassembly and turnover must concomitantly occur. To date there have been limited studies on proteins that negatively regulate cytoskeletal dynamics in T cells, but recent evidence indicates that both cofilin and Coronin1 might be important for T cell function in this regard (Foger *et al.*, 2006; Nishita *et al.*, 2005). In response to chemokine, the actin-severing protein cofilin is spatiotemporally controlled by LIM domain kinase (LIMK) and slingshot phosphatase (SSH1L). LIMK-mediated phosphorylation inhibits cofilin, thereby promoting F-actin assembly and the formation of multiple cellular protrusions, while SSH1L-dependent dephosphorylation (activation) of cofilin then restricts this actin polymerization to one leading edge (Nishita *et al.*, 2005). Both ROCK and PAK can activate LIMK in T cells, suggesting they are important for F-actin turnover at the uropod and leading edge, respectively. In addition, Coronin1, which binds to the ARP2/3 complex and inhibits ARP2/3-mediated actin polymerization, has emerged as a regulator of steady-state F-actin in lymphocytes. Recent analysis of *coronin1*$^{-/-}$ T cells indicates defects in migration, homing, and polarization in response to chemokines (Foger *et al.*, 2006). Thus, negatively acting cytoskeletal regulatory proteins are essential for migration, and the continued study of these molecules is key to our understanding of the dynamic process of cellular locomotion.

7. CONCLUSION

More than a quarter of a century has passed since the discovery that cytoskeletal polarization at the T cell–APC synapse correlated with productive immune responses. We now appreciate the essential role of

F-actin and microtubules in the regulation of IS formation, signaling, secretion, migration, and adhesion, and over the past decade, there has been an explosion of information regarding cytoskeletal regulation in T cells. Despite our progress in understanding these complex cytoskeletal regulatory networks, there are many remaining questions to be answered. We have yet to distinguish the compositional requirements for the diverse F-actin conformations (branched vs. linear F-actin) in the regulation of the various T cell functions, or the way in which these various filamentous architectures are integrated during T cell activation. Although we do have some understanding of how distinct actin-nucleating and -regulatory proteins cooperate to orchestrate the formation of complex morphological structures such as lamellipodia, filopodia, and microvilli, we still need to determine the unique spatiotemporal activation of each cytoskeletal regulator in order to better appreciate their relative contributions. For instance, while WAVE2 and WASP both stimulate ARP2/3-dependent nucleation, these two molecules demonstrate distinct subcellular distribution, and consequently, differential effects on ARP2/3-mediated processes. This distinction is likely a result of their dynamic sequestration into specific multimolecular regulatory complexes throughout the process of cellular activation. Also, despite the fact that the mechanisms by which chemokine receptors and integrins orchestrate morphological changes during migration are beginning to take shape, there are many questions that remain regarding signaling during chemotaxis. For example, although we have identified important RAC-GEFs during migration, candidate chemokine-activated CDC42- and RHOA-GEFs remain to be characterized. Moreover, the study of other small GTPases is of central interest, as well as a more in depth understanding of downstream effects mediated by these GTPases, which will likely shed light on unique aspects of cytoskeletal control over T cell function. Additionally, a more mechanistic understanding of how the polarity network dictates the subdomains of activated T cells should help us to better understand the functional relevance of this molecular compartmentalization. In this regard, what is the functional significance of T cell polarization during IS formation and how does the T cell cytoskeleton control this process? Despite the description of the IS nearly a decade ago, we still do not clearly understand the role of this structure in regulating T cell immune responses or how F-actin-regulatory proteins contribute to the segregation of molecules within the IS. The answers to these questions will inevitably be answered, and will provide important novel mechanistic insight into how these two cytoskeletal systems are regulated and how they shape the T cell response.

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CHAPTER 2

HLA Class II Transgenic Mice Mimic Human Inflammatory Diseases

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Abstract

Population studies have shown that among all the genetic factors linked with autoimmune disease development, MHC class II genes on chromosome 6 accounts for majority of familial clustering in the common autoimmune diseases. Despite the highly polymorphic nature of HLA class II genes, majority of autoimmune diseases are linked to a limited set of class II-DR or -DQ alleles. Thus a more detailed study of these HLA-DR and -DQ alleles were needed to understand their role in genetic predisposition and pathogenesis of autoimmune diseases. Although *in vitro* studies using class-II restricted CD4 T cells and purified class II molecules have helped us in understanding some aspects of HLA class-II association with disease, it is difficult to study the role of class II genes *in vivo* because of heterogeneity of human population, complexity of MHC, and strong linkage disequilibrium among different class II genes.

To overcome this problem, we pioneered the generation of HLA-class II transgenic mice to study role of these molecule in inflammatory disease. These HLA class II transgenic mice were used to develop novel *in vivo* disease model for common autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, insulin-dependent diabetes mellitus, myasthenia gravis, celiac disease, autoimmune relapsing polychondritis, autoimmune myocarditis, thyroiditis, uveitis, as well as other inflammatory disease such as allergy, tuberculosis and toxic shock syndrome. As the T-cell repertoire in these humanized HLA transgenic mice are shaped by human class II molecules, they show the same HLA restriction as humans, implicate potential triggering mechanism and autoantigens, and identify similar antigenic epitopes seen in human. This review describes the value of these humanized transgenic mice in deciphering role of HLA class II molecules in immunopathogenesis of inflammatory diseases.

1. INTRODUCTION

The Major Histocompatibility Complex (MHC) in the human, the HLA genes, has been mapped to the 6th chromosome. It comprises three classes of genes: class I, class II, and class III. The class I genes are HLA-A, -B, and, -C, which code for the 45,000 mw class I heavy chain. The class I molecules are formed by the association of the heavy chain with a 12,000 mw β2 m microglobulin. The class II genes, HLA-DR, -DQ, and -DP, code for a 33,000 mw alpha chain and a 28,000 mw beta chain. The class III genes code for complement components, tumor necrosis factor, etc. The HLA class I and class II molecules shape the T-cell repertoire in thymus. The class I molecules bind 8–9 amino acid self peptides in thymus and initiate the positive and negative selection of CD8 T cells. The class II molecules present 14–16 amino acid self peptides, resulting in positive and negative selection of CD4 T cells. High-affinity interactions of the T-cell receptor with MHC plus peptide result in negative selection, while the low-affinity interaction leads to positive selection. Positively selected, class I restricted CD8 T cells and class II restricted CD4 T cells in the periphery interact with MHC molecules presenting endogenously and exogenously processed peptides to initiate the immune response. The MHC genes are closely linked with rare recombination such that they are inherited enblock, referred to as ''linkage disequilibrium.'' The MHC genes are the most polymorphic genes in the mammalian genome. There are over 300 alleles of the DRβ gene, and over 100 alleles of the DQ and DP alpha and beta genes. DRα is a nonpolymorphic gene. Thus, every human has a unique combination of genes, referred to as a haplotype, since they inherit one set of alleles from each parent. The primary function of MHC molecules is to activate T cells and to help B cells to produce

antibodies to clear infectious agents and malignant self tissue, and protect autoimmunity by negative selection of autoreactive T cells.

Population studies have shown that predisposition to almost all human autoimmune diseases is linked to HLA genes, primarily the class II genes. Among these linkages, three MHC class II haplotypes stand out as the most autoimmune prone genes. These are HLA-DQ2/DR3, HLA-DQ6/DR2, and HLA-DQ8/DR4. These three haplotypes account for almost 90% of all autoimmune diseases. Considering there are potentially thousands of MHC class II haplotypes in the human population, it is indeed very intriguing why these three haplotypes are prone to autoimmunity. If these genes were ''bad'' genes, they would have been eliminated during evolution. On the contrary, these three haplotypes have the highest gene frequency, suggesting they are ''good'' genes and critical for the survival of the species. The current theory is that during thousands of years of evolution, these genes underwent multiple mutational events to make these molecules better able to present multiple peptides from infectious agents to activate large numbers of CD4 T cells to clear infections. Unfortunately, during thymic education these molecules also present multiple self peptides that positively select a high proportion of autoreactive CD4 T cells, which initiate autoimmunity. These class II genes also show extensive polymorphism such that there are several unique subtypes present in different geographical regions and in ethnic populations selected by environmental pressure. To understand the role played by HLA class II genes in predisposition, onset, progression, severity, and modulation of human inflammatory diseases, we embarked on a pioneering project to generate transgenic mice expressing human class II genes predisposed to human diseases. This chapter will describe the exciting new findings derived from these humanized disease models.

2. EXPRESSION AND FUNCTION OF HLA-DQ TRANSGENES IN MICE

Transgenic mice were generated by using genomic DNA of HLA-DQ6 and -DQ8 α and β genes isolated from human cell lines. The DQ6β transgenic mice were generated by microinjecting the linearized 40 kb cosmid clone containing 34 kb DQβ1*0601 genomic DNA isolated from HLA homozygous human B cell line AKIBA (Zhou *et al*., 1991). Thus, the target gene contained not only the coding sequences of the DQ6β chain, but also up to 10 kb of promoter region upstream and 10 kb of regulatory element downstream. This will enable the human gene to be expressed and regulated similar to human since they will have all the DNA binding sites, tissue specificity, and regulatory factors. The DQ6α*0103 gene construct

derived from the AKIBA cell line was microinjected to generate the DQ6α transgenic mice. The DQ8 α and β genes were isolated from cosmids containing a 30 kb DNA fragment and a 38 kb DNA fragment from the PRIES cell line (Okada *et al.*, 1985). The cosmid inserts were digested, purified, and microinjected into F1 mice. The transgene positive founders were identified by Southern blot analysis of tail DNA and subsequently mated to B10 mice to generate transgenic lines expressing DQ8 α and β genes (Cheng *et al.*, 1996). Tests with the progeny of the founder mice showed stable transmission of the gene transcripts. Northern blot analysis showed the expression of the transgenes in thymus, spleen, kidney, and lung, but not in liver and heart. Thus, we were successful in generating transgenic mice expressing the DQ6 and DQ8 α and β genes. Considering the early 1990s, when transgenic technology was still in its infancy, this was quite an accomplishment.

2.1. Expression and characterization of HLA-DQ molecules

To enable the pairing of the DQ α and β chains to express the molecule on the cell surface, transgenic mice expressing DQ6 α and β genes and DQ8 a and β genes were mated together, and offspring expressing both the α and β genes were selected for breeding the transgenic line. The offspring were screened for HLA-DQ expression by flow cytometry analysis of PBL using anti-HLA-DQ specific monoclonal antibodies Leu10 and IVD12. The DQ molecules were found in 20–30% of spleen cells and lymph node cells. The level of DQ expression can be markedly increased by *in vitro* stimulation with mouse recombinant IL-12 and LPS, suggesting expression on B cells. Thymic staining showed that the DQ molecules were mainly found in the medulla. The expression of endogenous mouse class II molecules remained similar to the parent strain.

2.2. DQ molecules can present viral superantigens in the thymus to delete T cells

The mouse genome contains several genes coding for the mammary tumor virus (MTV). These genes code for several viral antigens. Some of them have been designated ''superantigens'' since they interact and activate a large number of T cells (Choi *et al.*, 1991). These viral superantigens do not go through antigen processing and bind outside the MHC class II groove, and the MHC bound to a viral superantigen can interact with the T-cell receptor Vβ chains. The MTV genes are expressed in thymus. Since MTV superantigens are endogenous molecules, they will be recognized as self antigens. Their high-affinity interaction with the T-cell receptor Vβ chain will result in the clonal deletion of those T cells. This may be a mechanism by which the viral antigens protect themselves from

elimination. Prior to the identification of the MTV genes and their products, these genes were referred to as the ''minor lymphocyte stimulating'' locus (MLS). We tested whether the DQ transgenic molecules expressed in thymus can present viral superantigens and interact with the T-cell receptor Vβ chain. T cells expressing TCR Vβ genes Vβ5, Vβ6, Vβ8, and Vβ11 were eliminated in the DQ positive mice compared with their negative full-sibs, suggesting that transgenic DQ molecule may play a role in the thymic clonal deletion of T cells expressing specific TCR Vβ genes (Zhou *et al*., 1992). Thus, the DQ transgenic molecules were functional in the mouse thymus.

2.3. DQ restricted antigen specific T cells missing in the periphery

Next, we needed to determine whether we could generate HLA-DQ restricted antigen specific T-cell response in the periphery. In thymus, viral superantigens, regardless of their antigen specificity, bind to class II molecules outside the pocket and delete selected T cells expressing certain TCR Vβ genes. We wanted to know whether HLA-DQ molecules bind self-peptides to positively select DQ restricted T cells, which migrate to the periphery. The transgenic mice were immunized with several antigens that had been implicated in autoimmune diseases, such as type II collagen in arthritis, myelin basic protein in MS, and GAD in diabetes. The mice generated T-cell response to most of the antigens. We tested the HLA restriction of the T-cell response by blocking with anti-DQ specific monoclonal antibodies. None of the T-cell responses were blocked by anti-DQ antibodies while all the T-cell responses were blocked by antibodies against the endogenous mouse class II molecules H2-A. This suggested that all the T cells in these mice were restricted by endogenous mouse class II molecules and not by the transgenic human DQ molecules. This was confirmed when DQ restricted peptides did not generate T-cell response. Thus, the CD4 T cells in thymus were ignoring the DQ molecules, and primarily interacting with the endogenous class II molecules to generate the peripheral T-cell repertoire. There were two possibilities. Either the mouse CD4 T cells are incapable of interacting with human class II molecules or the mouse CD4 T cells had a higher affinity for endogenous mouse class II molecules and low affinity to the human transgenic DQ molecule. The CD4 T cells interact with class II molecules in the second domain of β chain between residues 110 and 140. We found that this sequence was identical between the human DQ molecules and mouse H2-A molecules. Thus, we decided that the mouse CD4 molecules can indeed interact with human DQ molecules, but the affinity of the mouse CD4 molecules for mouse class II molecules was several fold

higher, resulting in very few DQ restricted CD4 T cells in the periphery. We had only one option, to get rid of the mouse class II molecules.

2.4. Generation of HLA/DQ positive, H2-A class II negative (Aβ°) mice

We decided to express the HLA class II transgene in animals rendered deficient of mouse class II expression through gene targeting (Cosgrove *et al.*, 1991). Expression of only the human class II molecules in thymus should lead to the development of DQ restricted CD4 T cells. In these mice the CD4 T cells do not have a choice, either they interact with human class II and live or they will die. With this hypothesis in mind, we introduced the human DQ8 and DQ6 genes into mouse class II deficient H2-Aβ° mice by mating. The Aβ gene in this mouse was mutated and is nonfunctional. Since these mice were of the $H2^b$ haplotype they had a natural nonfunctional Eα gene. These knockout mice do express the endogenous mouse class II Aα and Eβ gene. Mice expressing the HLA-DQ genes and lacking endogenous mouse class II expression were intercrossed to produce the DQ6.Aβ° and DQ8.Aβ° mice. These mice expressed the DQ molecule in up to 25% of the peripheral blood lymphocytes. No endogenous mouse class II A or class II E molecules were detected. Further studies also showed that no hybrid HLA-DQβAα or HLA-DQαEβ molecules were generated in these mice.

2.5. Selection of peripheral CD4 positive T cells in HLA-DQ.Aβ° mice

A hallmark feature of class II deficient H2-Aβ° mice is a paucity of peripheral CD4 positive T cells. In general, H2-Aβ° animals contained less than 3% of CD4 positive cells within the lymph nodes and the majority of these cells expressed the CD44 (PGP-1) antigen. To determine if expression of the human HLA-DQ molecules in H2-Aβ° mice induces selection of CD4 positive T cells, lymph node cells were analyzed. HLA-DQ transgenic mice expressing only the DQα genes or the DQβ genes did not show expansion of the CD4 T-cell population. Mice expressing both the DQ α and β genes showed between 10% and 15% CD4 T cells, suggesting positive selection of CD4 T cells in the context of the HLA-DQ molecules (Nabozny *et al.*, 1996). Analysis of Vβ TCR expression within the CD4 positive population showed that the DQ positive Aβ° mice expressed a variety of Vβ TCRs. In addition, distinct differences in the level of some CD4 positive/Vβ TCR positive cells such as Vβ4, Vβ5, Vβ6, Vβ8.2, and Vβ14 were detected between the DQ positive and DQ negative mice, suggesting positive and negative selection. The DQ

transgenic mice were able to generate DQ restricted T-cell response to Type II collagen.

2.6. HLA-DQ mice present bacterial superantigens to activate T cells

Bacterial superantigens such as SEB toxins from *Staphylococcal aureus* bind to MHC class II molecules outside the antigen binding groove and contact T cells with specific TCR Vβ chains and activate many T cells. Studies have shown that while SEB binds with high affinity to human class II molecules to activate a substantial number of T cells, the binding of SEB to mouse class II molecules are much lower and activate only a small proportion of these cells. To determine whether the HLA class II molecules expressed in our transgenic mice, function similar to the class II molecules in the mouse or to the human class II molecules, the DQ6 and DQ8 transgenic mice were stimulated with SEB and MAM, a mycoplasma superantigen. SEB and MAM induced *in vitro* proliferation of splenocytes from DQ6 and DQ8 transgenic mice, several fold higher than B10 mice (Cole *et al*., 1997). These studies clearly showed that with reference to bacterial toxins, the HLA class II molecules in the transgenic mice function very similar to the way they function in the human system, and not like the endogenous mouse class II molecules.

2.7. HLA-DQ.Aβ° transgenic mice predict epitopes on *Plasmodium falciparum* for production of vaccines against malaria

To develop a vaccine for malaria that would have a broad use in the world, well-defined conservative epitopes that induce T-cell responses across many haplotypes need to be identified (Pimtanothai *et al*., 2000). Studies in Dr. Carolyn Hurley's lab (Georgetown University) had shown four conserved T-cell epitopes using computer algorithms to predict potential T-cell epitopes from conserved regions of blood stage proteins with vaccine potential. One of those peptides, Abra14, showed high level binding to many DQ molecules. To determine whether the binding studies correlate with T-cell response to the peptides, they injected HLA-DQ8 transgenic mice with various peptides (Pimtanothai *et al*., 2000). Only Abra14 induced proliferative responses in the DQ8 mice. Immunization of this peptide in the DQ8 mice also resulted in production of high levels of Th1 cytokines such as IFN-γ, IL-2, and IL-12. Thus, the studies with DQ8 transgenic mice implicate Abra14 as a major epitope for inclusion in a vaccine construct for malaria.

3. HLA-DQ TRANSGENIC MICE AS MODELS FOR HUMAN ALLERGIES

Nearly 20% of the general population is genetically predisposed to developing hypersensitivity to various environmental stimuli. The HLA molecules have been implicated in several measures of the allergic phenotype and asthma in many studies. Strong relationships between the immune response to several highly purified allergens and specific HLA class II genes have been published. Several murine models of antigen-induced pulmonary eosinophilia and airway hyper-reactivity (AHR) have been developed to study the role of B cells, T cells, mast cells, NK cells, dendritic cells, Ig and its receptor, cytokines, leukotrines, and costimulatory molecules. In many of these animal models the antigen that induces asthma is ovalbumin, not a human allergen. Second, these animals have mouse MHC class II molecules. We initiated extensive studies to understand the role of human HLA class II molecules and their response to known human allergens.

3.1. HLA-DQ restricted antigenic epitopes on house dust allergens

House dust mites (HDM) are a significant source of indoor allergens causing symptoms ranging from atopic dermatitis and extensive asthma, to allergic rhinitis in 10% of individuals. The mite *Dermatophagoides pteronyssines* (DerP), one of the causative agents, is the most frequently implicated allergen in patients with respiratory allergy (O'Brien *et al.*, 1992). Most of the allergy response in HDM allergic patients is directed against the group 2 allergens – DerP2. Recombinant DerP2 is a potent inducer of immediate hypersensitivity reactions in HDM allergy patients. Even though potential T-cell epitopes on DerP2 molecules have been identified using human lymphoid tissue, it is complicated due, in part, to the heterozygosity of the human population, and the difficulty and expense of obtaining and maintaining DerP specific human T-cell lines.

To determine whether HLA-DQ transgenic mice can respond to a human allergen, DerP extracts were immunized and the draining lymph node cells were challenged *in vitro* (Neeno *et al.*, 1996). A strong DQ restricted CD4 T-cell mediated response was detected. RNA was isolated from the challenged cells and analyzed for the presence of lymphokine mRNA. Large amounts of IL-2 and IFN-γ were identified with lesser amounts of IL-4 and IL-6. Thus, it can be concluded that the response to DerP2 is mediated by both Th1 and Th2 cells.

To identify specific DerP2 epitopes processed and presented by DQ8 molecules, draining lymph nodes from mice injected with the whole DerP extract were challenged *in vitro* with overlapping synthetic peptides

representing the 129 amino acids constituting the major HDM allergen. Three peptides showed high T-cell response identifying potential epitopes in sequence 61–80, 91–110, and 111–129. There were also some minor epitopes. To further confirm the presentation and response to these three epitopes, the peptides were immunized into the mice and tested for T-cell proliferation. All three peptides generated high T-cell response. The DQ8 molecules present primarily three C-terminal peptides. On the other hand, the DQ6 transgenic mice identified two new epitopes in regions 11–30 and 21–40 in addition to the 50–70 peptide. Thus, each HLA-DQ molecule presents unique determinants of DerP2 antigen. This demonstrated that the HLA class II transgenic mice can be used to identify potential antigenic epitopes on human allergens to develop altered peptide ligands to desensitize allergic patients.

3.2. Short ragweed allergen induces eosinophilic lung disease

The association of HLA haplotypes and ragweed allergy was the first human immune response gene to be recognized (Levine *et al.*, 1972). A HLA restriction of Ig reaction to antigen 5 is well documented. Eosinophils play an important role in the pathogenesis of allergic airway inflammation. We investigated the genetic and molecular basis of immune responsiveness to short ragweed (*Ambrosia artemisiifollia*) and giant ragweed (*Ambrosia trifida*) in DQ6.Aβ° and DQ8.Aβ° mice (Chapoval *et al.*, 1998). Panels of overlapping peptides spanning the *Amb a*5 and *Amb t*5 Ags were synthesized. Mice were immunized with whole short ragweed (SRW) extract or individual peptides s.c. and lymph node cells (LNC) were challenged *in vitro*. Strong T-cell responses to SRW extract were measured in both HLA-DQ transgenic mice, while controls were unresponsive. IL-5 and IL-10 were the primary cytokines produced by *in vitro* challenged LNC of SRW-primed transgenic mice. HLA-DQ6-restricted T-cell responses were detected to three peptides of *Amb t5* (1–20, 11–30, 21–40) and two peptides (1–20 and 11–30) on A*mb a5*. In contrast, LNC of HLA-DQ8 mice did not recognize peptide 11–30 of *Amb t5* Ag, but recognized several *Amb a5* determinants.

We used the ragweed antigen extract to generate a humanized mouse model for asthma (Chapoval *et al.*, 1999). Macrophages and B cells purified by broncho-alveolar lavage (BAL) expressed HLA-DQ antigens. HLA-DQ6 and HLA-DQ8 mice were sensitized by intraperitoneal injection of 40 mg of short ragweed extract. A booster injection was given 7 days later. On day 14, mice were challenged intranasally 2 times, 6 h apart by application of a dialyzed extract in the nostrils under light anesthesia. Mice were euthanized 48 h after the last intranasal challenge and BAL fluids were collected. The total number of cells increased six-fold in the DQ6 mice and three-fold in the HLA-DQ8 transgenic mice compared with that in controls. There was also significant infiltration of eosinophils into

the DQ6 trasngenic mice than those into the HLA-DQ8 transgenic mice. B6 mice had very little eosinophilic infiltration. This demonstrated that the expression of HLA-DQ6 gene causes the eosinophilic infiltration in the BAL of these mice sensitized to short ragweed. *In vivo* treatment of the HLA-DQ6 mice with anti-DQ monoclonal antibodies inhibits the airway eosinophilia, confirming the role of HLA-DQ molecules. A significant increase in total protein level and IL-5 was observed in the HLA-DQ6 mice when compared with controls.

The extent of the allergic airway inflammation induced by SRW exposure was further studied by histological analysis. The lungs of SRW treated HLA-DQ6 mice contained multifocal dense inflammatory infiltrates consisting primarily of eosinophils. Epithelial damage including hypertrophy, sloughing of epithelial cells, and increased spaces between them was observed. The control mice showed no inflammation. Systemic anti-DQ monoclonal antibody treatment of DQ6 mice significantly decreased the lung inflammation. Massive perivascular and peribronchial eosinophilic infiltration was found in HLA-DQ6 mice. Using high-end magnification, margination and migration of eosinophils through the blood vessel wall to the epithelial and the granular release of airway mucosa in the epithelium were detected. As supporting data for eosinophil activation, granular release and positive staining of infiltrating macrophages for major basic protein as a result of this uptake was found. The lung disease resulted in decreased expression of the HLA-DQ molecules in lung tissue. The T cells infiltrating the bronchial tissues were found to be CD4 T cells. HLA-DQ mice developed a strong airway hyper-responsiveness 48 h after intranasal challenge with allergenic extract, suggesting the requirement of specific DQ molecules in the induction of AHR *in vivo*. The SRW specific antibodies in HLA-DQ6 mice were composed mainly of IgG1, IgG2b, and IgG3 whereas in controls, only IgG3 was found, confirming that these mice generate Th2 dependent antibody responses. They also have anti-ragweed IgM and IgG3 antibodies. SRW boosted HLA-DQ6 mice showed a significant increase in total IgE compared with the pre-immune controls and was more pronounced 48 h after challenge. Thus, we developed an *in vivo* model for human allergies using short ragweed extract as a natural airborne allergen instead of ovalbumin, which has been used in most studies involving murine models of asthma.

3.3. Cockroach allergen-induced eosinophilic airway inflammation

Cockroach (CR) allergen has long been recognized as a major indoor allergen, particularly in inner city children with asthma. The raising morbidity and mortality of asthma in this population may be explained by the high levels of cockroach allergen exposure in their homes. The

German cockroach (*Blattella germanica*) is prevalent particularly in large, crowded cities in the Southern United States and in tropical countries throughout the world (Ledford, 1994). Inhalation of German CR allergen induces Ig antibody production in the development of asthma in genetically predisposed individuals. We initiated studies to develop an asthma model for an indoor allergen, German cockroach. The major allergen of German cockroach is designated BLA-G5 (Stewart and Thompson, 1996). The mice were either challenged with the CR extract or individual synthetic peptides representing the BLA-G5 (Papouchado *et al.*, 2000). Strong T-cell responses to CR extract was detected in the DQ6 and DQ8 transgenic mice. We found that expression of the human CD4 transgene in the context of a mouse CD4 knockout resulted in a much higher response. Thus, the rest of the studies were done in HLA-DQ8 and DQ6 transgenic mice expressing the human CD4 in the absence of the mouse CD4. Using overlapping synthetic peptides, we identified the T-cell response primarily to two epitopes—residues 91–110 and residues 161–180.

Using the same procedures that have been used for short ragweed, we developed an *in vivo* model for cockroach allergen (Papouchado *et al.*, 2001). Mice were immunized and challenged intranasally to cockroach allergen extract. The mice developed bronchio-alveolar lavage fluid eosinophilia and pulmonary eosinophilia. This was accompanied by an increase in total protein levels of IL-5 and IL-13. There were also elevated levels of cockroach specific serum IgG1 and serum IgE. Histological analysis revealed peribronchial and perivascular eosinophilic inflammation in cockroach treated mice (Fig. 2.1). Other pathologic changes in the airways were epithelial cell hypertrophy and mucous production. Treatment with anti-DQ monoclonal antibody significantly reduced pulmonary and BAL eosinophilia in cockroach allergen sensitized mice. Control mice showed no disease.

In conclusion, these studies have shown that HLA-class II molecules play an essential role in the development of allergic diseases and related manifestations of allergic airway disease, which are significantly reduced after treatment with anti-DQ mAb. Therefore, our transgenic mice offer the opportunity to investigate the role of distinct HLA molecules in allergen sensitivity and may be useful for developing therapeutic strategies against allergic diseases, including asthma.

4. EXPRESSION AND FUNCTION OF HLA-DR TRANSGENIC MICE

4.1. HLA-DR2 mice

HLA-DR2 transgenic mice were produced by microinjecting into (SWRxB10.M)F2 embryos a linearized 34 kb DNA fragment containing the entire DRB1*1502 gene isolated from the HLA homozygous B cell line

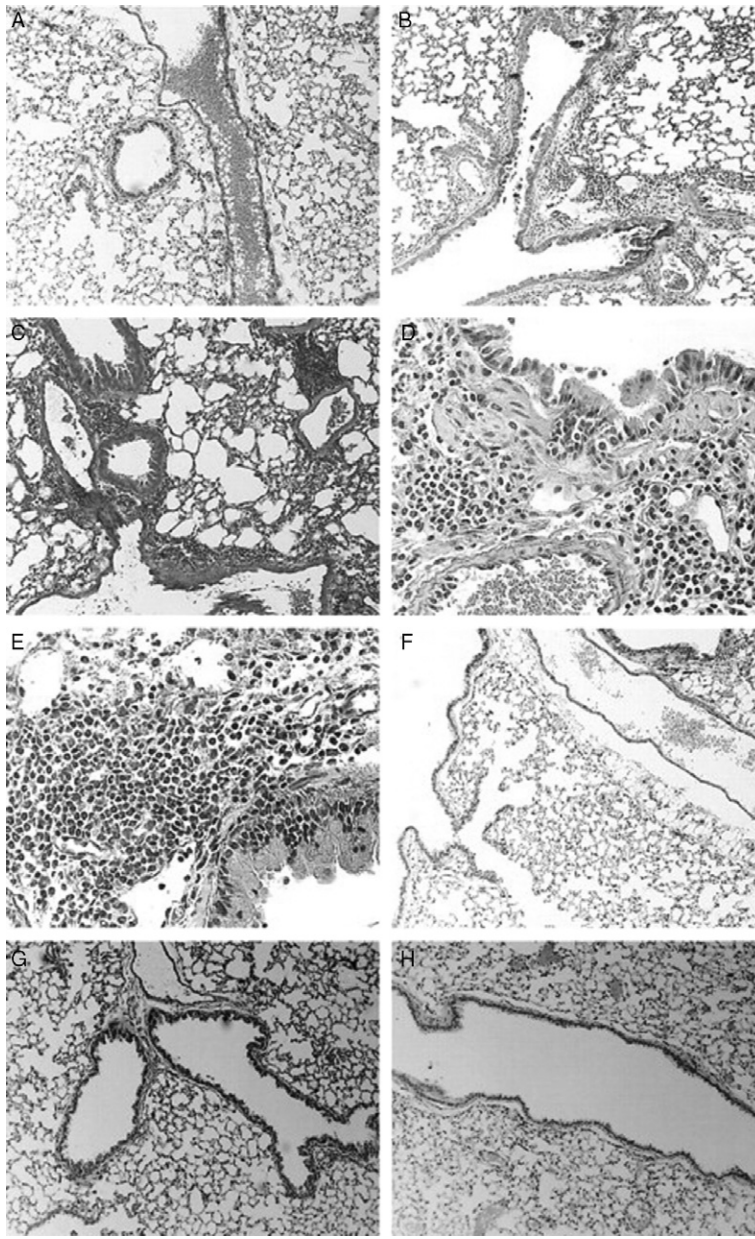

FIGURE 2.1 Cockroach allergen induces increased pathology in HLA transgenic mice. Formalin-fixed lung sections taken from PBS-treated HLA-DQ8/hCD4$^+$ mice (A, ×10) represented normal lung histology. In contrast, lungs taken from CRa-treated HLA-DQ8/hCD4$^+$ (B, ×10) and HLA-DQ6/hCD4$^+$ (C, ×10) tg mice showed a dense peribronchial and perivascular inflammatory infiltrate. Higher magnification (×40) revealed that

AKIBA (Gonzalez-Gay *et al.*, 1996). The founders were identified by PCR reaction and backcrossed to B10 mice to generate two lines of DR2 transgenic mice—a congenic B10.DR2 line and an intercross DR2 line. The DRB1*1502 transgenic mice were then mated either to an $E\alpha^k$ transgenic mouse or to a DRα transgenic mouse for cell surface expression. DRβ chain can pair with either the mouse Eα or the DRα since they are 99% homologous. Expression on peripheral blood cells was done by flow cytometry using a DRB1 specific L227 monoclonal antibody. Approximately 40% of PBL expressed the DR2 molecule. Since the mice used to produce the DR2 transgenic mice lack endogenous mouse class II H2-E genes, the DR2 molecule was expressed and are functional in these mice. Even though these mice expressed the $H2\text{-}A^f$ gene products, we could generate DR2 restricted T-cell response in these mice.

4.2. HLA-DR3 mice

The DR3 transgenic mice were generated in Dr. Gunther Hammerling's laboratory (Strauss *et al.*, 1994). A DRα genomic fragment and a DRB1*0301 gene fragment were co-injected into F2 embryos and backcrossed to B10.M mice. The transgenic mice were embryo derived in the Mayo pathogen-free mouse facility and bred as B10.M (DR3) line.

4.3. HLA-DR4 mice

We first tried to generate a DR4 transgenic mouse using a DRB1*0401 gene isolated from a human cosmid generated from PRIES cell line (Okada *et al.*, 1985) The mice did not express the DRB1*0401 gene. We found that the DRB1*0401 gene in the cosmid provided to us was truncated such that it did not have sufficient upstream promoter regions for proper orientation and expression of the gene. Since we could not find any other genomic fragment containing an intact DRB1*0401 gene, we decided to generate a cDNA construct. The DRB1*0401 gene cDNA was generated by the RT-PCR method using mRNA isolated from the PRIES cell line. Since cDNAse can be mutated, we decided to generate a DR4

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the infiltrate contained predominantly eosinophils, and there were epithelial cell hypertrophy and mucus hyperproduction (D, E). Minimal inflammation consisting primarily of mononuclear cells was detected in tg mice expressing only HLA-DQ8 molecule (without CD4) (F, ×10). A considerable reduction in the degree of inflammation in both HLA-DQ8/$hCD4^+$ (G, ×10) and HLA-DQ6/$hCD4^+$ (H, ×10) is observed after treatment with anti-DQ mAb. (Reproduced from *J Immunol*, Papouchado *et al.*, 2001, 167, 4627, with permission from *The American Association of Immunologists, Inc.*)

gene construct, which would have an optimum interaction with mouse CD4 molecule. The interaction sites of CD4 on the MHC class II molecule was mapped between amino acid 137 and 143 in the β2 domain in the mouse. Studies with the human DR4 molecule suggested that residue 110 in the class II b domain was also critical. Comparing the amino acids in this region between human DQ, DR, and mouse H-2A and H-2E, we decided that residues 110 and 139 may be the key to better interaction of DR molecules with mouse CD4. We introduced two substitutions into the DR4β cDNA gene construct. The Gln at position 110 and Lys at position 139 were substituted by Asn and Thr, respectively. The specific site-directed mutagenesis was done by using the overlap PCR method (Ho *et al.*, 1989). The altered DR4β cDNA was cloned into the pD01-5 expression vector downstream of the H2-Eα promoter and rabbit β-globulin intron (Kouskoff *et al.*, 1993). The gene construct was double-digested to remove the plasmid sequence and microinjected into fertilized eggs from the (SWRxB10)F1 mice. Mice carrying the transgene were identified with Southern blot analysis and two lines were generated—an intercrossed line and a B10 congenic line. Differences were found in the TCR Vβ expression of $CD4^+$ T cells in the transgene positive mice, indicating positive and negative selection had occurred. Thus, the altered DR4 molecule was interacting efficiently with the mouse CD4 T cells. We were able to generate DR4 restricted T cells specific for MBP and GAD65 showing that the DR4 molecule in these mice was functional. Two other laboratories also generated viable DR4 transgenic mice by substituting the α2/β2 domain of the HLA-DR gene with a mouse H-2E α2/β2 domain (Fugger *et al.*, 1994; Ito *et al.*, 1996). In the second study, the human CD4 gene was introduced into the HLA-DR4 transgenic mice to obtain DR4 restricted T-cell response. In both cases, the DR4 restricted T-cell response was much lower in frequency and magnitude.

4.4. DR.Aβ° mice

The DR transgenic mice expressed the DR molecules and were able to generate DR-restricted T cells against foreign antigens and peptides. Since these mice are on the B10.M background, the DR molecules replace the nonfunctional endogenous H2-E molecules. But, these mice do express the mouse H2-A molecule which not only plays a role in the shaping of the T-cell repertoire in thymus but can also present antigens in the periphery. Thus, the immune responses in these mice are mediated both by mouse class II H2-A and human class II DR. It would be very difficult to evaluate the role of HLA-DR molecules in a disease model in these mice due to the interference from endogenous mouse class II molecules. We introduced these DR transgenic mice into our class II knockout Aβ° mice. The DR3.Aβ° mice had very good expression of DR3 molecules, and we

were able to generate DR3-restricted antigen-specific T cells in these mice. On the other hand, in both the DR2 transgenic mice and the DR4 transgenic mice, the expression of the DR molecules were much lower. On further analysis, we found that the DR2 and DR4 transgenic mice were expressing a hybrid H2-Eβ/DRα molecule since the Aβ° knockout mice express the endogenous Eβ gene. It was also very difficult to generate DR2- and DR4-restricted T cells in these mice. On the other hand, we detected very low amounts of the Eβ/DRα molecule in the DR3 transgenic mice. This is probably because in the DR3 transgenic mice the DR3 gene have a much higher copy number and the DRb molecules are more dominant than the mouse Eb molecules. Thus, the DR2.Aβ° and DR4.Aβ° mice will not work as a model for autoimmune diseases because of the expression of the endogenous H2-Eβ molecule which is known to be protective in many autoimmune diseases.

4.5. Mice lacking all conventional class II genes

While we were trying to figure out how we were going to generate functional DR2 and DR4 transgenic mice for autoimmune disease models, Drs. Chris Benoist and Diane Mathis came to our rescue again. They generated a new class II knockout mouse ($MHC^{\Delta/\Delta}$) which lacked all endogenous mouse class II genes by deleting the entire 80 kb class II region engineered by homologous recombination and Cre recombinase-made excision (Madsen *et al.*, 1999). We mated all of our DR transgenic mice to the MHC $^{\Delta/\Delta}$mice. These mice will be referred to as the AE° mice. The DR.AE° mice developed normally and had no gross phenotypical abnormalities. Thymus, spleen, and lymph nodes were normal in size and cell numbers, indicating the DR class II transgenes in these mice act similarly to mouse endogenous class II genes in maintaining T-cell homeostasis in thymus and periphery. Lymphocytes from spleen, thymus, and lymph nodes were examined for expression of DR by flow cytometry. The spleen had a very high expression, with the lymph nodes intermediate, and thymus had a low expression. The cellular architecture of the cortex and medulla (thymus), red and white pulps in the spleen, and B and T cell areas of the lymph nodes were similar to wild-type mice. Cell type analysis showed that there was normal T-cell differentiation to both CD4 and CD8 lineage with normal numbers of CD4/CD8 cells in different tissues. The CD4 to CD8 ratio was also comparable to other strains of mice together with the diverse T-cell Vβ repertoire. T cells expressing Vβ3 and Vβ17 were absent, while Vβ5, Vβ6, Vβ8, Vβ10, and Vβ11 were preferentially expressed. The higher numbers of Vβ6 and Vβ8 cells confirmed the absence of the endogenous mouse H2-E molecules. Other cell types such as B cells, macrophages, and DC were also similar. MHC class II expression was also detected in lower levels in the liver and

kidney, but not in the brain or spinal cord tissue sections. Expression of DR2 and DR4 increased considerably in the AE° mice. We were able to generate DR-restricted antigen specific T cells in these mice. Thus, the DR molecules in these mice were functional.

4.5.1. The new AE° mice express HLA class II on T cells

The major differences between human class II molecules and mouse class II molecules are that human class II molecules are expressed on T cells, especially activated T cells, while mouse T cells do not express class II. In our analysis of the DR.AE° mice, we were surprised to find expression of the DR molecules on CD3 cells, suggesting expression on T cells. We analyzed the expression of the DR on CD4 T cells by both flow cytometry as well as confocal laser scanning microscope. We observed class II expression on CD4 T cells from DR. AE° mice while no expression of class II was found on CD4 T cells from B10 mice or DR.Aβ° mice. Approximately 7–15% of CD4 T cells expressed class II on their cell surface. To further confirm the expression of HLA-DR on CD4 T cells, immunofluorescent staining was done on frozen tissue sections from thymus and spleen. CD4 T cells were found to overlap with HLA-DR (Fig. 2.2A). Similarly, DQ molecules were also expressed on T cells in DQ.AE° mice. The level of expression is increased when the T cells from these mice are activated *in vitro* with superantigen SEB. The level of DQ and DR expression also increased on memory T cells.

To exclude the possibility that class II on T cells is acquired from surrounding cells, we analyzed the HLA-DR expression at mRNA level by RT PCR. Splenocytes in naïve DR.AE° mice were plated on Petri dishes to remove adherent monocytes and DC population, followed by isolation of $CD4^+$ T cells from nonadherent populations. RT-PCR analysis showed that HLA-DR was expressed at the mRNA level on $CD4^+$ T cells from DR. AE° mice, but not on $CD4^+$ T cells from DR.Aβ° mice. Thus, the RT-PCR demonstrated that class II genes are expressed on CD4 T cells (Fig. 2.2B). The expression of DR molecules on mouse T cells is the intrinsic property of the DR gene. The mouse class II genes may have an inhibitory signal for expression on T cells. Thus, when human class II is expressed in Aβ° mice, presence of mouse Aα and Eβ gene might inhibit expression on T cells. Transgenic mice generated with human class II cDNA constructs have lower expression on T cells, suggesting part of this signal may be from the upstream or downstream of the coding sequence.

4.5.2. $CD4^+$ T Cells expressing HLA-DR can present peptide

Since CD4 T cells express both TCR and class II on their cell surface, they should be able to present antigen. To test this hypothesis, lymph node $CD4^+$ T cells were isolated from mice immunized with various peptides and tested *in vitro* for their ability to present antigen. $CD4^+$ T cells were

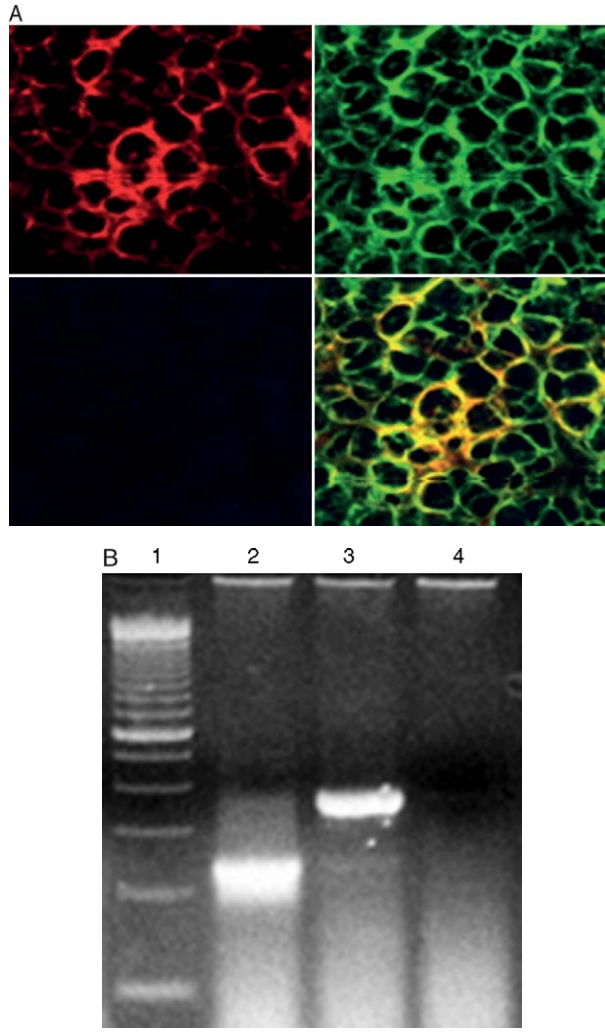

FIGURE 2.2 MHC class II is expressed on mouse $CD4^+$ T cells. (A) A representative fluorescence micrographs showing expression of class II on murine $CD4^+$ T cells in the spleen of naïve DR3.AEo. Frozen sections were stained with FITC-HLA-DR and Texas red-anti-CD4. Four panels within the micrograph show that the subset of splenocytes co-expressed both HLA-DR as well as murine CD4 T-cell surface marker. (B) MHC class II (HLA-DR) was expressed in murine $CD4^+$ T cells at mRNA levels. $CD4^+$ T cells were sorted using FACS sorter and the resultant $>$ 96% pure CD4 T-cell population (1×10^6 cells/well) were analyzed for expression of HLA-DR and GADPH mRNA expression by RT-PCR. Lane 1, 100-bp DNA ladder; lane 2, GADPH; lane 3, HLA-DR ($CD4^+$ T cells from DR3.AEo mice); and lane 4, HLA-DR ($CD4^+$ T cells from DR3.Abo mice). (Reproduced from *Eur J Immunol,* Mangalam *et al*., 2006, 36, 3656, with permission from *Wiley-VCH Verlag GmbH & Co. KGaA*.)

isolated from nonadherent lymph node cells to exclude the possibility of contamination from monocytes and dendritic cell populations. $CD4^+$ T cells from DR.AE° mice, but not from DR.Aβ° or B10 mice, presented DR-restricted peptides, and induced strong T-cell proliferation. This response was mediated through HLA-DR as blocking of class II leads to abrogation of T-cell response. This was antigen specific since only the specific DR-restricted peptides were presented. Although the DR + T cells can present peptides, they were unable to present whole antigens, suggesting a defect in processing of antigens.

5. HLA CLASS II TRANSGENIC MICE IDENTIFY THE SAME DETERMINANTS OF *M. TUBERCULOSIS*-DERIVED PROTEINS AS HUMANS

To demonstrate that the HLA class II transgenic mice reproduce the human immune response, we collaborated with Drs. Geluk and Ottenhoff (Leiden, Netherlands) to study *Mycobacterium tuberculosis*-derived proteins for epitope mapping. HLA class II polymorphism plays a major role in dictating the specificity and magnitude of the human CD4 positive response to mycobacterial antigens (Ottenhoff *et al.*, 1985). For example, HLA-DR3 is associated with the high response to tuberculosis and leprosy with strong T-cell activity to mycobacterial antigens *in vitro* and *in vivo* (Ottenhoff *et al.*, 1991). Importantly, HLA class II-restricted human T cells frequently respond to the BCG heat shock protein HSP65 (Ottenhoff *et al.*, 1988). HLA class II restricted T cells in the human identify specific epitopes on HSP65. Human DR3 restricted T cells recognize one epitope on HSP65, P1-20. To determine whether HLA class II transgenic mice identify the same epitopes of HSP65 from *M. tuberculosis* proteins, they were injected with BCG. The DR3 and DQ8 transgenic mice showed T-cell response primarily to the HSP65 of BCG, similar to that shown by humans (Geluk *et al.*, 1998a). Epitope mapping of mouse DR3 restricted T-cells reactive to HSP65 showed identification of one epitope P1-20, the same epitope recognized by DR3 restricted T cells from tuberculosis/leprosy patients (Table 2.1). Among the 54 peptides tested, P1-20 is the only high binding peptide by DR3. Low binding peptides, 41–60 and 171–190 did not induce T-cell proliferation in the DR3 transgenic mice similar to what was previously observed in human patients. Thus, the DR3 molecule in the transgenic mice binds the same high-affinity peptide as human DR3 molecules to initiate the T-cell response. These results imply that the major MHC class II antigen processing pathways (endosomal, lysosomal proteases such as cathepsin) and peptide/loading systems (Ii chain, H2M/HLA-DM in mice) can cooperate efficiently across the species barrier with human HLA molecules. Thus,

TABLE 2.1 Comparison of T-cell response and HLA-DR3 binding of hsp65-derived peptides in human and HLA-DR3.Aβ° mice (Geluk *et al.*, 1998b)

	Recognition by HLA-DR3 restricted T cells*		
Hsp65	Humans	HLA-DR3.Aβ° mice	HLA-DR3 binding affinity, IC_{50} μM**
1–20	+ + +	+ + +	0.06
41–60	−	−	0.3
511–530	−	−	0.7
201–220	−	−	1.3
281–300	−	−	1.7
211–230	−	−	2
241–260	−	−	2
481–500	−	−	2.6
261–280	−	−	5
81–100	−	−	6.5
181–200	−	−	9
321–340	−	−	14
191–210	−	−	19
341–360	−	−	20
401–420	−	−	20
441–460	−	−	22
491–510	−	−	22
301–320	−	−	25
171–190	−	+	26
331–350	−	−	28
31–50	−	−	30
All other peptides	−	−	>100

* Results for hsp-65-reactive T cells are shown for reference only. + + + indicates SI (stimulation index) 4; + indicates $2 \leq SI \leq 3$; − indicates $SI < 2$.
** Peptide binding affinity (IC_{50}) was defined as high affinity (<1 μM), intermediate-affinity (1–10 μM), weak affinity (10–100 μM), or nonbinding (>100 μM).

the processing of an antigen, binding of a peptide, and T-cell activation are identical in mice and man.

T cells from BCG immunized HLA.DQ8.Aβ° mice also responded to BCG and to HSP65, but did not recognize peptide P1-20 (Geluk *et al.*, 1998a). Instead, they identify six other peptides covering three different regions comprising amino acid 171–200 (a low binder for DR3) 311–340, and 411–440. Thus, three naturally processed antigenic determinants are antigenic in the context of DQ8 molecule, predicting that DQ8 restricted T cells in human *M. tuberculosis*-infected patients would recognize these same three epitopes. To determine what would happen in a

heterozygous individual, double transgenic DQ8/DR3 mice were tested for response to HSP65. The humanized mice recognized all four epitopes recognized by the two parents, suggesting there is no competition at the level of T-cell epitope formation.

Another mycobacteria-derived protein AG85 has been proposed as an important candidate for vaccine production. AG55 is frequently recognized by human T cells. DR3.Aβ° mice immunized with BCG also showed response to AG85 (Geluk *et al.*, 1998b). The epitopes for AG85 protein were mapped by using 28 peptides covering the entire *M. tuberculosis* 85B sequence. T-cell reactivity was observed to only one single peptide epitope, P51-70, which also happened to be the highest binding epitope for DR3. To determine whether P51-70 is also recognized by DR3 restricted AG85 reactive human T cells, four AG85 reactive T cell lines from three HLA-DR3 positive and one HLA-DR3 negative individuals were generated by stimulation with *M. tuberculosis*, and were tested for proliferation to all overlapping 85B peptides. All the DR3 positive T cell lines efficiently recognized B51-70. The HLA-DR3 negative T cell line responded to a different epitope B71-90. Thus, HLA-DR3 restricted human T cells from vaccinated or infected individuals recognize precisely the same epitope as HLA-DR3 restricted T cells from BCG or HSP65 immunized DR3.Aβ° mice, showing that HLA transgenic mice can be used as an efficient model to define naturally processed epitopes for human T cells.

6. COLLAGEN-INDUCED ARTHRITIS—MODEL FOR HUMAN RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation of synovial lining of joints. This disease has a worldwide distribution, affecting approximately 1% of the population, and leads to severe disability. Predisposition to rheumatoid arthritis has been linked to the MHC class II HLA-DR4 (Stastny, 1978). There are at least 44 different DR4 subtypes known, which differ from each other at one to three amino acids in the third hypervariable region (amino acids 67–74). Among the HLA-DR4 genes, DRB1*0401 (Dw4), DRB1*0404 (Dw14), and DRB1*0405 (Dw15) alleles confer genetic predisposition to RA while DRB1*0402 (Dw10) does not (Gregersen *et al.*, 1986). Other alleles, notably DRB1*01, DRB1*1402, and DRB1*10, have been reported with predisposition to RA in certain ethnic populations (Taneja *et al.*, 1996; Willkens *et al.*, 1991; Yelamos *et al.*, 1993). HLA-DR4 gene occurs in linkage with DQB1*0301 (DQ7) and DQB1*0302 (DQ8) genes. RA patients in certain ethnic groups were found to be predominantly of the DQ8/DR4 haplotype and not DQ7/DR4 (Taneja *et al.*, 1992; Yelamos *et al.*, 1993) while an increased frequency of a DQ7 was found in other RA patients (Lanchbury *et al.*,

1989; Singal *et al.*, 1987). DQB1*0301 (DQ7) allele in American Indians of the Yakima nations and DQ9 (DQB1*0303/DQA1*0301) in RA patients in Chile differ from DQ8 only at amino acid position, 57 and 26 of the beta chain (Gonzalez *et al.*, 1992; Sattar *et al.*, 1990). These data support a role for HLA-DQ polymorphism in genetic predisposition to RA. Thus, the HLA class II contribution to RA predisposition is the result of the interaction between HLA-DQ and HLA-DR molecules on both haplotypes carried by an individual.

6.1. Collagen-induced arthritis—a model for RA

RA is characterized by synovial inflammation and erosion of bone and cartilage leading to destruction of joints. One of the sites of the major inflammatory damage is the diarthrodial joints, where the synovitis progresses to damage the cartilage and the subchondral bone. Articular cartilage is a highly differentiated connective tissue characterized by a dense hypocellular and avascular matrix. Type II collagen constitutes 80–90% of total collagen content of hyaline cartilage found in joints and is a genetically conserved sequestered protein and thus could be an autoantigen when presented in an appropriate immunogenetic context. Patients with RA have been shown to harbor B- and T-cell reactivity to CII, and accumulation of CII-reactive T cells in RA synovial fluid suggest that autoreactivity to collagen might be important in pathogenesis (He *et al.*, 2000; Londei *et al.*, 1989; Tarkowski *et al.*, 1989).

Experimental inflammatory arthritis that shares several clinical, hematologic, serological, and radiographic features with RA in humans can be induced in rats (Stuart *et al.*, 1979; Trentham *et al.*, 1977), mice (Courtenay *et al.*, 1980; Wooley *et al.*, 1981), and non-human primates (Gonnerman *et al.*, 1984). Upon immunization with heterologous type II collagen, rodents develop severe arthritis in some of the strains. Collagen-induced arthritis (CIA) appears to represent an experimental autoimmune disease dependent upon the immune response to a tissue restricted, sequestered, organ protein where the immune and autoimmune responses are under immunogenetic regulation. The varying immune reactivity to type II collagen observed between different mouse strains, therefore, provides a model to examine how the development of an immune response to foreign/self antigens may progress to an autoimmune response and subsequently to autoimmune disease.

In 1981 we were the first to report that CIA was linked to the MHC in mice (Wooley *et al.*, 1981). Subsequently, the susceptibility gene was mapped to the H2-A locus. Evolutionary studies and intron/exon organization of the genes, primary amino acid sequences, as well as function suggest that human and mouse class II genes evolved from a common ancestral class II gene with the mouse H2-A genes being homologous to

human DQ genes and the mouse H2E genes homologous to human DR genes. In mouse, susceptibility to collagen-induced arthritis (CIA) is determined by the HLA-DQ homologous mouse H2-A genes and not by DR homologous H2-E genes (Wooley *et al.*, 1981). The studies in mouse to determine the role of H2-E genes in CIA has shown that polymorphism within H2-Eβ genes modulate the disease (Gonzalez-Gay *et al.*, 1994; Taneja *et al.*, 1997). Certain H2-E alleles are protective; others are either neutral or permissive. Studies with mice expressing the polymorphic Eβ genes as well as mice with recombination within the Eβ genes narrowed down the protective region within Eβ gene to the sequence 67–74, the same as the ''Shared epitope'' in RA susceptibility (Gonzalez-Gay *et al.*, 1995). Further, the sequence of a protective $E\beta^{d}$ gene is very similar to the RA resistant DRB1*0402 gene while the permissive $E\beta^{b,k}$ genes are homologous to the RA susceptible DRB1*0401/*0404 gene.

6.2. HLA-DQ transgenic mice as models for rheumatoid arthritis

The first model of autoimmunity to determine the role of human class II molecules was established by using transgenic mice carrying genes from RA susceptible haplotype DQA1*0301, DQB1*0302 (DQ8) and an RA resistant haplotype DQB1*0601/DQA1*0103 (DQ6) and lacking endogenous mouse class II molecules ($A\beta^{\circ}$) (Bradley *et al.*, 1997; Nabozny *et al.*, 1996). Upon immunization with bovine type II collagen, both strains showed a moderate T-cell response that was CD4 mediated and DQ-restricted. Approximately 4 weeks after immunization, DQ8 transgenic mice generated autoantibodies to mouse type II collagen, while DQ6 mice did not show any autoantibodies. HLA-DQ8 transgenic mice developed severe inflammation and swelling, which progressed to severe arthritis and then to joint deformity. Histological examination of the arthritic hind limbs showed cellular infiltration, marked synovitis consisting of synovial cell hyperplasia, erosion of articular cartilage and subchondral bone. About 75% of the DQ8 transgenic mice developed severe arthritis while about 15% of DQ6 transgenic mice developed mild arthritis (Fig. 2.3). Thus, disease in these DQ mice was similar to the human linkage studies in RA. We generated double transgenic mice, DQ8/DQ6, to simulate human heterozygosity of a susceptible and resistant haplotype. These mice showed similar incidence but developed moderate CIA when compared with the severe arthritis observed in DQ8 transgenic mice. These observations contributed to the concept that polymorphism in DQB1 genes determines incidence, onset, and severity of collagen-induced arthritis (Bradley *et al.*, 1997).

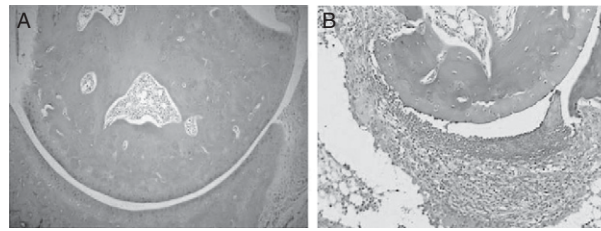

FIGURE 2.3 HLADQ8.Aβ° mice are susceptible to collagen-induced arthritis. Cross-sections of the hind foot from transgene negative littermate, Aβ° mouse (A) and DQ8. Aβ° mouse show DQ8 mice develop arthritis while transgene negative mice are resistant to arthritis. A normal synovial lining is observed in Aβ° mouse (A) while DQ8.Aβ° mice (B) show mononuclear cell infiltration of the synovium with pannus formation and cartilage and subchondral bone erosions.

6.3. HLA-DR transgenic mice as models of rheumatoid arthritis

To understand the role of RA associated DR alleles in generating a pathogenic immune response that could lead to arthritis, transgenic mice expressing DR4 and human CD4 were generated (Andersson *et al.*, 1998). Even though DR4 was shown to be functional in this mouse, they did not develop arthritis until they were mated to DBA/1 mice to have a susceptible background. Another problem with these mice was that they expressed hCD4 on all cell types. DR1 and DR4 transgenic mice were developed by introducing chimeric (human/mouse) HLA-DRB1*0101/IE and DRB1*0401/IE gene construct into CIA resistant B10.M mouse (Woods *et al.*, 1994). Inflammatory arthritis resembling rheumatoid arthritis can be induced in these DR1 and DR4 transgenic mice following immunization with CII (Rosloniec *et al.*, 1997, 1998). An immunodominant epitope identified from human type II collagen was found to be DR1 and DR4-restricted (Rosloniec *et al.*, 2004). However, it was difficult to decipher the immune response in these mice because endogenous mouse class II molecules (IA^f) were also present, and could shape the T-cell repertoire.

DRB1*0401 mice were generated in complete MHC knockout mice (AE°), that lacked all four classical murine chains, Aα, Aβ, Eα, Eβ. The only class II molecules expressed in these transgenic mice are DR4 (EαDRB1*0401). The DR molecule is expressed at higher levels in AE° mice compared with Aβ° transgenic mice. The major difference between RA and models of CIA has been that while RA shows a significant gender bias, most of the CIA models do not. Immunization of DRB1*0401.AE° mice led to the development of arthritis in around 40% of mice. However, disease developed predominantly in female mice, 52% female and 15% male mice, (approximately 3F : 1M ratio) similar to that of human disease. However, there was no significant difference in the severity of arthritis

between the two genders. This suggested that DR4 renders susceptibility to arthritis in female mice. Histopathology studies of the paws showed that while DQ8 mice had pathology in all the joints, DR4 mice showed severe infiltration and erosive arthritis in the digits of the paws.

To understand the reason for gender-bias in disease development we analyzed antigen specific T- and B-cell responses. Female mice responded with much stronger T-cell response to CII and DR4-restricted CII-derived peptide, 254–273, and produced increased amounts of inflammatory cytokines, IFN-γ, than male mice did. One possible reason for this stronger response could be the high cellularity and increased number of $CD4^+$ cells and B cells observed in female mice. RA patients generally produce autoantibodies such as RF and anti-CCP (Ferucci *et al.*, 2005). We determined whether DR4.AE° also produce those antibodies. All arthritic mice developed anticollagen antibodies in response to immunizing and self-collagen. DR4.AE° mice produced rheumatoid factors, IgG and IgM, and anti-CCP antibodies. Female mice produced significantly higher amounts of autoantibodies than male mice did and the levels of RF correlated with the severity of disease. This could account for gender-bias and earlier onset of arthritis in female mice. DQ8 mice do not show gender-bias in CIA thus suggesting that DR4 renders susceptibility to develop arthritis in females. Thus this model mimics human disease and could be utilized to answer an important question about increased autoimmunity in women.

6.4. Role of DR4 "Shared Epitope" in CIA

According to the shared epitope hypothesis, individuals expressing HLA alleles sharing sequence QKRAA (DRB1*0401) are susceptible to develop arthritis. On the other hand, motif I/D/E/A (DRB1*0402) at 67,70,71,74 was found to be associated with nonsusceptibility to RA. Although the mechanisms by which these alleles predispose to RA is not known, positive selection of potentially autoreactive T cells by susceptible class II alleles, or conversely negative selection of autoreactive T cells by non-susceptible alleles may be playing a role. To delineate the role of shared epitope in RA, we generated DRB1*0402.AE° mice (manuscript submitted). The DRB1*0402.AE° mice are resistant to CIA. DRB1*0402 mice have fewer number of cells in thymus than *0401 mice do, suggesting a higher rate of clonal deletion. Thus in individuals expressing *0402 gene, pathogenic autoreactive cells may have been deleted in thymus. This is supported by the observations of lower levels of autoantibodies in *0402 mice compared with *0401 mice. Also, T-cell response to CII and its derived immunodominant peptide, 254–273, is much lower in *0402 mice. However, *0402 mice are not defective in immune response and can respond very effectively to other antigens.

6.5. RA susceptible/resistant haplotype in CIA

DR2 (DRB1*1502) and DR3 (DRB1*0301) mice are resistant to CIA similar to human RA. To simulate human DQ/DR haplotypes linked to RA susceptibility and resistance, we generated DQ/DR double transgenic mice. Mice expressing DQ8.DR2 were resistant to develop arthritis while DQ8.DR3 mice were susceptible (Taneja *et al.*, 1998). In humans DR2 is associated with protection from RA (Taneja *et al.*, 1992) while DR3 is neutral. DQ8.DRB1*0401.AE° mice were susceptible to CIA while DQ8.DRB1*0402.AE° mice were resistant suggesting *0401 is permissive while*0402 protects DQ8 mice from developing arthritis (Taneja *et al.*, submitted for publication). These studies show that DRB1 polymorphism modulate DQ8-restricted arthritis.

Arthritis observed in DQ8.DRB1*0401 mice showed similarity with human RA as arthritic mice produced both IgG and IgM rheumatoid factors, and showed erosive disease in paws. The disease occurred with increased incidence and was much more severe than that observed in DRB1*0401 mice and involved big and small joints (Fig. 2.4). Since double transgenic mice can respond to both DR and DQ restricted CII-derived peptides, that could increase the immune response leading to increased incidence of arthritis in DQ8.DRB1*0401 mice compared with DRB1*0401 mice. Also, DQ8.DRB1*0401 showed gender-bias with predominantly female mice developing arthritis. Thus gender bias mediated by DR4 (DRB1*0401) is dominant.

To understand the mechanism of protection in DQ8.DRB1*0402.AE° mice, we analyzed immune response to CII in these transgenic mice. DQ8.DRB1*0401 mice mounted a stronger response to CII and derived peptides and produced higher amounts of proinflammatory cytokines. Also,

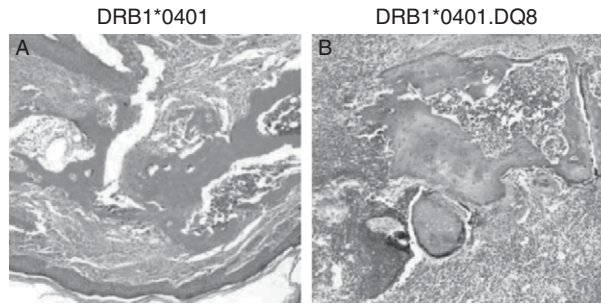

FIGURE 2.4 DRB1*0401.DQ8.AE° double transgenic mice with CIA develop more severe pathology in joints then DRB1*0401.AE° mice. Haematoxylin and eosin staining of section of an arthritic (A) digit of the paw of DRB1*0401.AE° mouse showing infiltration of cells in the synovium and arthritis and (B) paw of DRB1*0401.DQ8.AE° mouse showing erosive arthritis with cartilage and subchondral bone destruction.

anti-CII antibodies to self CII and other autoantibodies was higher in DQ8.DRB1*0401.AE° mice compared with DQ8.DRB1*0402.AE° mice. We determined whether lower immune response to CII was due to regulatory cells that could inhibit T-cell responses or due to activation induced cell death. Resistant mice had higher number of regulatory T cells compared with susceptible mice. Also, DQ8.DRB1*0402 mice showed higher activation-induced cell death after challenge with CII, suggesting that a lower threshold of immune response with lower autoantibodies and increased regulatory cells prevent DQ8.DRB1*0402 mice to develop CIA.

On the basis of these studies it can be hypothesized that HLA molecules play a critical role in positive and negative selection of T cells in thymus. Weak interaction between DR-derived peptides and DQ can lead to positive selection of autoreactive T cells like in RA susceptible DQ8.DRB1*0401. In the periphery, the autoreactive cells can respond to antigens that mimic an autoantigen and generate an autoreactive response. In case of RA resistant haplotype, a strong affinity of DRB1*0402-derived peptides with DQ can lead to negative selection of autoreactive T cells or can lead to selection of T regulatory cells. The transgenic mice expressing DQ8.DRB1*0401 simulate human RA susceptible haplotype and develop arthritis that shares similarities with human disease. On the other hand, DQ8.DRB1*0402 mice simulate a RA resistant haplotype and are protected from arthritis. These studies suggest that these transgenic mice provide a good model to study the mechanism of pathogenesis as well as protection. Also, since these mice develop arthritis predominantly in female mice, they will be good models to study why there is increased autoimmunity in women. Table 2.2 summarizes the similarities and differences between human RA and mouse CIA.

7. EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS: A MODEL FOR MULTIPLE SCLEROSIS

Multiple sclerosis (MS), a chronic inflammatory and demyelinating disease of the central nervous system (CNS), is hypothesized to be mediated by autoreactive T-cells against a variety of myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocytic glycoprotein (MOG) (Steinman, 1996; Zamvil and Steinman, 1990). Similar to other autoimmune diseases, MS has been strongly associated with certain MHC class I and II molecules. The HLA class II region of the MHC on chromosome 6p21 accounts for majority of familial clustering in MS and is by far the major susceptibility locus. Initial reports documented a weak association of HLA class I antigens- HLA-A3 and -B7 with MS (Bertrams and Kuwert, 1972), but eventually a much stronger

TABLE 2.2 Similarities and differences between rheumatoid arthritis and collagen-induced arthritis

		CIA	
	RA	Aβ°	AE°
Clinical phenotype			
Swelling	+	+	+
Deformities	+	+	+
Gender bias	+	−	+
Histopathology			
Pannus formation	+	+	+
bone erosions	+	+	+
Immune response			
CII reactive T cells	+	+	+
Class II expression on T cells	+	−	+
Autoantibodies			
Antibodies to CII	+	+	+
Rheumatoid factor	+	+	+
Anti-citrullinated peptide	+	−	+

association was shown with HLA-DR2 (DRB1*1501)/DQ6 (DQB1*0602) in Caucasian MS patients (Tiwari and Terasaki, 1981; Weinshenker *et al.*, 1998). Although HLA-DR2 has a strong association with MS in Caucasian population, other HLA-DR genes, such as HLA-DR1, -DR3, -DR4, and -DR6 are linked in certain ethnic populations (Kurdi *et al.*, 1977; Marrosu *et al.*, 1988, 1997). Positive association of DR3 allele with MS is less frequent but has been reported from different parts of the world, including USA, Canada, Mexico, and Europe (Alvarado-de la Barrera *et al.*, 2000; Marrosu *et al.*, 1997; Weinshenker *et al.*, 1998). Role of DR and DQ genes in MS has been difficult to assess in past because of heterozygosity in patient populations and linkage disequilibrium between MS associated HLA-DR and -DQ genes. Several studies in MS have raised the question of whether it is a single allele, a combination of two, or a complex haplotype that confers an increased risk for disease susceptibility (Barcellos *et al.*, 2003; Haines *et al.*, 2002).

Now, two animal models are present to study MS: experimental autoimmune encephalomyelitis (EAE) and viral induced demyelination model such as Theiler's Murine Encephalomyelitis Virus (TMEV). The hypothesis that MS is an autoimmune disease comes from studies of EAE (Gold *et al.*, 2000; Swanborg, 2001). EAE can be induced in various inbred animal strains by inoculation of whole myelin or defined myelin proteins such as MBP, MOG, and PLP in complete Freund's adjuvant. Attempts to

transfer EAE by humoral or cellular components in mice have suggested that it is a cell mediated and not an antibody-mediated disease (Ando *et al.*, 1989; Pettinelli and McFarlin, 1981). The characteristics of encephalitogenic MBP, MOG, and PLP-specific T cells have been described in detail with respect to activation requirements, fine specificity, MHC restriction, TCR usage, and cytokine profiles. These elegant studies documented that encephalitogenic T cells are $CD4^+$, T helper (Th1)-type cells secreting TNF-α/β and IFN-γ (Ando *et al.*, 1989; Zamvil and Steinman, 1990). Depending on the animal strains used, encephalitogenic T cells are specific for short peptides derived from MBP, MOG, or PLP that is recognized in the context of MHC class II (Encinas and Kuchroo, 1999). Several experimental immunotherapies have been successfully used in EAE, including MHC blockade (blocking peptide, peptide/MHC complexes) (Brocke *et al.*, 1996; Sharma *et al.*, 1991) TCR peptide vaccination, TCR antagonistic peptides, and oral tolerization with autoantigens (Howell *et al.*, 1989; Kuchroo *et al.*, 1994). Although successful in preventing or treating ongoing EAE, most of these therapies have had limited success in treating human MS patients. A mouse EAE model where autoreactive T-cell repertoire is selected and shaped by human MHC class II molecule will provide new information on human MS.

7.1. Mouse myelin extract induced EAE in HLA class II transgenic mice

As a first step to determine whether the HLA class II transgenic mice can generate humanized models for MS, we tested the T-cell proliferative responses of HLA-Transgenic mice associated with MS (-DR2, -DR3, DR4, DQ6, and -DQ8) against a mouse myelin extract prepared from brains and spinal cords of normal mice over sucrose gradients (Das *et al.*, 2000). All the HLA class II Transgenic mice responded to the mouse myelin extract to various degrees with DR2 and DR3 transgenic mice showing the highest response. We were able to induce EAE only in DR2 (50%) and DR3 (60%) transgenic mice. Histological analysis revealed extensive inflammation and demyelination in the spinal cords of these mice. Following the initial acute phase of the disease, the majority of DR3 mice went into remission and did not relapse for the remainder of the test period. Both HLA-DQ6 and -DQ8 transgenic mice were resistant to EAE. These studies showed that HLA class II transgenic mice could indeed serve as an excellent humanized model of multiple sclerosis and reproduce human linkage data. Furthermore, double transgenic mice expressing both DR3 and DQ8 got severe EAE. Myelin extract consists of number of myelin antigens such as MBP, PLP, MOG, CRYAB, MAG, CNP and S 100. Auto-reactive T-cells reactive with some of these antigens have been reported from MS patients; however it is not clear which of

these antigens play an important role in initiation of MS. There are two possibilities i) MS is initiated by auto-reactive T cells against one of these myelin antigens and later in disease, epitope spreading leads to development of T-cell response against other myelin antigen or ii) disease can be initiated by anyone of these myelin antigens. To test these two hypotheses EAE was induced in different HLA transgenic mice expressing HLA-DR2, -DR3, DR4, DQ6 and DQ8 molecules using the three major myelin antigens MBP, PLP and MOG.

7.2. Myelin basic protein (MBP)-induced EAE

MBP is second most abundant protein in CNS and has been studied extensively to understand immunopathogenesis of MS. Studies in MS patients have suggested that MBP-specific T lymphocyte repertoire in multiple sclerosis are restricted within the amino acids 38–59, 80–105, 108–131, 131–153 and 141–160. These MBP epitopes were recognized in the context of DRB1*0101, DRB5*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*1402, and DRB3*0102 molecules. Among these, $MBP_{84–102}$ has been shown to be most dominant epitope and was restricted to HLA-DR2. In our HLA class II Transgenic mice, only DR2 transgenic mice responded to this peptide. All the others were non-responders, demonstrating the specificity of the HLA class II molecules in these mice. Using overlapping peptides encompassing the entire human MBP sequence, we identified number of MBP epitopes in these HLA class II transgenic mice. The DR2 transgenic mice recognized several MBP peptides including 84–102, while the DQ6 transgenic mice recognized a single MBP peptide. The DR3 transgenic mice responded to two specific MBP peptides 31–50 and 131–150, while DQ8 transgenic mice gave very low response to three peptides 71–90, 131–150, and 141–160 (Table 2.3). Most of the epitope(s) of MBP recognized by our class II Transgenic mice were same as reported earlier from T-cell responses of MS patients. Thus epitope mapping data validated use of HLA class II transgenic mice for studying MS. Madsen *et al*. (Madsen *et al*., 1999) generated a transgenic mice, expressing three human components involved in T-cell recognition of an MS-relevant MBP antigen presented by the HLA-DR2 molecule: *DRA*0101/DRB1*1501* (HLA-DR2), a T-cell receptor (TCR) from an MS-patient-derived T-cell clone specific for the HLA-DR2 bound immunodominant MBP 84–102 peptide and the human CD4 coreceptor. Although spontaneous disease was observed only in 4% of mice, immunization with MBP leads to development of clinical disease in 85–90% of transgenic mice. Crossing this triple transgenic line with $Rag2^{-/-}$ mice lead to spontaneous disease development in 100% of mice, suggesting role of Tregs in prevention of disease in non$Rag2^{-/-}$ background mice. This model was further refined by Ellmerich *et al*. (2005),

TABLE 2.3 Myelin specific T-cell epitope recognized by different HLA class II molecules*

Myelin antigen		HLA transgenic mice					
		DR1	DR2	DR3	DR4	DQ6	DQ8
PLP	1–20						
	21–40						
	31–50						
	41–60						
	51–70						
	71–90						
	81–100						
	91–110						
	101–120						
	139–154						
	178–197						
	188–207						
	198–217						
	208–207						
	264–277						
MBP	1–20						
	11–30						
	21–40						
	31–50						
	41–60						
	51–70						
	61–80						
	71–90						
	81–100						
	91–110						
	101–120						
	111–130						
	121–140						
	131–150						
	139–154						
	141–160						
	151–170						

(*Continued*)

TABLE 2.3 (*continued*)

Myelin antigen		HLA transgenic mice					
		DR1	DR2	DR3	DR4	DQ6	DQ8
MOG	1–20						
	11–30						
	21–40						
	31–50						
	41–60						
	51–70						
	61–80						
	71–90						
	81–100						
	91–110						
	101–120						
	111–130						
	121–140						
	131–150						
	139–154						
	141–160						
	151–170						

* Data for table are derived from the following studies: PLP epitopes (Mangalam *et al.*, 2004), MBP epitopes (Sireci *et al.*, 2003, unpublished data from our lab), and MOG epitopes (Khare *et al.*, 2003)

who generated a humanized transgenic mice, strongly expressing HLA-DR2 with an MS-derived $MBP_{85–99}$ specific TCR. These $MBP_{85–99}$/DR2 transgenic mice spontaneously develop paralysis even on a RAG-2 wild-type background. Disease was characterized by demyelination and axonal degeneration in CNS, and correlated with inter- and intra molecular spread of the T-cell response to HLA-DR15-restricted epitopes of myelin basic protein, myelin oligodendrocyte glycoprotein, and αB-crystallin. MBP has also been shown to be immunogenic in context of HLA-DR4 as adoptive transfer of CD4 T cells from double transgenic mice expressing HLA-DR4 (DRB1*0401) and TCR specific for $MBP_{111–129}$ into DR4 transgenic mice induced EAE (Quandt *et al.*, 2004). Furthermore, $MBP_{87–106}$ peptide has been shown to induce a mild EAE in HLA-DR4 Transgenic mice (Ito *et al.*, 1996). Sireci *et al.* (2003) used DR1 transgenic mice to identify immunodominant epitope of MBP restricted to HLA-DRB1*0101. In DR1 (DRB1*0101) transgenic lines $MBP_{13–33}$ epitope induced a strong Th1

response accompanied by high T-cell precursor frequency and caused mild EAE, while the $MBP_{139–154}$ induced a weak response. MBP $peptide_{87–106}$ showed very low affinity for DR1 and did not induce any T-cell response or disease in DR1 transgenic mice. Thus studies with HLA class II transgenic mice has reestablished role of MBP as an important autoantigen in MS.

7.3. Proteolipid protein (PLP)

Proteolipid protein (PLP), is the most abundant protein constituting the CNS myelin (<50%), and PLP reactive T cells have been identified in both MS patients and normal controls. The major T-cell epitopes has been shown to be restricted to PLP31-70, 90–120, 140–160, 178–227 and 263–276. Since, PLP molecule is very hydrophobic forming insoluble high molecular weight aggregates in aqueous solution, it is difficult to do studies with whole protein. Therefore, we synthesized 20 amino acid peptides (overlapping by 10) of the entire sequence of the human PLP molecule (human and mouse PLP are 100% conserved). T-cell responses of varying degrees were detected against PLP peptides 31–50, 41–60, 51–70, 91–110, 101–120, 141–160, 178–197, 188–207, 208–227 and 263–276 among the HLA class II Transgenic mice (Table 2.3). The majority of epitopes identified largely encompassed regions previously reported to be immunogenic in humans. More importantly not all epitopes were restricted to all class II molecules. While, some peptides elicited response specific to particular HLA class II allele, others were promiscuous. $PLP_{138–153}$ was immunogenic in DQ6 and DQ8 transgenic mice but not in DR2, DR3 and DR4 transgenic mice. Some peptides elicited responses that were specific for individual HLA molecules. PLP peptides 1–20 and 141–160 were immunogenic only in DQ8 mice. In addition, we observed both DQ and DR molecules restricted response to $PLP_{91–110}$ whereas responses to 263–276 residue of PLP were restricted only to DR molecule. Similar observation has also been reported in a study of MS patients from Japan (Minohara *et al.*, 2001). Other epitopes (41–60, 91–110, 178–197, 208–227) were promiscuous in inducing T-cell responses in all of the transgenic mouse lines.

7.3.1. $PLP_{91–110}$-induced EAE in HLA-DR3 transgenic mice

Among all these immunogenic epitopes, $PLP_{91–110}$ and $PLP_{178–197}$ have been shown to be encephalitogenic in HLA class II transgenic mice. First, Ito *et al.* (1996) showed that $PLP_{175–192}$ can induce strong proliferative response and EAE in HLA-DR4 transgenic mice. Earlier, PLP epitope 89–116 has been shown to elicit vigorous T-cell responses in human MS patients (Ohashi *et al.*, 1995; Trotter *et al.*, 1998). An overlapping sequence of $PLP_{95–116}$ has been reported to be restricted by HLA-DR and -DQ using T cell lines from MS patients (Minohara *et al.*, 2001). Moreover $PLP_{95–116}$

specific T cell lines elicited atypical autoimmune encephalitis in RAG-$2^{-/-}$, HLA-DR2 tg mice on adoptive transfer (Kawamura *et al.*, 2000). In view of the responses elicited by PLP peptide 91–110 and its homology to aforementioned residues 89–116 and 95–116, peptide 91–110 was assessed for encephalitogenic potential. $PLP_{91–110}$ peptide induced severe clinical EAE only in DR3 Transgenic mice (60%) (Mangalam *et al.*, 2004). The inability of $PLP_{91–110}$ to induce disease in DR2 Transgenic mice might be attributed to presence of regulatory T cells, as in previous study by Kawamura *et al.* (2000), disease was observed in DR2 Transgenic mice on $RAG2^{-/-}$ background. Thus, we were able to generate an animal model of MS in HLA class II transgenic mice expressing only human class II in the absence of endogenous mouse class II molecules and on a RAG2 sufficient background. DR3 transgenic mice with clinical signs of EAE showed diffuse meningeal infiltrates in both the spinal cord and the brain. In addition, occasional sections of the spinal cord showed paragonal mononuclear cell infiltrates that were closely associated with the meningeal infiltrates. In the brain, mononuclear cell infiltrates were seen primarily in the meningeal surfaces of the brain stem, cerebellum, and surrounding the ventricles. Demyelination was observed in the spinal cord white matter immediately below the surface of the core. T cells from DR3 transgenic mice immunized with PLP showed a typical Th1 cytokine profile characterized by high levels of IL-6, IL-12, TNF-α, and IFN-γ. High IL-10 levels were observed in T cells from HLA-DR2 Transgenic mice, suggesting that regulatory T cells might be responsible for resistance of these mice to develop EAE. Moderate to low levels of IL-2, IL-6, IL-12, and TNF-α were produced by the other transgenic mice, while IL-4 and IL-5 were very low.

7.3.2. Delineation of the minimal encephalitogenic PLP epitope and critical residues

Using single amino acid truncation, we narrowed down residues necessary for binding to HLA-DR3 molecule as amino acid 97–108 of PLP (Mangalam *et al.*, 2005). Immunization of DR3 transgenic mice with minimal epitope $PLP_{97–108}$ led to induction of EAE. Alanine substitutions suggested that amino acid residues isoleucine, aspartate, and lysine at positions 99, 102 and 104 are the anchor residues and fits MHC class II pocket P1, P4, and P6 respectively. Presence of Isoleucine as 1st anchor residue was expected as a large hydrophobic amino acid form first anchor residue at P1 pocket in all –DR as well as mouse class II molecule. However, the P1 pocket in DRB1*0301 is shallow as compared to deep pockets seen in other class II molecules such as (DRB1*0101, DRB1*0401, DRB*1101), due to presence of a valine at DRh86 as compared to a glycine. This may explain the presence of amino acids with aliphatic side chains and absence of bulky amino acids with aromatic side chains like tyrosine and tryptophan in DRB1*0301 binding peptide. An acidic amino acid

like aspartate (D) is preferred at the P4 pocket of DR3 molecule due to the presence of a positively charged lysine (K) at h74. Presence of aspartate in the P6 pockets makes it acidic in DR3 and in most of the cases; lysine (K) is preferred at this position because of its positive charge.

7.4. Myelin oligodendrocyte gycoprotein (MOG)

MOG is a minor component of CNS myelin, which is exposed on the surface of the outer most laminae of the myelin sheath (Brunner *et al.*, 1989). It is a 218 amino acid glycoprotein with an extracellular Ig-like domain encompassing amino acids 1–125,which is encoded within the distal region of MHC in humans, mice, and rats (Pham-Dinh *et al.*, 1993, 1995). In a study by Kerlero de Rosbo *et al.* (1993), about 50% of MS patients showed peripheral mononuclear cells directed towards MOG rather than MBP or PLP. Extensive studies in the last decade have established MOG as an important and potential target antigen in MS pathogenesis. MOG is the only identified myelin autoantigen that triggers not only an encephalogenetic T-cell response, but can also induce pathogenic demyelinating autoantibody responses in rodents and primates.

Autoimmune response to MOG in MS patients has been shown to be directed against three major epitopes, amino acids 1–22, 34–56, and 64–96. Anti-MOG antibodies have been reported in the serum and cerebral spinal fluids of MS patients. Recombinant MOG (rMOG) corresponding to the extracellular domain of rat MOG (amino acids 1–125) was prepared (Amor *et al.*, 1994), which had 96% homology with human and mouse MOG. For fine mapping of a MOG epitope, a panel of 16 overlapping 20 mer (overlapping by 10 amino acids) peptide were synthesized. All transgenic mice showed a strong proliferative response with the recombinant MOG (Khare *et al.*, 2003). T-cell autoreactivity to MOG was directed against peptides 1–20, 31–50, 61–80, and 91–110, of which three are also immunodominant epitopes for MOG in MS (Table 2.3). The DR2 transgenic mice recognized several epitopes within MOG including peptides 1–20, 31–80, and 91–110, while DQ6 (*0601) a protective allele in MS, recognized two main regions- 71–110 in extracellular and 131–150 in intracellular region. Maximum number of MOG specific T-cell epitopes were observed in DR3 and DQ6 (DQB1*0604) transgenic mice with immunodominant regions spread over whole length of MOG protein and included both extra as well as intracellular regions of protein. Both DR4 and DQ8 recognized 2–3 epitopes, with former responding to MOG peptides 31–60 and 81–110, while DQ8 transgenic mice showed three immunodominant regions 61–80, 81–100, and 121–160.

All transgenic mice (DR2,-DR3, -DR4, DQ6, and -DQ8) showed a strong B cell response to MOG protein, with major B cell epitopes located within amino acids 1–30, 51–80, and 101–120 of human MOG.

Interestingly, two of these three epitopes have been reported earlier in MS. Most of the transgenic mice recognized similar B cell epitopes except DQB1*0604 which only recognized a unique epitope 81–100.

7.4.1. MOG-Induced EAE

HLA-DR2 (DRB1*1502).Aβ° transgenic mice were susceptible to recombinant MOG-induced EAE with a 60% penetration rate. Transgenic mice with EAE showed typical course of ascending paralysis accompanied with severe weight loss. Following initial acute phase of disease, the majority of the DR2 mice went into partial remission and did not relapse during the remainder of the test period. No disease was seen in -DR3, -DR4, -DQ6, or -DQ8 transgenic mice. Histopathology of brain and spinal cord revealed moderate degrees of inflammation in the white matter parenchyma and meninges of spinal cords and brain of the DR2 mice.

MOG-specific T cells from DR2 mice produced high amounts of IFN-γ and IL-6, with very low amounts of IL-4, IL-12 and a moderate amount of IL-10. LNCs of DQ8.Aβ° mice produced low amounts of all cytokines except IL-10. DQ6.Aβ° mice produce high levels of IL-6, IL-10, and IFN-γ and moderate levels of IL-4 and IL-12. Thus, all transgenic mice produced both Th1 and Th2 cytokines after rMOG stimulation, although levels of Th1 cytokines were higher than Th2 cytokines in most of the mice. Altered cytokine levels produced by T cells in these mice could be merely reflecting their increased proliferation response to rMOG. While increase in Th1 cytokines may be correlated to disease severity, it cannot be discounted that the increase in Th2 cytokines (IL-10) may also influence disease development and progression. These data indicated that predominant primed T-cell population was of Th1 phenotype, although Th2 cells were also stimulated.

Rich *et al*. (2004) also showed that HLA-DR2 (DRB1*1501) transgenic mice are susceptible to murine $MOG_{35–55}$-induced EAE. HLA-DR2 Transgenic mice with EAE showed a typical Th1 cytokine profile and white matter lesions. hMOG-35-55 peptide, which differs from mMOG-35-55 peptide by a proline for serine substitution at position 42, was also immunogenic, but not encephalitogenic. HLA-DRB1*1503 transgenic mice was also susceptible to MOG-induced EAE and show similar disease incidence and severity. Thus all DR2 subtypes DRB1*1501, DRB1*1502, and DRB1*1503 are susceptible to MOG-induced EAE. All three subtypes produced both Th1 and Th2 cytokines with predominance of Th1 cytokines. However, maximum CNS histopathology was observed in DRB1*1501 mice with EAE.

Recently Forsthuber *et al*. (2001) have shown that $MOG_{97–108}$ epitope is an immunodominant T-cell determinant in context of HLA-DR4 (DRB1*0401) molecule and can induce severe disease in HLA-DR4 transgenic mice. Mice with EAE showed perivascular and periventricular

inflammatory infiltrates in brain sections consisting of lymphocytes, macrophages, and occasional neutrophils. They also showed that HLA-DR4 + human B cell present the same peptide of MOG.

7.5. Role of HLA-DQ on disease susceptible HLA-DR transgenic mice

Studies with single Transgenic mice have suggested that $PLP_{91–110}$ can induce EAE in HLA-DR3 transgenic mice (Mangalam *et al.*, 2004) while MOG and MBP antigens induced EAE in HLA-DR2 (Khare *et al.*, 2005; Madsen *et al.*, 1999) and -DR4 transgenic mice (Klehm *et al.*, 2004). None of the studies so far have shown induction of EAE in HLA-DQ transgenic mice. Thus, prevalent data suggests that HLA-DR genes are primarily responsible for susceptibility to MS. However HLA-DQ genes could play a role in modulating the disease in association with DR genes. The frequency, progression and severity of disease in human patients differ depending upon the haplotype. HLA haplotype DQ6 (DQB1*0602)/DR2 (DRB1*1501) predispose to MS while DQ6 (DQB1*0601)/DR2 (DRB1*1502) is resistant suggesting DQ6 (DQB1*0601) may be protective in MS (Amirzargar *et al.*, 1998; Serjeantson *et al.*, 1992). Our data showing susceptibility of DRB1*1502 to EAE also suggests that DQB1*0601 must be protective. Linkage studies suggests that DQ8 (DQB1*0302)/DR4(DRB1*0401) haplotype predispose to MS. Therefore we investigated the role of HLA-DQ6 (DQB1*0601) and HLA-DQ8 (DQB1*0302) gene(s) in disease susceptible HLA-DR3 and -DR2 transgenic mice.

7.5.1. DQ6 (DQB1*0601) is protective in EAE

We generated double transgenic mice expressing protective MS allele DQB1*0601 on disease susceptible HLA-DR3 background to determine whether presence of DQB1*0601 can lead to a protective phenotype on DR3 background. Using $PLP_{91–110}$ -EAE model, we observed that introduction of DQ6 (DQB1*0601) on disease susceptible DR3 (DRB1*0301) transgenic mice caused decrease in disease incidence suggesting a protective effect of the DQ6 allele. The protective effect of DQ6 was due to anti-inflammatory effect of IFN-γ, which paradoxically is considered a pro-inflammatory cytokine. Blockade of IFN-γ with neutralizing antibody against IFN-γ abrogated this protective effect of DQ6 (Mangalam *et al.*, 2008). Similar to DR3 transgenic mice, presence of DQ6 with DR2 lead to decrease in disease incidence as DQ6/DR2 (0601) mice showed a very low incidence of MOG-induced EAE (Khare *et al.*, 2005). DQ6/DR2 transgenic mice showed mild inflammation without any demyelination. Thus, our HLA class II transgenic data demonstrated that DQ6 (DQB1*0601) is protective in EAE.

7.5.2. DQ8 (DQB1*0302) exacerbates EAE

We also investigated role of HLA-DQ8 on $PLP_{91–110}$ induced EAE in DR3 Transgenic mice. Introduction of DQ8 onto DR3 transgenic mice led to higher disease incidence (100% in DR3/DQ8 vs 65% in DR3) as well as increased disease severity (average clinical score 4.2 in DR3/DQ8 vs 2.9 in DR3). We further characterized that the increased susceptibility in DR3/DQ8 transgenic mice was due to increased production of pro-inflammatory cytokine IL-17 by DQ8 specific T cells. These double transgenic mice (HLA-DR3/DQ8) with EAE also showed increased inflammation and demyelination in CNS tissue as compared to single DR3 transgenic mice. We also observed that inflammatory cells inside CNS undergo increased apoptosis in DR3 transgenic mice explaining milder CNS pathology in these mice.

Similar to DR3 transgenic mice, presence of DQ8 with DR2 lead to increased disease incidence as well as severity. MOG-induced EAE was characterized by severe weight loss and a typical course of ascending paralysis with majority of sick mice showing paralysis of both hind as well as forelimb. The majority of affected DR2/DQ8.Aβ° mice never went into remission. While DR2 transgenic mice with EAE showed mild CNS pathology, the inflammatory infiltrates were more extensive in the spinal cord and brain of the DR2/DQ8 mice with EAE. In the DR2 mice, the infiltrates tended to localize in perivascular regions whereas in the DR2/DQ8 mice inflammatory cells infiltrated wildly throughout the whole white matter. In the brain, mononuclear cell infiltrates were seen in the meningeal surfaces of the brain stem, cerebellum, cortex, and stratum. These perivascular and periventricular infiltrates consist of lymphocytes, macrophages, and neutrophils. The presence of persistent neurological defects observed in DR2/DQ8.Aβ° mice was associated with the demyelination in the spinal cord. Thus our double transgenic mouse model of EAE provides a novel tool to study linkage disequilibrium and shows that HLA-DQ molecule might modulate disease in MS-susceptible HLA-DR allele by functional epistatic interaction.

Similar scenario might be true in MS where both DQ and DR are linked with MS but individual role of these genes are not clear. Our data suggests that presence of certain DR gene(s) predispose an individual to MS and polymorphism in DQ gene(s) might play a modulating role.

7.6. A new humanized HLA transgenic mouse model of multiple sclerosis expressing class II on mouse CD4 T cells

7.6.1. Severe EAE in AE°.DR3 mice:

As mentioned previously, we have introduced the new complete mouse class II knockout AE° into all our transgenic mice. These mice express class II on T cells which can present peptide antigen. To analyze

encephalitogenic ability of $PLP_{91–110}$ peptide, the new transgenic mice expressing DR3, DR2, DQ6 or DQ8 were immunized with PLP peptide using standard protocol and disease was monitored. $PLP_{91–110}$ peptide-induced severe EAE, only in DR3.AE° transgenic mice characterized by ascending paralysis (Mangalam *et al.*, 2006). EAE in HLA-DR3.AE° mice was characterized by an early onset of disease and increased disease incidence as compared to DR3.Aβ° transgenic mice. Mice with EAE showed typical Th1 type cytokine pattern with DR3.AE° mice showing higher levels of pro-inflammatory cytokine IL-17 as compared to DR3.Aβ° transgenic mice. No disease was seen in other class II transgenic mice expressing HLA-DR2, -DQ6 or -DQ8 suggesting that this peptide is encephalitogenic only in context of HLA-DR3 molecule. In addition, CNS pathology of DR3.AE° mice showed severe inflammation and demyelination in CNS similar to MS (Fig. 2.5). While DR3.Aβ° mice showed mild brain pathology, wide spread inflammation and demyelination was observed in cerebellum, brain-stem, cortex, corpus callosum and meninges of DR3.AE° mice. Similarly more inflammation and demyelination was observed in spinal cord of DR3.AE° mice with EAE as compared to DR3.Aβ° mice. Analysis of epitope spreading suggested extensive inter as well as intra molecular epitope spreading in DR3.AE° mice with EAE as compared to DR3.Aβ° mice with disease. DR3.AE° mice with EAE recognized extra PLP epitopes such as $PLP_{41–60}$, $PLP_{101–120}$, $PLP_{111–130}$ and $PLP_{267–277}$ besides showing T-cell response to PLP epitope 141–160, 178–197, 188–207, 208–227 and rMOG.

Expression of MHC class II inside CNS may be the first step for a proper T-cell activation. While class II molecules are not expressed inside healthy CNS, MS patients and mice with EAE show expression of class II inside CNS. We found that DR3.AE° mice with EAE showed strong

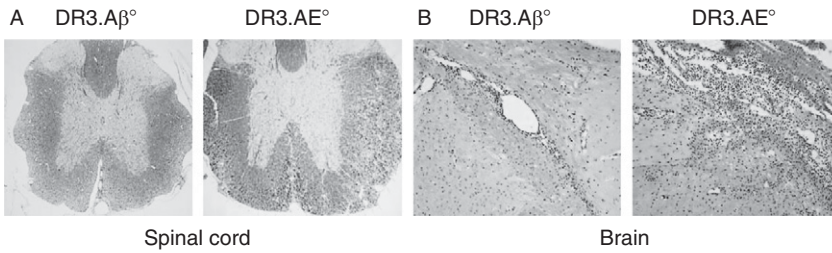

FIGURE 2.5 DR3.AE° transgenic mice with EAE show more severe CNS pathology then DR3.Aβ° mice. Haematoxylin and eosin staining of section of a spinal cord (A) and brain (B) showing inflammation with severe demyelination in DR3.AE° when compared with mild inflammation in DR3.Aβ°. Mice immunized with $PLP_{91–110}$. (Reproduced from *Eur J Immunol,* Mangalam *et al.*, 2006, 36, 3656, with permission from *Wiley-VCH Verlag GmbH & Co. KGaA.*)

HLA-DR expression in resident brain and spinal cord cells. We also isolated class II + $CD4^+$ T cells from affected CNS tissue, further suggesting a role of these T-cell subsets in disease pathogenesis. Furthermore, our bone marrow chimera experiments confirmed important role of class II + CD4 + T cells in disease pathogenesis as DR3.Aβ° mice reconstituted with BMCs from DR3.AE° mice developed more severe disease as well as CNS pathology similar to DR3.AE° transgenic mice.

7.6.2. Role of class II + $CD4^+$ T cells in MS

The role of class II + $CD4^+$ T-cells in the immunopathogenesis of MS or other diseases have not been well understood due to the lack of a proper animal model. It is well established that presentation of self antigens to autoreactive T-cells inside the CNS (Slavin *et al.*, 2001; Tompkins *et al.*, 2002) plays an important role in the initiation and the propagation of immune responses resulting in inflammation and subsequent demyelination. We hypothesize that activated $CD4^+$ T cells expressing class II can recognize myelin antigen inside CNS and thus perpetuate the local inflammatory response (Fig. 2.6). Direct recognition and presentation of antigens at the site of injury by class II + $CD4^+$ T cells might lead to proliferation of antigen-specific T cells or epitope spreading. Besides, they can modulate the inflammatory response by secreting pro-inflammatory cytokines. These pro-inflammatory cytokines could directly act on glial cells or in turn could recruit more inflammatory cells to the site of injury. Thus class II + $CD4^+$ T cells can play multiple roles in pathogenesis of demyelination and expression of class II on T cells results in a pathologic scenario much more similar to human MS. Thus EAE in DR3.AE° transgenic mice may simulate immuno-pathogenesis of MS in human.

It is well established that most of the autoimmune diseases are the result of an aberrant immune response to self antigens, controlled by MHC molecules. Thus MHC molecules are central to etiology of most inflammatory autoimmune disease and it is not surprising that majority of these show strong association with MHC class II gene(s). Thus, studies of these HLA class II gene(s) first in isolation (DR or DQ transgenic mice) and then together in different combination (double or triple transgenic mice expressing different DQ or DR) have given us a unique tool to dissect out the role of individual HLA class II molecule in disease pathogenesis. Thus data generated from HLA class II transgenic mice have helped us in understanding many aspects of immunopathogenesis of MS, which were not possible by use of conventional mouse models such as role of disease associated DR and DQ genes and how they interact with each other. Data generated from HLA transgenic mice suggests that MS can be initiated by anyone of major myelin antigen (PLP, MOG or MBP) and choice of initiating agent is dependent on presence of particular MHC class II molecules. While DR2 Transgenic mice are susceptible to

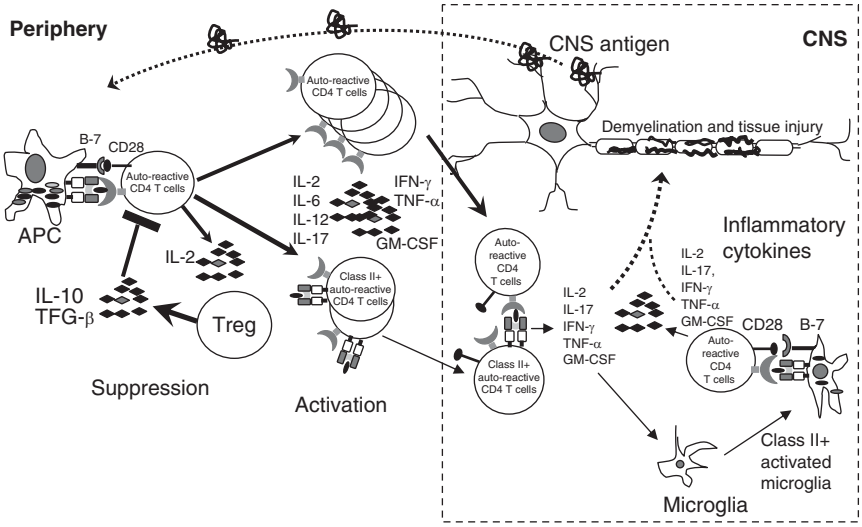

FIGURE 2.6 A model for role of class-II + $CD4^+$ T cells in pathogenesis of MS. In MS susceptible individuals, myelin antigens are presented by disease-associated class II alleles such as HLA-DR3 or HLA-DR2, leading to activation of myelin antigen specific auto-reactive T cells. The expansion of these autoreactive T cells in periphery can be suppressed by regulatory T cells (both $CD4^+$as well as CD8 Tregs). However, if the autoreactive T cells overcome this suppression step, there is an expansion of these autoreactive T cells. In our new HLA class II transgenic mice, subset of activated T cells also express class II on their cell surface, similar to activated human T cells. We hypothesize that these class II + $CD4^+$ T cells play an important role in antigen presentation inside the CNS as activation of auto-reactive T cells inside the CNS is required for development of EAE/MS. Direct recognition and presentation of antigens at the site of injury by classII + $CD4^+$ T cells might lead to proliferation of antigen-specific T cells or epitope spreading. The pro-inflammatory cytokine (e.g., GM-CSF) secreted by these activated T cells leads to activation of microglia, which then further perpetuate the local inflammatory response either by presenting antigens to T cells or secreting inflammatory cytokines. Thus classII + $CD4^+$ T cells can play multiple roles in the pathogenesis of demyelination and expression of class II on T cells, resulting in a pathologic scenario much more similar to human MS.

MOG-induced EAE, PLP can induce disease in DR3 Transgenic mice and MBP can induce mild disease in DR1 and DR4 transgenic mice. Data from double transgenic mice suggests that presence of other class II molecule can modulate disease positively or negatively when present with disease susceptible class II molecules. Presence of DQ6 (DQB1*0601) play a protective role in MS as they reduce disease incidence in susceptible class II transgenic mice, whereas presence of DQ8 (DQB1*0302) on disease susceptible class II background increase disease incidence and severity. Since most human population are heterozygous expressing two DQ and DR

genes, the onset, progression, severity and modulation of MS in these patients will vary depending upon the contribution of different HLA genes. Obviously other MHC linked genes and non MHC genes and environment will determine the ultimate phenotype of disease in each individual.

8. SPONTANEOUS MODELS FOR HUMAN TYPE I DIABETES

Type 1 diabetes (T1D), also called insulin-dependent diabetes mellitus (IDDM), is an autoimmune disease, characterized by immune-mediated destruction of β cells, which are the endocrine unit of the islets of Langerhans that produce the hormone, insulin. This results in insulin deficiency, hyperglycemia and associated side effects (Jahromi and Eisenbarth, 2007; Wilson and Eisenbarth, 1990). Like most other autoimmune diseases, there is a strong genetic association with incidence of T1D. However, lack of complete concordance in identical twins, higher incidence in certain geographical areas, an overall increase in incidence, and other related observations suggest a critical role for yet undefined environmental influences on the pathogenesis of T1D. The non-obese diabetic (NOD) strain of mice spontaneously develops autoimmune diabetes and shares several features of human T1D, including the genetic and environmental aspects (Wicker *et al.*, 2005). While MHC and several non-MHC genes contribute to the pathogenesis of T1D in humans and NOD mice, the MHC region confers the highest genetic risk for T1D. Certain MHC haplotypes such as the HLA-DQ8/DR4 or HLA-DQ2/DR3 are found at significantly higher rates in patients with T1D than in control population. At the same time, inheritance of certain haplotype such as DQ6/DR2 seems to confer dominant protection from T1D even in the presence of predisposing haplotype (Todd and Wicker, 2001). Similarly, the NOD mice express the unique H2-A^{g7} class II molecule which is not expressed in other conventional inbred mice strains and transgenic expression of other MHC class II molecules in NOD mice significantly protects from spontaneous diabetes, suggesting MHC class II molecules can predispose to or protect from T1D (Wicker *et al.*, 1995). Given the dominant roles of MHC class II molecules and the MHC class II-restricted $CD4^+$ T cells in the immunopathogenesis of T1D, delineating the exact mechanisms by which certain MHC class II molecules predispose to development of T1D have been difficult to pinpoint. However, several biological as well as computational experiments have provided mechanistic evidence supporting such associations.

HLA-DQ8, believed to be the highest contributor of genetic risk for T1D, has a strong structural and functional homology with the NOD MHC class II molecule, H2-A^{g7}. While the HLA-DQ and H2-A molecules

not associated with predisposition to T1D express the charged residue, aspartic acid at position 57 of the β chain, HLA-DQ8 has serine and I-A^{g7} has alanine at this position. Normally, the charged aspartic acid at position 57 on the β chain forms a salt bridge with arginine located at position 76 in the α chain of the class II molecule. Absence of aspartic acid in HLA-DQ8 and H2-A^{g7} changes the property of the peptide binding cleft, favoring the binding of antigenic peptides with negative charges particularly at position 9 or 10. X-ray crystallography and molecular modeling studies have shown that the non-charged aa at ß 57 in HLA-DQ8 and H2-A^{g7} results in a bigger P9 pocket in their peptide-binding cleft, capable of accommodating peptides with bulkier side chains (Godkin *et al.*, 1997; Lee *et al.*, 2001). This altered peptide presentation by HLA-DQ8 and H2-A^{g7} is thought to be important for the diabetogenic potential of HLA-DQ8 and H2-A^{g7}. Transgenic expression of H2-A^{g7} ß chain with substitutions at these two sites reduces the incidence of autoimmune diabetes in NOD mice (Lund *et al.*, 1990).

8.1. HLA class II transgenic mice models

Availability of mice transgenically expressing HLA molecules (either class I or II molecules) of interest either alone or in combination has tremendously helped in elucidating HLA association with various human autoimmune diseases including T1D. HLA-DQ8 and DR4 transgenic mice have helped in identifying the T-cell epitopes on islet autoantigens presented by these class II molecules (Herman *et al.*, 1999; Patel *et al.*, 1997; Raju *et al.*, 1997; Wicker *et al.*, 1996). However, with respect to T1D, the foremost important question to study using these mice was to verify whether transgenic expression of HLA-DQ8 and other disease-associated HLA class II molecules would lead to break down of tolerance to islet autoantigens, cause β cell destruction and culminate in hyperglycemia. We have shown that transgenic expression of T1D-associated class II molecules *per se* resulted in breakdown of tolerance to GAD65 (GAD65 is one of the autoantigens targeted by autoantibodies and T cells in T1D). Transgenic mice expressing HLA-DQ8 and/or HLA-DR3 showed spontaneous proliferative T-cell responses to native GAD65 as well as its peptides and produced proinflammatory cytokines during this process (Abraham *et al.*, 2000, 2001). In addition, immunization of HLA-DQ8 transgenic mice with GAD65 peptides elicited a robust T-cell response. Adoptive transfer of these GAD65-reactive, HLA-DQ8-restricted $CD4^+$ T cells in to naïve HLA-DQ8 recipients could cause insulitis but not diabetes (Wen *et al.*, 1998). HLA-DQ8 can also positively select $CD4^+$ T cells expressing the BDC2.5 TCR (specific for an islet autoantigen presented in the context of H2-A^{g7}) and cause diabetes in HLA-DQ8-BDC2.5 transgenic mice (Wen *et al.*, 2002). These observations strengthened the notion that

HLA-DQ8 and HLA-DR3 are pro-diabetogenic, islet-autoreactive T cells are present among the peripheral T-cell pool and antigen-specific immune responses could be elicited by appropriate stimulation. Nevertheless, full-blown diabetes was seldom seen in these mice, probably due to absence of certain additional genetic factors and/or environmental stimuli. Provision of certain local immunological stimuli, such as the co-stimulatory molecule, B7.1, was sufficient to precipitate spontaneous clinical hyperglycemia in naïve HLA-DQ8 transgenic mice.

HLA-DQ8, but not HLA-DQ6 transgenic mice or C57Bl/6 mice expressing B7.1 on the β cells of islets of Langerhans under the control of rat insulin promoter (RIP) developed spontaneous diabetes (Rajagopalan *et al.*, 2003; Wen *et al.*, 2000). However, a HLA-DQ8 transgenic line expressing the proinflammatory cytokine, GM-CSF also under the control of RIP did not develop spontaneous diabetes (Rajagopalan *et al.*, 2007). This suggested the requirement for both susceptible HLA class II allele and the appropriate immunological stimulus to precipitate diabetes. Splenocytes from diabetic HLA-DQ8.RIP-B7 transgenic mice could proliferate in response to purified islets and were able to induce hyperglycemia in normal HLA-DQ8.RIP-B7 recipients following adoptive transfer, confirming that the disease is HLA-DQ8 restricted and T cell mediated (Wen *et al.*, 2000). Infiltration of islets with T and B cells also indicated an immune mediated event (Fig. 2.7). Interestingly, RIP-B7.1 transgenic mice expressing HLA-DR4 (DRB1*0401) had a low incidence of spontaneous diabetes when compared to HLA-DQ8.RIP-B7 mice. The incidence of diabetes in RIP-B7.1 mice expressing both HLA-DR4 and HLA-DQ8 was reduced to the levels seen in HLA-DR4 transgenic mice, indicating that HLA-DR4 might play a regulatory role. Purified T cells from HLA-DQ8.RIP-B7 transgenic mice produced more of Th1 type cytokine, IFN-γ and less of Th2-type cytokine, IL-4 upon stimulation with GAD65 *in vitro* (Wen *et al.*, 2001). However, T cells from HLA-DR4.RIP-B7 and HLA-DR4.DQ8.RIP-B7 transgenic mice produced more of IL-4 and less of IFN-γ, providing mechanisms behind DR4-mediated immune regulation (Wen *et al.*, 2001). The immunoregulatory role of HLA-DR4 was confirmed using a different mouse model of T1D (Gebe *et al.*, 2007a, b). HLA-DR4 transgenic mice also transgenic for the α and β chains of a TCR recognizing a HLA-DR4-restricted, GAD65 peptide (555–567)-specific, human $CD4^+$ T-cell clone developed insulitis, showed impaired glucose tolerance but did not develop spontaneous hyperglycemia. Notably, the TCR-HLA-DR4 double transgenic mice harbored significantly elevated numbers of Foxp3 + $CD4^+$ $CD25^+$ regulatory T cells, possibly explaining the mechanism of immunoregulation by HLA-DR4. Unlike HLA-DR4, HLA-DR3 did not down regulate the incidence of T1D in RIP-B7 transgenic mice (Rajagopalan *et al.*, 2003). HLA-DR3.RIP-B7 mice had T1D incidence comparable to that of HLA-DQ8.RIP-B7 transgenic mice and HLA-DR3.

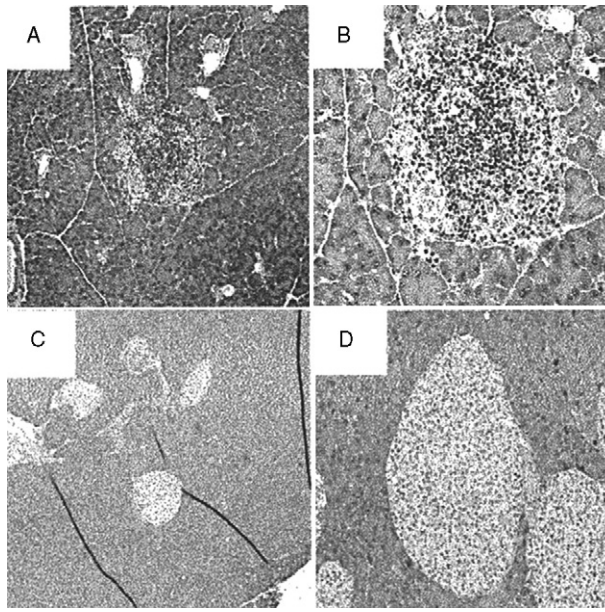

FIGURE 2.7 HLA-DQ8.RIP-B7-1 transgenic mice develop spontaneous diabetes. Pancreata from a hyperglycemic mouse and an age- and sex-matched euglycemic littermate were fixed in buffered formalin. Thin sections were stained with hematoxylin/eosin and evaluated microscopically. (A, B) Hyperglycemic DQ8.RIP-B7-1 mice. (C and D) Non-diabetic littermate. Magnification: A and B, × 10; C and D, × 20. (Reproduced from *Int Immunol*, Rajagopalan *et al.*, 2003, 15, 1035, with permission from *Oxford University Press*.)

DQ8.RIP-B7 double transgenic mice had slightly higher incidence of T1D compared to the two parental lines. Thus, different HLA-DR molecules seem to have differential effects on the immunopathogenesis of T1D, similar to the observations made in human T1D (Redondo and Eisenbarth, 2002; Wolf *et al.*, 1984).

Thus, studies using HLA class II transgenic mice suggest that transgenic expression of T1D-associated class II molecules can lead to generation of autoreactive T cells. While these T autoreactive cells somehow remain ignorant of the autoantigen in vivo, appropriate inflammatory stimuli (such as B7.1 or TNF-α) can lead to breakdown of this ignorance and result in β cell destruction and hyperglycemia. A similar scenario can be applicable to human T1D. Not all individuals expressing the T1D-associated haplotypes develop the clinical disease. Indeed studies have shown that healthy individuals do harbor autoreactive T cells against multiple autoantigens. However, some inflammatory stimuli (such as infection), could break this tolerance and render these ignorant

autoreactive T cells pathogenic. The nature of the inflammatory stimuli necessary to break this tolerance could be determined by other genetic events in that individual (other IDDM alleles). For example, molecules such as CTLA4, PTPN22, IL-2 and Foxp-3 play important roles in regulation of the immune response. Polymorphisms/mutations in these genes are known to be associated with T1D. Therefore, expression of T1D-disease associated HLA haplotype(s) together with polymorphic variations at one or more of these loci could thereby determine the threshold of the inflammatory stimuli required to break self tolerance and result in autoimmune disease. Selective manipulation of the HLA class II transgenic mice at one or more of these non-MHC loci could help to recapitulate the human disease and facilitate better understanding of the disease process.

9. EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

Myasthenia gravis (MG) is an important model for the study of molecular mechanisms of autoimmunity since a target autoantigen is known (nicotinic acetylcholine receptor-AChR), is sequenced and well characterized (Lindstrom *et al*., 1988). Muscle AChR is formed by four homologous subunits (Conti-Tronconi *et al*., 1994). AChR specific $CD4^+$ T cells from MG patients are known to recognize several epitopes on all the muscle AChR subunits (Protti *et al*., 1993). MG is an antibody mediated disease as antibodies have been shown to reduce the number of available muscle AChRs (Drachman, 1994). $CD4^+$ T cells are required for generation of the high-affinity IgG antibodies that bind muscle AChR (Lennon *et al*., 1976). Initial epidemiological studies demonstrated that HLA-DR3 is linked to MG in Caucasians (Naeim *et al*., 1978). Even though HLA-DQ polymorphism in MG pathogenesis has been found, the role of individual HLA-DQ genes is not very clear (Bell *et al*., 1986). Several different DQ genes have been implicated in MG in different ethnic populations and geographical locations. In mice, immunization of the Torpedo acetylcholine receptor generates autoimmune myasthenia gravis very similar to the human disease. Strong MHC linkage has been found in the mouse model with the H2b haplotype being the most susceptible strain (Christadoss, 1989). Using a mutation within the Aβ gene of the $H2^b$ haplotype, which differs from the parent at 3 amino acid residues 67, 70, and 71, the binding region of the autoantigen peptide has been narrowed down (Infante *et al*., 1991). In order to determine the role of HLA-DQ polymorphism in myasthenia gravis, HLA-DQ6 and DQ8 transgenic mice were tested in the mouse model of experimental autoimmune myasthenia gravis (EAMG). Torpedo acetylcholine receptor purified from the Torpedo electric eel was injected into the mice subcutaneously. The mice were given two boosters at four week intervals. Muscle weakness was assessed every week in a blind

study and graded from 0 to 3. The disease was confirmed in mice showing muscle weakness by measuring the neuromuscular transmission failure in the junction. Antibodies against the acetylcholine receptor were also measured in all the mice.

HLA-DQ8 transgenic mice were highly susceptible to EAMG (Raju *et al.*, 1998). Clinical symptoms were observed three weeks after the third immunization and antibody titers remained high after a second immunization. There were clear differences in the forces evoked by muscle stimulation versus nerve stimulation, reflecting extensive neuromuscular transmission failure. HLA-DQ6 mice showed only moderate susceptibility to EAMG. The Aβ° mice did not show any clinical signs of muscle weakness, confirming the role of the transgenic HLA-DQ molecules in the disease process. Our studies confirmed the role of the DQ8/DR4 haplotype in human MG linkage studies. The DQ6 mice (DQB1*0601) have a much milder disease, whereas the human linkage was reported with DQB1*0604. To comprehend the reduced susceptibility of HLA-DQ6 transgenic mice in comparison with HLA-DQ8 mice, cytokine responses to AChR were examined. DQ6 mice exhibited significantly reduced *in vitro* lymphoproliferative and cytokine responses to AchR, compared to DQ8 mice. Resistance of DQ6 transgenic mice to EAMG was linked to a dramatic suppression of AchR-specific IFN-γ, IL-2, and IL-10 productions by AChR-primed lymph node cells (Poussin *et al.*, 2001; Raju *et al.*, 2002).

Since HLA-DR3 has the highest relative risk for MG, we looked at the susceptibility of DR3 transgenic mice in EAMG. 90% of the HLA-DR3 transgenic mice developed clinical signs of EAMG (Raju *et al.*, 2001). The DR3 mice showed a severe neuromuscular transmission failure. We analyzed the major epitopes on the human acetylcholine receptor which may be involved in the induction of MG. The DR3 transgenic mice were immunized with hAChR alpha chain extracellular region peptides. We found significant immune response to a limited number of sequence regions, 31–50, 41–60, 141–160, 171–190, 181–200, and 191–210. Peptides 141–160 and 171–190 induced consistently high T-cell response. The sequence of the peptide 141–160 is homologous between mouse and human AChR alpha chain, while the sequence 171–190 differs from the mouse at two residues. To understand the minimal T-cell determinant on the peptide 141–160, the peptide was increasingly truncated from carboxy and amino terminal by two amino acids. The DR3 mice were immunized with a full length peptide and lymph node cells were *in vitro* challenged with each of the truncated peptides. On the basis of these studies, we found that sequence 149–158 encompasses the minimal T-cell determinant. This ten amino acid peptide contains tryptophan at position 1 and aspartic acid a negatively charged residue at position 4. Truncation analysis of the peptide 171–190 showed a 12 amino acid minimal epitope 175–186. Next, we studied the binding affinity of a panel of overlapping

human AChRα subunit peptide for DR3 molecule. We found 5 high-affinity binding peptides. Among the 5, three peptides 36–49, 145–163, and 195–212 were the same peptides that also gave strong T-cell response in DR3. The peptides 141–160 and 171–190 also gave high T-cell proliferation in HLA-DQ8 and HLA-DQ6 mice. Thus, these two epitopes may be the promiscuous epitope on the AChRα subunit stimulating T-cell responses in Myasthenia gravis.

10. HLA AND GLUTEN SENSITIVITY-CELIAC DISEASE AND *DERMATITIS HERPETIFORMIS*

In order to understand the mechanisms involved in the initiation of celiac disease, *in vivo* (animal) models with significant similarities to human disease need to be generated. The ideal animal model of celiac disease would be one in which both the initiation and disease progression could be studied. With such a model, potential treatments could be administered and their effectiveness determined before clinical trials are conducted on the celiac patients (Stazi and Mantovani, 2002). The elements that need to be present in such an animal model are those that are typically associated with celiac disease. These would include: marked villous atrophy, increased numbers of intra-epithelial lymphocytes (IELs), gluten sensitivity that can be resolved with a gluten free diet, a tight association with the MHC II, and circulating IgA antibodies directed against tissue transglutaminase (tTG).

The tight association between HLA class II and celiac disease, specifically HLA-DQ2 and HLA-DQ8 is well established. To determine if the DQ8 transgenic mice could recognize gluten in an MHC class II specific manner, we immunized the transgenic mice with gluten in complete Freund's adjuvant. Spleen cells were isolated and proliferation assays were performed. Using a monoclonal antibody directed against the beta chain of the DQ molecule in inhibition assays, it was determined that these mice can recognize gluten in the context of DQ8. In addition, the response was dependent upon the specific HLA allele because HLA-DQ6 transgenic mice did not respond as well to gluten as the DQ8 transgenic mice (Black *et al*., 2002). With respect to recognition of deamidated gliadin, proliferation assays performed on lymphocytes isolated from the spleen of HLA-DQ8 transgenic mice gave results similar to those using human lymphocytes from celiac patients. ELISA assays performed on sera from gluten challenged mice also determined that significant levels of gliadin specific IgG existed within the sera of immunized mice. These results therefore demonstrate that strong DQ8 specific T and B cell responses against gliadin are generated in these HLA-DQ8 transgenic mice, lending support for the use of these mice as models for celiac disease.

We have also used these mice to demonstrate that the route of administration affects which epitopes of gliadin are recognized by the DQ8 molecule. Specifically, an oral route of gluten administration led to a strong response against two non-deamidated α-gliadin peptides P13 (aa120–139) and p23 (aa220–239). As ''native'' peptides of α-gliadin, these may have an important role in the initiation of disease (Senger *et al.*, 2005). Surprisingly though, histopathology of the small bowel of these mice revealed normal bowel architecture with no villous atrophy or crypt hyperplasia despite the different route of administration. They also did not produce any IgA antibodies against tTG. This would indicate that the autoimmune component of celiac disease was lacking in these mice, despite the strong and varied responses against gliadin and deamidated gliadin epitopes. Thus, in these transgenic mice, DQ8 alone conferred gluten sensitivity but not symptomatic autoimmune disease. Therefore, an element of predisposition towards autoimmunity needed to be introduced into the model.

10.1. Gluten sensitive blistering in NOD.DQ8.Aβ° mice

Non-obese diabetic mice (NOD) spontaneously develop insulin dependent diabetes and thus have been a useful animal model in the study of the autoimmune disease, IDDM. A number of studies have demonstrated that these mice generate a limited immune response towards gluten as well. Based on this data, the NOD background was introduced into the DQ8 transgenic mice under the theory that the introduction of a genetic background associated with autoimmunity may allow symptomatic celiac disease to develop.

Interestingly, some of the NOD-DQ8 transgenic mice that had been sensitized to gluten and later fed gluten developed blistering on the ears (Marietta *et al.*, 2004) (Fig. 2.8). The blisters developed as small white papules which progressed to form erythematous erosions that subsequently scabbed. Hematoxylin and eosin staining of skin biopsies from the ears revealed subepidermal blisters with upper to mid dermal inflammatory infiltrate. The infiltration consisted of neutrophils, eosinophils, histiocytes, and lymphocytes. Direct immunofluorescence analysis of the perilesional skin biopsies also showed granular IgA deposition at the basement membrane and the tips of the dermal papillae. No IgA deposits were observed on areas that did not blister, such as the skin on the back of the mice. When these mice were placed onto a gluten free diet, the blistering resolved in 2–3 weeks. Administration of dapsone resulted in a faster resolution, similar to that observed in patients. All of these symptoms are similar to those observed in *Dermatitis herpetiformis* (DH), which is the skin manifestation of celiac disease. Thus, this was characterized as the first animal model of DH.

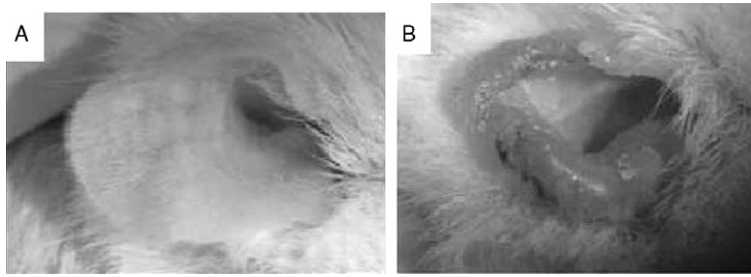

FIGURE 2.8 Gluten dependent cutaneous blistering in NOD.DQ8.Aβ° transgenic mice. (A) Normal unaffected ear. (B) Blistered ear of an NOD.DQ8.Aβ° mouse sensitized to gluten. (Reproduced from *J Clin Invest*, Marietta *et al.*, 2004, 114, 1090, with permission from *American Society for Clinical Investigation*.)

10.2. Gluten sensitive enteropathy on NOD.DQ8.Aβ° mice

At the same time some NOD.DQ8.Aβ° mice, after gluten sensitization and oral challenge with gluten, lost weight and demonstrated an overall appearance of fatigue. These mice produced IgA antibodies to gliadin and also IgA against tissue transglutaminase (Black *et al.*, 2002). When examined histologically, the small intestine had an increased number of lymphocytes and some decrease in the villous height. There was also a direct correlation between the presence of circulating IgA against tTG and the presence of enteropathy. Thus, the addition of the NOD background to the HLA-DQ8 transgenic mouse led to the development of symptomatic disease in these mice.

The results with the NOD.DQ8.Aβ° mice would demonstrate that genes present in the NOD background are necessary for the development of symptoms similar to celiac disease and *Dermatitis herpetiformis* in DQ8 transgenic mice. Many genes present in the Idd loci of the NOD background could be contributing to this phenomenon. The genes most likely to be contributing would be those that predispose the nod mice towards developing autoimmunity. Supporting this theory is the fact that patients with celiac disease and *Dermatitis herpetiformis* are also predisposed towards developing other autoimmune diseases such as diabetes and thyroiditis (Schuppan and Hahn, 2001). The ultimate goal for generating an animal model of celiac disease is to use them for testing novel therapies. Administration of α-gliadin intranasally before parenteral injection of gliadin decreased the T-cell response towards gliadin in these gluten sensitive HLA-DQ8 mice (Senger *et al.*, 2003). IFN-γ was significantly downregulated in this particular protocol for generating tolerance to gluten. This approach holds great promise as a therapy for both celiac disease and *Dermatitis herpetiformis*.

11. RELAPSING POLYCHONDRITIS

Relapsing polychondritis (RP) characterized with recurrent episodes of inflammation resulting in destruction of cartilaginous tissues, especially the pinnae, nose, and tracheobranchial cartilage inflammation of the outer ear is an autoimmune disease of unknown etiology (Zeuner *et al.*, 1997). Bilateral auricular chondritis is the most common clinical manifestations of RP with 90% of patients developing it (Kent *et al.*, 2004; Letko *et al.*, 2002). Auricular Chondritis is characterized by bilateral pain, erythema and inflammation of the cartilaginous tissue of the outer ear accompanied by a mononuclear infiltrate. RP patients also develop polyarthritis, nasal chondritis, and ocular/renal involvement. Mortality is significantly elevated with the onset of RP, with one fourth of the patients dying within 5 years of diagnosis because of the involvement of laryngotracheal cartilage and cardiovascular problems associated with RP. Predisposition to RP has been reported to be significantly associated with the presence of HLA-DR4, a class II allele known to be associated with rheumatoid arthritis (RA), although no predominance of DR4 subtypes has been observed (Buckner *et al.*, 2002; Lang *et al.*, 1993). Cell-mediated immunity and anti-CII–specific antibodies have been observed in both RA and RP patients (Foidart *et al.*, 1978; Terato *et al.*, 1990). In addition, circulating autoantibodies against type IX and type XI collagens are also observed in patients (Alsalameh *et al.*, 1993; Yang *et al.*, 1993). Type II collagen is a putative autoantigen for both RA and RP, although the antigen specificity is different. T cells directed against type II collagen (CII) with specificity to peptide 261–273 have been identified in a patient with RP (Buckner *et al.*, 2002).

Models of RP in susceptible strains of mice following immunization with CII have been reported (Cremer *et al.*, 1981; McCune *et al.*, 1982). A mouse model of autoimmune ear disease required both immunization with CII and trauma to the ear to induce inflammatory response (Yoo *et al.*, 1994). Some strains of rats and mice have been shown to develop only the respiratory features of RP, as seen by immunizing animals with matrilin-1, protein predominantly expressed in tracheal cartilage (Hansson *et al.*, 1999). In this model, the trachea was affected with no involvement of joints and outer ears. In some rats, auricular chondritis may appear spontaneously without other features of polychondritis (Chiu and Lee, 1984; McEwen and Barsoum, 1990).

11.1. DQ8/DQ6.Aβ° transgenic mice

Transgenic mice expressing both DQ8 (DQA1*0301/ DQB1*0302) and DQ6 (DQA1*0103/ DQB1*0601) molecules deficient in endogenous class II molecules were shown to develop experimental polychondritis,

exhibiting both polyarthritis and auricular chondritis following immunization with type II collagen (Bradley *et al.*, 1998). Auricular chondritis, however, was seen in only 25% of the immunized animals even though this was not seen in parental strain. A strong anti-CII-specific humoral and cellular response was observed in primed mice. Since neither DQ6 nor DQ8 transgenic mice develop auricular chondritis, it is likely that a mixed haplotype DQA1*0103/DQB1*0302 molecule is able to present new epitopes of CII not involved in RA, or present a cross reactive epitope leading to stimulation of T cells autoreactive for Type IX and/or XI collagen homologous with type CII. Studies in our laboratory showed that double transgenic mice do have anti-type IX collagen antibodies. Recently, mice expressing alpha chain of DQ6 and beta chain of DQ8 molecule have been shown to develop spontaneous auricular chondritis in 30% of mice (Lamoureux *et al.*, 2006). The disease is remitting/relapsing as shown in humans and occurs more often in female mice. In humans a role of such hybrid class II molecules has not been shown in pathogenesis. These mice provide an excellent tool to unravel the genetic predisposition in other autoimmune disorders where unique mixed haplotypes might be involved.

11.2. NOD.DQ8.Aβ° mice

We described an experimental model of polychondritis in which presentation of an autoantigen by DQ8 molecule leads to development of polyarthritis and chondritis (Taneja *et al.*, 2003). DQ8 transgene in B10 background lacking endogenous class II molecules develop polyarthritis but no chondritis. However when DQ8 mice were bred on NOD background, immunization with CII led to development of polychondritis, auricular chondritis and polyarthritis, in 85% of animals with clinical and histological similarities to relapsing polychondritis in humans. NOD.DQ8 mice generated T-cell response and antibodies to CII and type IX collagen while B10.DQ8 mice only generated response to CII. Further, immunization of NOD.DQ8 mice with CIX led to auricular chondritis without arthritis, suggesting CIX as an autoantigen for polychondritis. NOD.DQ8 mice have higher number of activated T cells and lower number of regulatory T cells than B10.DQ8 mice. Also, activated B cells expressing CD25 were much higher in NOD.DQ8 mice compared to B10.DQ8 mice which could explain higher levels of autoantibodies in NOD.DQ8 mice. Transgene negative littermates did not develop any disease. This suggested that while DQ8 is necessary for presentation of an autoantigen in this model, non-MHC genes also play a role.

Polychondritis observed in NOD.DQ8 mice shares similarities with RP (Fig. 2.9). As observed in patients, NOD.DQ8 mice developed auricular chondritis as first sign of clinical disease that was characterized by

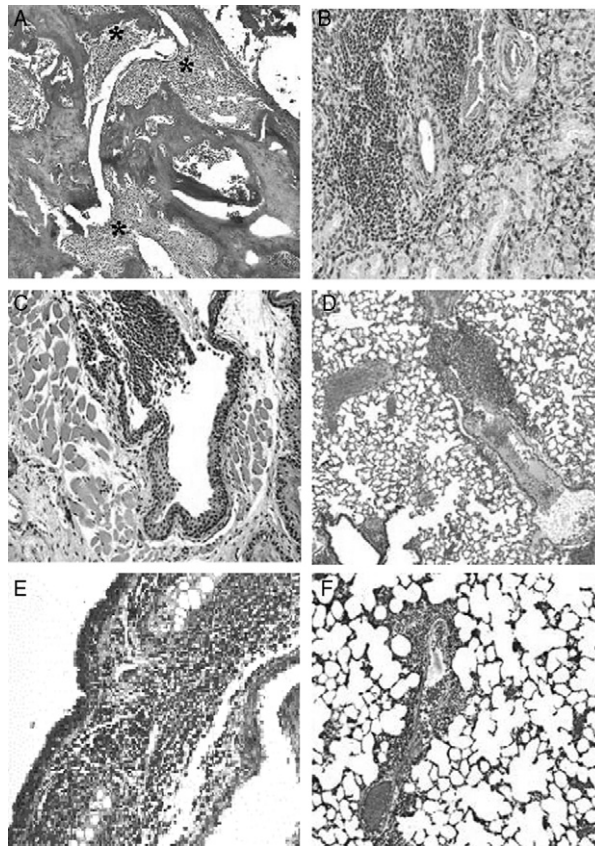

FIGURE 2.9 CII induces relapsing polychondritis in NOD.DQ8.Aβ° transgenic mice. (A) Representative inflammatory arthritis of knee from a NOD.DQ8.Aβ° mouse. Significant mononuclear infiltration with cartilage destruction and pannus formation is seen (*). (B) Salivary glands showing mononuclear infiltrate suggestive of sialadentitis. (C) Infiltration with mononuclear cells of trachea. (D) A section of lung shows perivascular infiltration in lungs. Accustain Trichrome Staining of frozen sections of trachea (E) and lungs (F). Only trachea showed deposition of collagen as seen by the blue color. All micrographs are at ×100 magnification, E and F are at ×200 magnification. (Reproduced from *J Clin Invest.*,Taneja *et al.*, 2003, 112, 1843, with permission from *American Society for Clinical Investigation*.)

bilateral erythema and swelling of the outer ears with massive infiltration of mononuclear cells. More $CD4^+$ cells are seen at the initiation of disease thus suggesting that presentation of an epitope to CD4 T cells might be important for pathogenesis. Involvement of B cells occurs later in the course of disease with B cells resembling lymphoid follicles. Auricular chondritis was followed by polyarthritis characterized by infiltration of

mononuclear cells and pannus formation in around 70% of mice. Histopathology of various organs showed infiltration of mononuclear cells in trachea and perivascular region of lungs with collagen deposition in trachea in some mice. Thus, these mice develop all histopathological manifestations seen in relapsing polychondritis patients. The NOD.DQ8 mice provide a model of RP with a high disease incidence and multiple organ involvement to investigate putative autoantigen.

Taken together these data show that like most of the autoimmune diseases both, MHC and non-MHC genes, are involved in pathogenesis of polychondritis. Since some of the genes are similar to those involved in RA, similar pathogenetic mechanisms may be true in pathogenesis of RP and RA. However, since polychondritis occurs at various locations, it is possible that several autoantigens may be involved.

12. SPONTANEOUS AUTOIMMUNE MYOCARDITIS

Heart disease is a major cause of mortality in advanced countries. Clinical manifestations of disease are varied and non-specific for diagnosis. Evidence of myocarditis has been demonstrated in 20% of autopsy cases of presumed healthy individuals below 40 years of age. Myocarditis is an inflammatory heart disease defined pathologically as mononuclear cell infiltration associated with myocyte necrosis and degeneration. It can progress to dilated cardiomyopathy (DCM), heart failure and death (Kim *et al*., 2002). A progression from viral myocarditis to dilated cardiomyopathy has been hypothesized even though the exact pathogenic mechanism remains uncertain (Rose, 2000). Several studies have indicated an aberrant immune response following viral-mediated damage to myocytes (Bachmaier *et al*., 2000; Olinde and O'Connell, 1994). A wide variety of pathogens such as viruses, bacteria and protozoa have been associated with inflammatory heart disease. Molecular mimicry, bystander activation, and viral persistence with or without epitope spreading are three mechanisms that can initiate immunoreactivity leading to autoimmune disease (Fujinami *et al*., 2006).

Genetic influence on the development of myocarditis is evident by familial aggregation and association of subsets of patients with HLA antigens. Presence of antibodies to cardiac antigens, association of DR4 with DCM and abnormal expression of HLA class II on cardiac endothelium underscore the influence of genetic factors in involvement of autoimmune processes (Limas, 1996; Lozano *et al*., 1997). Individuals with HLA-DR4/DR1 and HLA-DQ1, DQ5 have been shown to have increased susceptibility to myocarditis and dilated cardiomyopathy or both (Caforio *et al*., 2002). Patients with serum antibodies against β1-adenoreceptor had decreased frequency of DR3 compared to patients negative

for antibodies. HLA-DQB1*0602 has been associated with clinically overt disease and autoantibodies (Limas *et al.*, 1995).

As in humans, experimental myocarditis can be induced by infection with coxsackie B3 virus of susceptible mouse strains (reviewed in Penninger and Bachmaier, 2000). It is characterized by inflammation of the heart and myocyte damage. In viral-induced myocarditis models, age, sex and strain of mouse and virus affect the induction of disease. Studies in this model suggest that viral infection initiates the onset while T cells play an instrumental role in disease pathogenesis.

The pathogenesis of myocarditis is not fully understood, but there is sufficient evidence that autoimmune response to cardiac myosin following infection in human and mouse model may contribute to the disease process. Experimental autoimmune myocarditis resembling human DCM can also be induced in susceptible mice by immunization with purified foreign and murine cardiac specific α-myosin (Wang *et al.*, 1999). Role of HLA molecules was shown by HLA-DQ6 transgenic mouse in which myocarditis could be induced by immunization with peptides of cardiac myosin (Bachmaier *et al.*, 1999). All models of induced autoimmune myocarditis are strain-dependent, suggesting MHC class II as an important genetic factor for susceptibility. The histopathology in animals showed mononuclear infiltrates consisting majority of CD4 T cells and antigen-presenting cells with various other cell types. Inflammation of the heart muscle has been transferred to naïve animals by purified T cells from mice with active disease suggesting it to be a T-cell-mediated disease. MHC class II expression and other adhesion molecules have been shown to precede cellular infiltration of T lymphocytes into the heart (Smith and Allen, 1992).

12.1. Spontaneous myocarditis in NOD.DQ8.Aβ° mice

Backcrossing of HLA-DQ8 mice with NOD mice for up to 15 generations led to high mortality in female transgenic mice (Taneja *et al.*, 2007). Autopsy of these animals revealed several fold enlargement of the heart (Fig. 2.10). The echocardiography showed biventricular dilation with reduced systolic function in affected mice, which is similar to patients (Kim *et al.*, 2002). Histological examination of the NOD.DQ8 hearts revealed a widespread, mixed inflammatory infiltrate containing predominantly lymphocytes and histiocytes and lesser numbers of neutrophils and eosinophils. The inflammatory lesions were associated with myocyte destruction but no fibrosis, granuloma, or giant cells. There was a marked increase in the expression of DQ8 in these mice similar to what is known from biopsies of myocarditis patients (Caforio *et al.*, 2002). Another similarity with human patients was an

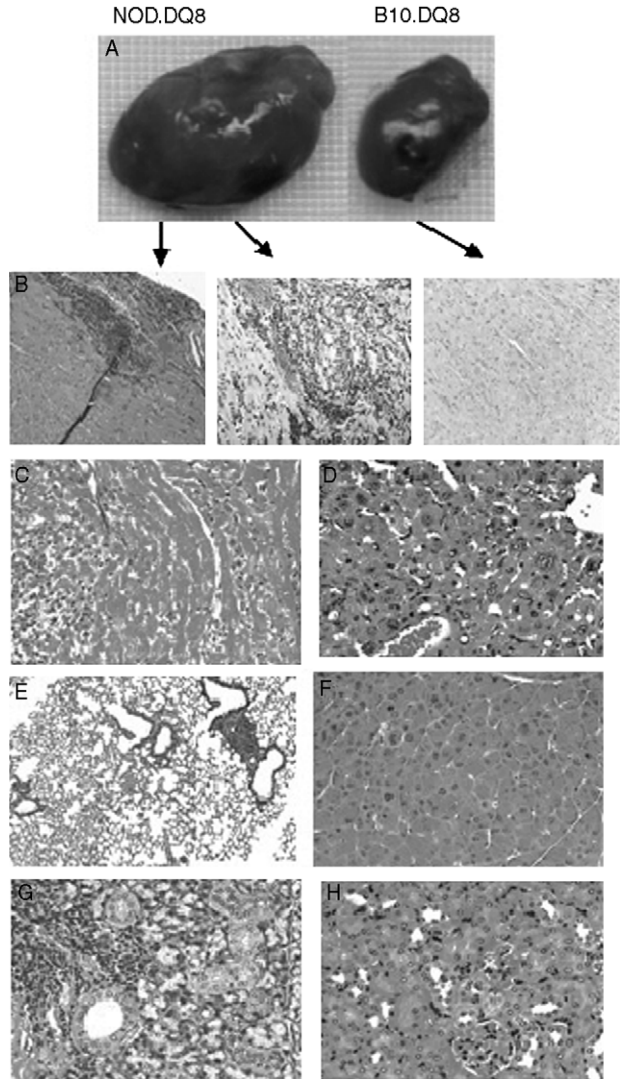

FIGURE 2.10 NOD.DQ8 mice develop myocarditis progressing to dilated cardiomyopathy. (A) 16 week old NOD.DQ8 mice with severe edema and enlarged heart compared to healthy age and sex matched B10.DQ8 mice. (B) Mononuclear infiltration observed in heart of NOD.DQ8 mouse first appeared as focal (left), which progressed to diffused with myocyte necrosis (middle) while B10.DQ8 (right) did not show any infiltration. Micrographs are at ×100 magnification. (C) Trichrome stain of heart section shows mononuclear infiltration but no fibrosis, ×400 magnification. Haematoxylin and Eosin stained sections of (D) Liver (E) Lungs (F) Pancreas (G) Salivary glands, and (H) Kidney. All Micrographs are ×400 magnification but that of panel E is at ×100. (Reproduced from *J Mol Cell Cardiol.*,Taneja *et al.*, 2007, 42, 1054, with permission from *Elsevier Limited*.)

increased level of anti-Tissue transglutamase antibodies in mice with myocarditis (Curione *et al.*, 1999; Frustaci *et al.*, 2002). Thus, DQ8 might be able to positively select T cells with specificity to cardiac antigens that are able to present self–peptides in heart of NOD.DQ8 mice. Spontaneous autoimmune myocarditis in NOD.DQ8 mice is the result of loss of tolerance to self-cardiac myosin leading to cellular and humoral response to it. Data from two other laboratories also observed the same phenomenon (Elliott *et al.*, 2003; Taylor *et al.*, 2004). Using various knockouts with DQ8 transgene and adoptive transfer, it was shown that CD4 is a major effector cell while CD8 initiates the disease (Hayward *et al.*, 2006).

DR3 transgene in NOD background did not lead to any spontaneous autoimmunity suggesting polymorphism in class II is important in predisposition to myocarditis. Lack of disease in B10.DQ8 mice demonstrates that NOD background influences the pathology. However, DQ8 is required since transgene negative littermates, NOD.DR3 and NOD mice do not develop this disease. The observations suggest that like other autoimmune disorders, disease expression in these mice requires both HLA-DQ8 and NOD-derived genes promoting loss of tolerance to self myosin.

13. EXPERIMENTAL AUTOIMMUNE THYROIDITIS (Adapted from Kong *et al.*, 2007)

In contrast to the well-established H2-linkage in susceptibility to murine Experimental autoimmune thyroiditis (EAT) for almost 30 years (Vladutiu and Rose, 1971), the HLA association to Graves' disease (GD), and in particular, Hashimoto's thyroiditis (HT) had remained controversial, even to the late '90s despite improved typing techniques and implied association with both HLA-DRB1*0301 (DR3) and DRB1*1101 (DR5) in Caucasians (Vaidya *et al.*, 2002). Delineation in polygenic patient populations was complicated by linkage disequilibrium of various DRB1 loci with particular HLA-DQ loci, ethnic variations, multiple epitopes on thyroid antigens and environmental factors, may contribute to the imprecise HLA association. In order to examine if the DRB1 locus was a determinant in EAT susceptibility, HLA-DR3 transgenic mice were analyzed for EAT. Thyroiditis was observed after either mouse (m) or human (h) thyroglobulin (Tg) immunization. None of the DR2 (DRB1*1502) or DR4 (DRB1*0401) transgenic mice responded to hTg or mTg immunization. DR3 + mice develop mild to severe thyroiditis with 100% incidence. Thus, DRB1 polymorphism determines susceptibility to EAT confirming DR3 association in patient studies (Kong *et al.*, 1996).

13.1. Class II gene interactions in double transgenic models

The multiple class II genes in humans may lead to enhancement or protection in autoimmune diseases (Todd *et al.*, 1988). Thus, co expression of double transgenes may alter the response of susceptibility alleles. DR3 transgenic mice were mated to DR2 and DR4 transgenic mice to generate double transgenic mice. After immunization with hTg and/or mTg, no significant reduction in thyroiditis incidence or severity can be discerned. Thus, DR2 and DR4 molecules are permissive for EAT, and neither enhance nor protect the disease. The presence of DQ6 in DR3/DQ6 double transgenic mice did not affect the strong susceptibility status of DR3 in response to either hTg or mTg induction. In contrast, the coexpression of DQ8, nonpermissive for mTg-induced EAT, significantly lowered the incidence and severity in B10.DR3, but not in autoimmunity-prone NOD background (Flynn *et al.*, 2002). However, after EAT induction with hTg, reduced thyroiditis severity was observed in NOD. DR3DQ8 double transgenic. The mechanisms of protection that seem to depend on the particular gene pairing and background genes remains to be unraveled.

13.2. Pathogenic htg peptides in HLA-DR3 transgenic mice

As with HT, EAT is a T-cell-mediated autoimmune disease, and the linear T-cell epitopes derived from Tg are presented by class II molecules, such as DR3. Tg is the largest autoantigen known, a 660 kDa homodimer of ~2,750 residues with no demonstrable immunodominant, thyroiditogenic T-cell epitopes (Carayanniotis and Kong, 2000); hTg shares ~73% homology with mTg (Caturegli *et al.*, 1997). To facilitate the identification of DR3-binding epitopes on hTg which are naturally processed, we screened computer-predicted, potential DR3-binding peptides which had been synthesized as 15–23 mers (Flynn *et al.*, 2004b). The peptides were added to cultured splenocytes from hTg-immunized DR3 $^{+}$ mice on either B10 or NOD background. Of the 39 soluble peptides, 4 stimulated hTg-primed cells from either background. The peptides were DR3-restricted, as hTg-primed cells from NOD.DQ8^{+}.Aβ$^{\circ}$ mice did not respond to the peptides. One 15 mer peptide, hTg2079, has been studied in more depth, as it activated sufficient T cells for thyroiditis transfer into 100% normal recipient mice, indicating that it is a thyroiditogenic peptide that is naturally processed. To determine if it could induce EAT, we immunized NOD/DR3^{+}.Aβ$^{\circ}$ mice with hTg2079 in either LPS or CFA as adjuvant and 50–70% of the mice developed mild thyroiditis. When peptide-primed cells were further expanded *in vitro*, thyroiditis increased to cover up to 35% of the gland. Interestingly, the identical sequence was found independently after sequencing peptides eluted from DR3-associated peptide complexes

isolated from thyroid infiltrates of a GD patient, confirming the natural ligand property of this peptide (Kong *et al.*, 2007). This correlation indicates the utility of HLA class II humanized models for study.

13.3. Replicating human thyroid autoimmune disease by immunization with human thyroid peroxidase and TSH receptor

These studies were carried out to determine if the DR3 gene encodes susceptibility to the other two major thyroid autoantigens, human thyroid peroxidase (hTPO) and human TSH receptor (hTSHR) and thereby serving as HT and GD models with the generation of thyroiditis and thyroid-stimulating antibodies (TSAbs) respectively. Because of the scarcity of sufficient quantities of highly purified hTPO protein free of Tg contamination (and hence best obtained as a recombinant protein to minimize the effect of minute contamination of Tg) with faithful pattern of glycosylation, immunizing regimes such as plasmid immunization was utilized. Similarly, as TSHR protein does not lead to the induction of TSAbs (Karras *et al.*, 2005), plasmid immunization approaches were applied to developing a GD model of $DR3^+$ mice.

13.3.1. Induction of autoimmune thyroiditis with hTPO plasmid DNA:

To enhance the immune response to hTPO DNA, NOD/$DR3^+$.Aβ° mice were co-immunized with GM-CSF or IL-12, using a standard protocol of three injections over 6 weeks after cardiotoxin treatment to enhance uptake (Banga, 2007). When the mice were sacrificed at wk 14, only co-immunization with GM-CSF produced detectable anti-TPO titers. Nevertheless, thyroiditis was observed at equivalent incidence of 23% in both GM-CSF and IL-12, a Th1 cytokine, treated groups with up to 15% thyroid involvement. Low anti-mTg levels were observed but did not correlate with anti-TPO or pathology incidence. Interestingly, the anti-TPO profile mimics those present in thyroid autoimmune disease with preference towards immunodominant epitopes. The presence of TPO Abs in $DR3^+$ mice with mixed background genes has recently been confirmed after immunization with adenovirus-expressing TPO DNA, indicating the importance of DR3 allelic control of susceptibility in thyroid autoimmunity (Flynn *et al.*, 2004a).

13.4. Induction of graves' hyperthyroidism and thyroiditis with hTSHR plasmid DNA

NOD/$DR3^+$.Aβ° mice were immunized with hTSHR plasmid DNA alone, or with GM-CSF or IL-4, a Th2 cytokine, plasmids, with the intent of promoting ab production. Serial bleeds on wks 8, 11, and 14 showed

20% of mice with TSAbs, 25% with elevated T_4 levels, and a subset with both signs of hyperthyroidism resulting in ~30% with some aspects of autoimmune hyperthyroidism. Interestingly, ~12% displayed thyroid infiltration with destruction and occasionally low anti-mTG titers. That hTPO and hTSHR immunizations can lead to thyroiditis and hyperthyroid syndrome demonstrates that DR3 is a susceptibility allele, and that DR3-transgenic mice could be a potential GD model. Plasmid DNA is generally less immunogenic than the protein itself. The recent demonstration of the efficiency of inducing hyperthyroidism following TSHR plasmid delivery by electroporation may lead to a highly efficient GD model of DR3-transgenic mice (Flynn *et al.*, 2007).

In conclusion, the applications of HLA class II transgenic models over the past decade have provided correlations between murine EAT and HT. That susceptible class II allele (HLA-DR3) can render EAT susceptibility to resistant strains underscores the importance of HLA class II gene inheritance. Subsequent single transgene expression in class II-negative recipients' permits clear delineation of HLA-DRB1 and -DQ polymorphism, obviating the problems of DR/DQ linkage disequilibrium in patient studies. Accordingly, DR3, but not DR2 and DR4, molecules are permissive for both hTg and mTg induction of EAT. Also, DR3 molecules are permissive for TPO plasmid DNA induction of thyroiditis with Ab profile to IRB-B, mimicking patient Abs. Moreover, TSHR plasmid DNA induces a hyperthyroid syndrome, providing a potential GD model. Thus, DR3 transgenic models are applicable to studies with all three major thyroid autoantigens. In contrast, DQ8 molecules are permissive only for hTg-induced EAT, albeit to a lesser extent than DR3, whereas DQ6 molecules do not respond to either hTg or mTg induction.

14. EXPERIMENTAL AUTOIMMUNE UVEITIS (Adapted from Pennesi *et al.*, 2003)

Experimental autoimmune Uveitis (EAU) is a T-cell-mediated autoimmune disease model that targets the neural retina and related tissues. EAU is induced by immunization with preparations of peripheral retinal antigens or their fragments of which the retinal soluble antigen (S-antigen), also known as arrestin, and the interphotoreceptor retinoid-binding protein (IRBP) are the best known. This experimental model is used to represent several human inflammatory diseases collectively known as uveitis, which are characterized by a frequent presence of immune response to retinal antigens, particularly S-Ag. EAU can also be induced by adoptive transfer of T cells from immunized recipients to naïve genetically compatible hosts. In most cases, the pathogenesis of the disease implicates TH1 type cytokine response (Caspi, 2002). Autoimmune

uveitis is a spectrum of diseases that show differences in clinical manifestations and cause differences in association with specific HLA loci. The associated class I or class II alleles may also depend on the ethnic origin of the population studied. HLA-DR4, DR3, and several DQ genes have been implicated (Pennesi and Caspi, 2002).

The antigen or antigens that might be involved in the etiology of human uveitis have not been elucidated. Susceptibility to EAU in the mice is dependent on the polymorphism in the MHC class II loci with both the H2-A and H2-E locus involved. To better understand the role of HLA molecules in the pathogenesis of uveitis, Dr. Rachel Caspi and her colleagues at the NIH undertook to develop a humanized model. EAU was induced in mice expressing HLA-DR3, -DR4, -DQ6, and -DQ6 in the absence of the endogenous class II (Aβ°) (Pennesi *et al*., 2003). Mice were injected subcutaneously with 200 μg of bovine IRBP, bovine S-Ag, or recombinant human S-Ag emulsified with CFA. All the transgenic mice immunized with IRBB developed EAU. The pathology showed mononuclear and polymorphonuclear cell infiltration, vetritis, choroiditis, and varying degrees of photoreceptor cell damage. Disease severity in individual mice varied from 0.5 to 3 and incidence was about 80%. The control Aβ° mice had no disease.

Uveitis patients frequently exhibit strong lymphocyte responses to S-antigen, but only sporadic responses to IRBP. Although S-antigen is uveitogenic, numerous strains of mice tested thus far have proved to be highly refractory to disease induction with S-antigen. Interestingly, HLA-DR3 tg mice immunized with bS-AG developed very severe disease with many individual mice reaching disease scores of 4 corresponding to complete destruction of the photoreceptor cell layer and extensive damage to the other retinal layers. DQ6 and DQ8 mice develop milder disease and lower incidence. DR3 and DQ8 mice also developed uveitis when injected with human S-antigen (hS-AG). This finding is of particular importance in view of the strong responses of human patients to S-antigens that are presumably developed as responses to the autologous human molecule. DR3 mice immunized with bS-AG produced high levels of IL-6, IFN-γ and TNF-α suggesting a predominant Th1 cytokine response profile. Epitope mapping for S-antigen showed peptide 291–310 to be immunodominant in the DR3 transgenic mice. It is of interest to note that peptide 291–310 of s-Ag partly overlaps the previously characterized S-AG peptide M303-320 and N281-302 which elicits responses in lymphocytes of patients affected by different uveitis diseases.

The EAU model in HLA class II transgenic mice described here provides evidence in favor of involvement of class II restricted responses in human uveitis. No less importantly, this new model suggests the same retinal antigens that are uveitogenic in animals may also be causally involved in human uveitic diseases and validates antigen specific

immunotherapies based on these antigens such as the recent oral tolerance trials in which uveitis patients were fed S-AG and appear to receive a clinical benefit. Although human patients appear to exhibit much more frequent cellular responses to S-AG than to IRBP, wild-type mice develop EAU with IRBP, but are highly resistant to EAU induced with S-AG. It has been proposed that the reason for this is central tolerance to S-antigen or its abundance in the murine thymus such that T cells capable of recognizing S-AG are efficiently deleted from their repertoire. The dramatic change in susceptibility of HLA-DR3 transgenic mice to the S-antigen suggests that in the context of the human class II molecules, the mice are not tolerant to the S-AG any more. In conclusion, this model, especially the EAU induced in DR3 mice with S-antigen, mimics the human disease.

15. BACTERIAL SUPERANTIGENS AND TOXIC SHOCK SYNDROME

The term ''superantigen'' was coined by Marrack and Kappler to designate the family of microbial proteins that can induce strong antigen-independent activation and proliferation of T cells (White *et al.*, 1989). While ''mitogens'' are polyclonal T-cell activators and do not have specificity for any TCR Vβ or Vα elements, ''superantigens'' activate T cells in a TCR Vβ, or in rare cases Vα-specific, manner (Petersson *et al.*, 2003). Superantigens are broadly classified into two types. ''Endogenous or self superantigens'' are those encoded by viruses integrated into genome (Choi *et al.*, 1991) and those of bacterial origin are called ''exogenous'' or ''bacterial superantigens (BSAg)'' (Proft and Fraser, 2003).

Bacterial superantigens, unlike conventional antigens, bind to the α or β chains of cell surface MHC class II molecules outside of the peptide-binding groove without undergoing processing, (Li *et al.*, 1999). Upon binding to MHC class II molecules, BSAg activate both $CD4^+$ and $CD8^+$ T cells bearing specific TCR Vβ (or Vα) elements, irrespective of their antigen-specificities. Thus, T-cell activation by BSAg is MHC class II dependent, MHC unrestricted, co-receptor independent and antigen non-specific (Bueno *et al.*, 2007). Because of these unique characteristics, BSAg robustly activate about 30–50% of all T cells (depending on the BSAg and their TCR preference). Activated T cells carry out their respective effector functions, such as cytokine production, cytotoxicity or other helper functions. BSAg can also activate and induce effector functions in APC by signaling through their MHC class II molecules (Dinges *et al.*, 2000; Li *et al.*, 1999). Thus, unlike certain microbial products which activate the innate immune system, BSAg can effectively activate adaptive immunity.

BSAg are exotoxins produced primarily by *Staphylococcus aureus* and *Streptococcus pyogenes*. Staphylococcal superantigens are named

staphylococcal enterotoxin (SE) A, B, C1, C2, C3, D, E, G through R, U and toxic shock syndrome toxin (TSST)-1. Superantigens produced by *S. pyogenes* are named streptococcal pyrogenic exotoxin (SPE) A, C, G through M, streptococcal superantigen A (SSA), streptococcal mitogenic exotoxin (SME) Z1 and SMEZ2. As evident from their nomenclature, many members of the staphylococcal superantigen family cause food poisoning. However, BSAg, by virtue of their mitogenic potential, have been implicated in a variety of other human diseases. These include toxic shock syndromes (menstrual or non-menstrual) (McCormick *et al.*, 2001), Kawasaki disease (Brogan *et al.*, 2003), rhinitis/nasal polyposis, atopic dermatitis, Wegener's granulomatosis (Popa *et al.*, 2002), neonatal toxic shock syndrome-like exanthematous disease (Takahashi, 2003), staphylococcal scarlet fever, certain vasculitis disorders, staphylococcal purpura fulminans (Kravitz *et al.*, 2005), cutaneous T-cell lymphomas (Jackow *et al.*, 1997) and even sudden infant death syndrome (Zorgani *et al.*, 1999). The unique property of superantigens is that they can activate T cells in the absence of cognate or specific antigens and render them as attractive candidates for autoimmunity (Wucherpfennig, 2001). The current review is restricted to the clinical relevance of staphylococcal and streptococcal superantigens as studied using HLA class II transgenic mice.

15.1. Animal models for studying superantigens

The conventional inbred strains of laboratory mice are unsuitable for studying BSAg-mediated diseases (McCormick *et al.*, 2001). BSAg mediate immunopathology by first binding to MHC class II molecules and subsequently activating T lymphocytes. However, BSAg interacts poorly with murine MHC class II molecules. Therefore, they fail to adequately activate murine T cells and are unable to induce immunopathology in mice. While extremely small quantities of superantigens can cause toxic shock in humans, even milligram quantities of superantigens fail to cause toxic shock in normal laboratory mice. Administration of sensitizing agents such as bacterial lipopolysaccharides or D-galactosamine is needed to induce superantigen-induced toxic shock in conventional laboratory mice. This potentially complicates the pathogenesis and therefore interpretation of data arising thereof (Silverstein, 2004). HLA class II transgenic mice lacking endogenous class II molecules respond robustly to BSAg and mimic human diseases caused by BSAg.

15.2. Enhanced immune response to SEB in HLA class II transgenic mice

When HLA class II transgenic mice were challenged with a single intraperitoneal injection of BSAg, they have a significant and rapid elevation of cytokines such as IL-2, IL-12, IFN-γ, and chemokines such as MCP-1, in

their sera. The profound systemic cytokine storm, greater peripheral T-cell expansion and severe thymocyte deletion in HLA class II transgenic mice (Fig. 2.11 A and B) underscore the fact that transgenic expression of human MHC class II molecules does indeed dramatically augments immune response to BSAg in mice (Rajagopalan *et al*., 2004, 2005).

15.3. HLA class II transgenic mice recapitulate superantigen-induced toxic shock without sensitizing agents

As discussed earlier, toxic shock syndrome (TSS) is an important systemic disease caused by BSAg. TSS can be either staphylococcal (menstrual or non-menstrual) or streptococcal in origin. All TSS follow a similar pathogenesis involving massive T-cell activation, and a systemic cytokine storm. This causes a capillary leak syndrome characterized by hypotension and multiorgan failure, termed a ''systemic inflammatory response syndrome'' (SIRS), which generally culminates in death (McCormick *et al*., 2001). HLA class II transgenic mice are readily susceptible to BSAg-induced TSS without any sensitization regimens. 100% of HLA-DR3 and HLA-DQ8 transgenic mice succumb to TSS at doses of BSAg at

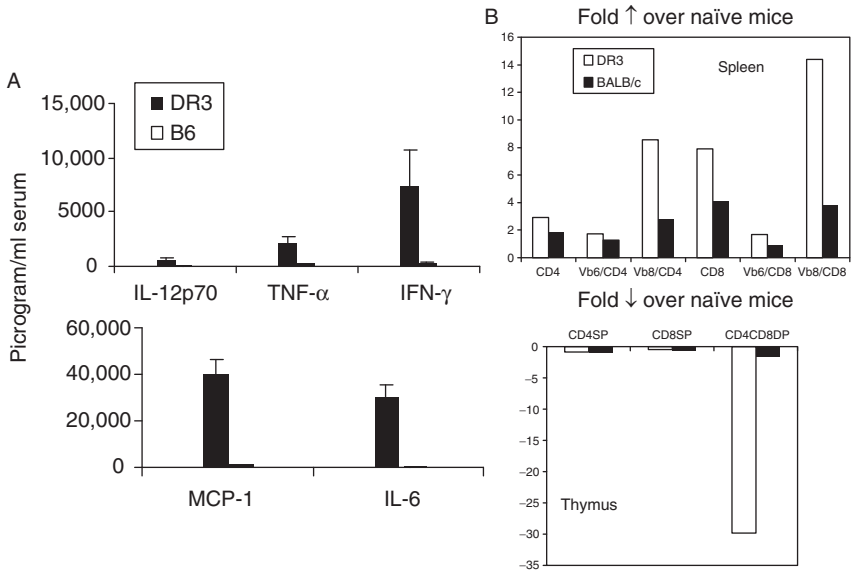

FIGURE 2.11 HLA Class II Transgenic Mice show enhanced immune response to SEB. C57BL/6 and HLA-DR3 mice were intraperitoneally challenged with SEB (10 μg). (A) Serum cytokines levels at 4 h post challenge were measured by cytometric bead assay. (B) Distribution of $CD4^+$ as well as $CD8^+$ T-cells expressing TCR Vβ6 or V β8 on day 3 post SEB challenge. Bars represent Mean ± SD from 5 mice/group.

which conventional mice are absolutely resistant. TSS in HLA class II transgenic mice is characterized by clinical symptoms akin to human patients, such as profound increase in systemic proinflammatory cytokine levels, hepatocyte damage, diarrhea and even hypotension (Boles *et al.*, 2003; DaSilva *et al.*, 2002; Rajagopalan *et al.*, 2002; Sriskandan *et al.*, 2001; Welcher *et al.*, 2002).

15.4. HLA class II polymorphism and superantigens

Isotypic and allotypic variations in HLA class II could influence BSAg binding and thereby potentially affect subsequent immune activation. Certain superantigens bind preferentially to HLA-DR isotype, while some superantigens bind predominantly to HLA-DQ isotype (Scholl *et al.*, 1990). Similar to isotypic variations, allotypic variations within HLA class II can also influence response to BSAg. For example, it has been shown that during group A streptococcal (GAS) infection in humans, while the DRB1*1501/DQB1*0602 haplotype was associated with protection, the DRB1*14/DQB1*0503 haplotype was associated with more severe systemic disease (Kotb *et al.*, 2002). Functional studies with mononuclear cells from these patients and healthy controls indicated that expression of protective haplotype correlated with a subdued proliferative/cytokine responses to streptococcal superantigens, whereas expression of DRB1*14/DQB1*0503 or the DRB1*07/DQB1*0201 was associated with an exaggerated response to streptococcal superantigens (Kotb *et al.*, 2002).

Role of HLA class II polymorphism on the outcome of GAS infection has been successfully proved using HLA class II Transgenic mice. Cells from HLA-DR4/DQ8 (GAS-neutral alleles) transgenic mice elicited a robust proliferative response to streptococcal superantigens when compared to the GAS-protective HLA-DQ6 allele (Nooh *et al.*, 2007). Further, upon in vivo challenge with a virulent GAS isolate, 80% of HLA-DR4/DQ8 double transgenic mice, but none of HLA-DQ6 transgenic mice succumbed to this challenge. Thus, HLA class II transgenic mice could facilitate a better understanding of the effects of polymorphic differences in HLA class II on the superantigen-mediated diseases. This could have several potential applications. Based on the patients' HLA profile the course of superantigen-mediated disease could be predicted and treatment could be ''tailored'' accordingly.

15.5. Use of HLA class II transgenic mice in biodefense research

While efforts are underway to harness the immunopotentiating ability of SEB in cancer therapy (Perabo *et al.*, 2005), the same immunostimulatory property of SEB has led to their use as agents of bioterrorism or biological

warfare (Madsen, 2001). Although development of bioweapons containing SEB has been disbanded in the United States, the possibility that SEB might be used as a bioweapon remains. Airway exposure to aerosolized SEB can induce TSS in HLA-DQ8 class II transgenic mice (Roy *et al*., 2005). This was characterized by a dramatic elevation of proinflammatory cytokines (such as IL-12, IFN-γ, and IL-6) in the serum, activation/proliferation of mature peripheral T cells and intense airway/lung inflammation (Roy *et al*., 2005). The inflammatory changes and the extent of T-cell expansion were directly dependent on the dose of superantigen administered; higher doses of which caused mortality in HLA class II transgenic mice but not in C57Bl/B6 mice. Our studies also showed for the first time that even though classified as an enterotoxin, SEB could act through nasal mucosa and could be involved in pathogenesis of toxic shock (Rajagopalan *et al*., 2006).

15.6. Chronic exposure to staphylococcal superantigens and inflammatory airway diseases

The TSS models described earlier result from acute exposure to BSAg through different routes. However, there is a definite possibility that chronic exposure to BSAg can happen. *S. aureus* colonizes nasopharynx and skin of normal humans with about 20–60% of the population being carriers of *S. aureus* (Chambers, 2001; Kluytmans *et al*., 1997). Studies have shown that similar to the pathogenic invasive strains, even *S. aureus* strains isolated from nasal/skin carriers also harbor genes encoding for superantigens (Kluytmans *et al*., 1997; Nashev *et al*., 2004). Considering these findings, it is possible that the nasal passage/skin is intermittently or chronically (according to the type of carriage) exposed to low amounts of staphylococcal superantigens. Given the pro-inflammatory effects of superantigens, this process could initiate and/or exacerbate inflammation of the airways (nasal polyposis, rhinosinusitis), lungs (asthma), and skin (atopic dermatitis) (Gould *et al*., 2007; Marone *et al*., 2007). Our experiments using HLA class II transgenic mice provide a formal proof implicating BSAg in certain allergic diseases.

Repeated exposure to small amounts of staphylococcal BSAg through nasal passage resulted in marked eosinophilic airway inflammation, bronchial goblet cell hyperplasia, and reversible airway hyperreactivity in HLA class II transgenic mice. Notably, the eosinophilic airway inflammation was more pronounced in HLA class II transgenic mice that are defective in STAT4 signaling molecule (Fig. 2.12), which, like atopic individuals, have a tendency towards increased production of Th2-type cytokines (Kaplan *et al*., 1996). This implies that airway exposure to BSAg may induce more severe eosinophilic airway inflammation in atopic individuals when compared with non-atopic individuals.

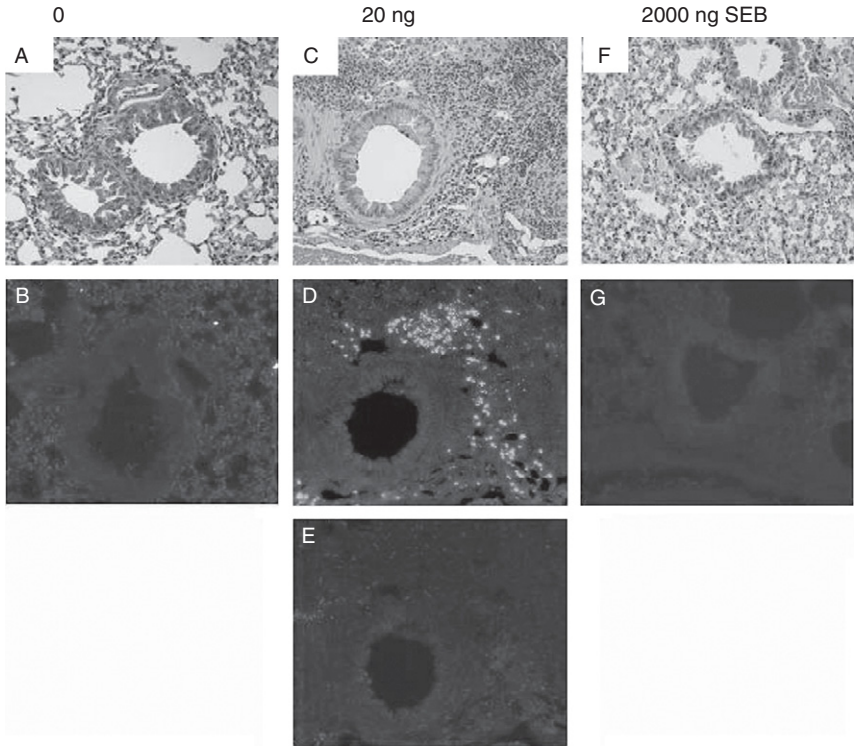

FIGURE 2.12 SEB-induced Airway inflammation in HLA-DR3 transgenic mice. Thin sections were stained in hematoxylin and eosin (A, C, and F), or the deparaffinized sections were stained with anti-MBP antibody (B, D, and G). While PBS-treated mice showed normal lung histology (A, B); mice receiving 20 ng of SEB showed marked mononuclear cell infiltration in the parenchyma as well as around the bronchi (C–E). Mice that received 2,000 ng of SEB showed severe damage to the alveoli as well as to the bronchi, with loss of normal lung architecture (F, G) 2,000 ng of SEB. (E) Lung section from mouse that received 20 ng of SEB treated with preimmune rabbit serum followed by fluorescein isothiocyanate-conjugated secondary anti-rabbit antibody showing the specificity of the anti-MBP antibody. Magnification, ×160. (Reproduced from *Infect Immun,* Rajagopalan *et al*., 2006, 74, 1284, with permission from *American Society of Microbiology*.)

16. CONCLUDING REMARKS

A big challenge in most human autoimmune disorders is the identification of an initiating autoantigen or autoantigens. MHC class II association with autoimmune disorders implies that autoantigen-specific T cells are important in the pathogenesis although the exact role of the MHC class II molecules in these disease processes is unknown. One way to study the role of

autoantigens in biologically relevant situation is to generate mice expressing human MHC alleles. While no single animal model perfectly mimics a human disease, experimental animal models afford opportunities to study the role of individual genes for investigating genetic, environmental, and pathogenic aspects of an autoimmune disease.

The HLA class II restricted diseases described here in the humanized transgenic mice clearly demonstrate that transgene encoded human MHC class II molecules are functional in mice, where they are involved in selection of T-cell repertoire and restrict T-cell responses to various antigens, leading to disease development. The humanized mice show a similar HLA-restriction in identifying antigenic epitopes as in humans, which clearly demonstrates their value in identification of autoantigens and mechanisms of pathogenesis. The HLA transgenic mice take us one step closer to understanding the role of HLA molecules, and accessory molecules involved in initiation and ensuing pathways after presentation of an autoantigen in inflammatory diseases. Finally, these mice can be used to try new therapeutic strategies such as immunotherapies, gene therapy, and vaccines, and evaluate their usefulness in human population.

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CHAPTER 3

Roles of Zinc and Zinc Signaling in Immunity: Zinc as an Intracellular Signaling Molecule

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Contents

Abstract

Zinc (Zn) is an essential nutrient required for cell growth, differentiation, and survival, and its deficiency causes growth retardation, immunodeficiency, and other health problems. Therefore, Zn

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homeostasis must be tightly controlled in individual cells. Zn is known to be important in the immune system, although its precise roles and mechanisms have not yet been resolved. Zn has been suggested to act as a kind of neurotransmitter. In addition, Zn has been shown to bind and affect the activity of several signaling molecules, such as protein tyrosine phosphatases (PTPs). However, it has not been known whether Zn itself might act as an intracellular signaling molecule, that is, a molecule whose intracellular status is altered in response to an extracellular stimulus, and that is capable of transducing the extracellular stimulus into an intracellular signaling event. Here we propose that Zn acts as a signaling molecule and that there are at least two kinds of Zn signaling: "late Zn signaling," which is dependent on a change in the expression profile of Zn transporters, and "early Zn signaling," which involves a "Zn wave" and is directly induced by an extracellular stimulus. We also review recent progress in uncovering the roles of Zn in the immune system.

1. INTRODUCTION

Zinc (Zn) is one of the essential trace elements. It is a structural constituent of a great number of proteins, including enzymes belonging to cellular signaling pathways and transcription factors, and it is essential for their biological activity (Prasad, 1995; Vallee and Auld, 1993). Patients with acrodermatitis enteropathica (AE) have unusually low serum concentrations of Zn, due to a point mutation in the Zn transporter ZIP4, which is expressed in the intestine, and suffer from severe skin disease and frequent infections (Wang *et al.*, 2002). In addition, Zn absorption during lactation is critical for the growth and survival of infants, and in mice, the gene encoding the Zn transporter ZnT4 is responsible for an inherited Zn deficiency in milk, the *lethal milk* (*lm*) mutant (Huang and Gitschier, 1997). A similar mutation in ZnT2 in humans also causes low Zn concentrations in milk (Chowanadisai *et al.*, 2006).

Zn has a variety of effects in the immune and nervous systems (Frederickson *et al.*, 2005; Rink and Gabriel, 2000). Zn-deficient mice have defects in natural killer cell-mediated cytotoxic activity, antibody-mediated responses, and host defense against pathogens and tumors (Fernandes *et al.*, 1979; Fraker *et al.*, 1982; Keen and Gershwin, 1990). On the other hand, Zn itself is cytotoxic; it induces apoptosis in T and B cells (Ibs and Rink, 2003; Telford and Fraker, 1995). Therefore, the intracellular Zn concentration must be tightly controlled, by Zn transporters, Zn-binding molecules, and Zn-sensing molecules (Andrews, 2001; Eide, 2004; Lichtlen and Schaffner, 2001a; Palmiter, 2004; Palmiter and Huang, 2004; Vallee, 1995).

Evidence suggests that Zn acts as a neurotransmitter (Colvin *et al.*, 2003; Frederickson, 2003; Ueno *et al.*, 2002). In neurons, exocytotic stimuli induce the release of Zn into the surrounding milieu, from which it is then taken up into the cytoplasm of neighboring cells through gated Zn-permeable channels (Wei *et al.*, 2004). Synoptically released Zn probably travels in this manner to adjacent postsynaptic neurons and glial cells and functions as a neurotransmitter (Hershfinkel *et al.*, 2001; Li *et al.*, 2001; Xie and Smart, 1994). Zn also mimics the actions of hormones, growth factors, and cytokines, suggesting it may affect intracellular signaling molecules (Beyersmann and Haase, 2001). In fact, Zn is a well-known inhibitor of protein tyrosine phosphatases (PTPs) (Brautigan *et al.*, 1981). Intriguingly, Zn binds several growth factors and cytokines directly and alters their structure, thereby modulating their biological outputs (Radhakrishnan *et al.*, 1996; Smith *et al.*, 1984).

Despite all these observations, it has been unclear if Zn functions as an intracellular signaling molecule, like calcium. We showed that the nuclear localization of the transcription factor Snail is dependent on the Zn transporter, ZIP6. This finding suggested that Zn plays a role in the nuclear localization of Snail and supported its possible identification as an intracellular signaling molecule (Yamashita *et al.*, 2004). This idea was further supported by the finding that toll-like receptor 4 (TLR4)-mediated dendritic cell maturation is, at least in part, dependent on an TLR4-induced decrease in intracellular free Zn (Kitamura *et al.*, 2006).

Although these results all suggest that Zn acts as an intracellular signaling molecule, the TLR4-mediated decrease in intracellular free Zn is dependent on a change in the expression profile of Zn transporters. Therefore, Zn's status as a second messenger was still uncertain. A second messenger is defined as a molecule whose intracellular status is directly altered by an extracellular stimulus and that can transduce the extracellular stimulus into intracellular signaling events. In 1957, Earl W. Sutherland and his colleagues found that cAMP functions as a second messenger. Calcium was the next second messenger to be identified. Today, only a limited number of second messengers and modes of signal transduction mechanisms have been identified; these include cAMP, calcium, NO, lipid mediators, protein phosphorylation and dephosphorylation, and nuclear receptors (Gomperts *et al.*, 2002). Our recent studies have provided evidence that Zn should be added to this list.

We recently found that FcεR1 stimulation induces an increase in intracellular free Zn, and we named this phenomenon the ''Zn wave'' (Yamasaki *et al.*, 2007). The Zn wave occurs several minutes after stimulation. We thus propose that intracellular Zn signaling occurs and can be classified into at least two categories: ''late Zn signaling,'' which is dependent on a transcriptional change in Zn transporter expression, and ''early Zn signaling,'' which involves the Zn wave and is directly induced

by an extracellular stimulus, such as Fc epsilon receptor I (FcεRI). In the latter situation, Zn acts as an intracellular second messenger capable of directly transducing an extracellular stimulus into intracellular signaling events.

In this review, we briefly describe the general aspects of Zn biology and then discuss the roles of Zn in the immune response. We emphasize Zn's newly discovered role as an intracellular signaling molecule.

2. Zn HOMEOSTASIS, Zn TRANSPORTERS, AND METALLOTHIONEINS IN HEALTH AND DISEASE

The ubiquity of Zn in subcellular metabolism suggests it has a role in many metabolic functions, and the effects of Zn deficiency provide evidence of its importance. The first major conceptual breakthrough came in 1961 (Prasad *et al.*, 1961) with the hypothesis that Zn deficiency was a major factor in the nutritional dwarfism syndrome observed in middle-Eastern countries. In 1974, the phenotypic expression of the rare autosomal recessively inherited disorder AE was found to be due to a defect in Zn metabolism (Moynahan, 1974). *ZIP4*, which encodes a ZIP/SLC39 Zn transporter family member that is expressed in the intestine, was later identified as the responsible gene for AE. This finding provided genetic evidence that intestinally absorbed Zn has important physiological roles (Wang *et al.*, 2002). Organs affected by severe Zn deficiency include those of the epidermal, gastrointestinal, central nervous, immune, skeletal, and reproductive systems (Hambidge and Walravens, 1982), suggesting that precise Zn homeostasis in these tissues is critical for proper early development. Furthermore, a Zn transporter ZnT4, which is expressed in breast epithelial cells, was identified as the gene responsible for the inherited Zn deficiency in *lm* mice, indicating that ZnT4 supplies the milk with Zn, which is critical for infant development (Huang and Gitschier, 1997). In humans, evidence suggests that ZnT2 has a similar role (Chowanadisai *et al.*, 2006).

These findings indicate that Zn homeostasis is important in normal physiology as well as in disease onset. The intracellular Zn concentration is tightly controlled by Zn importers (ZIPs/SLC39s) (Eide, 2004), exporters (ZNTs/SLC30s) (Palmiter and Huang, 2004), and Zn-binding proteins such as metallothioneins (Vallee, 1995). In addition, the gene expression of these proteins is regulated by Zn-sensing molecules, such as MTF-1, that maintain Zn homeostasis (Andrews, 2001). These Zn-controlling and Zn-controlled molecules have unique mechanisms, functions, and cellular locations (see reviews by Eide, 2006; Kambe *et al.*, 2004; Murgia *et al.*, 2006) (Fig. 3.1).

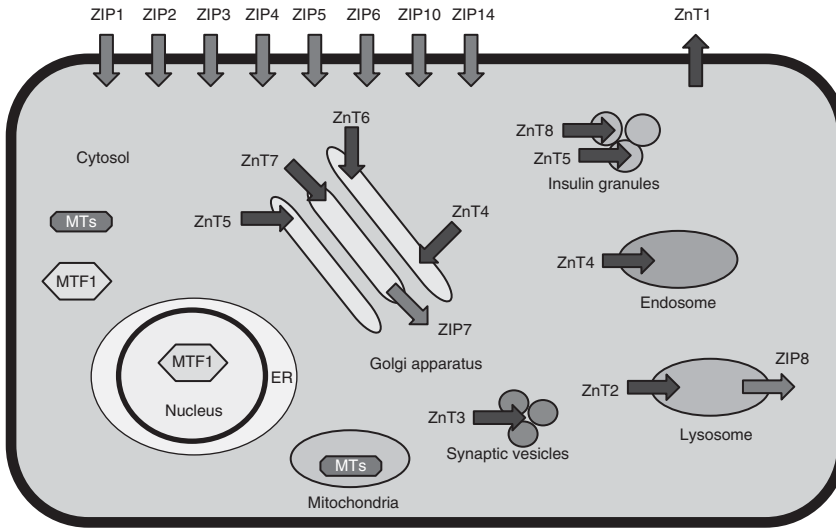

FIGURE 3.1 Subcellular localization of zinc transporters and metallothioneins. The illustrated subcellular localization and potential functions of the ZIP and ZnT family members are based on currently available information (Ref. Begum *et al*., 2002; Chimienti *et al*., 2004; Huang *et al*., 2005; Kambe *et al*., 2004; Kelleher and Lonnerdal, 2005; Taylor and Nicholson, 2003; Taylor *et al*., 2005; Wang *et al*., 2004). Arrows indicate the predicted direction of the Zn mobilization. MT, metallothionein; ER, endoplasmic reticulum; MTF-1, metal-responsive element-binding transcription factor-1.

2.1. ZIPs

ZIPs is an acronym for ''Zrt-Irt like proteins,'' which were first discovered in *Saccharomyces cerevisiae* (Zrt) and *Arabidopsis thaliana* (Irt) (Zhao and Eide, 1996) (Eide *et al*., 1996). Fourteen ZIP family members have been reported in mammals. Some ZIP knockout mice have been reported (Table 3.1). Each single-knockout mouse for *ZIP1*, *ZIP2*, or *ZIP3* shows abnormal embryogenesis only under zinc-limiting conditions. Homozygous *ZIP4* knockout mouse embryos die during early development, and *ZIP4* heterozygosity causes hypersensitivity to zinc deficiency, the same trend as seen in AE patients (the AE gene is *ZIP4)* (Kury *et al*., 2002; Wang *et al*., 2002). ZIP6/Liv1 of zebrafish was reported to control the epithelial–mesenchymal transition under STAT3 activation (Yamashita *et al*., 2004), and the Drosophila gene *fear of intimacy* (*foi*), which shares similarity with mammalian *ZIP6* and *ZIP10*, is essential for proper gonad formation, E-cadherin expression, and glial cell migration (Mathews *et al*., 2006; Pielage *et al*., 2004; Van Doren *et al*., 2003), suggesting that ZIP6/Liv1 and/or ZIP10 may have important roles in cell migration in general. In fact, ZIP10 is proposed to be involved in the invasive behavior of breast

TABLE 3.1 Phenotypical profile of mutants of Zn transporters and metallothioneins

Gene name	Protein name	Type	Phenotype	*Drosophila melanogaster* and *Caenorhabditis elegans*	References
MT-I MT-II	MT-I MT-II	KO	Defective in phagocytic and antigen-presenting activities of macrophages		Sugiura *et al.*, 2004
SLC39A1	ZIP1	KO	Abnormal morphogenesis of the embryonic zinc-limiting condition		Dufner-Beattie *et al.*, 2006
SLC39A2	ZIP2	KO	Abnormal morphogenesis of the embryo in zinc-limiting condition		Peters *et al.*, 2007
SLC39A3	ZIP3	KO	Abnormal morphogenesis of the embryo and depletion of thymic pre-T cells in zinc-limiting condition		Dufner-Beattie *et al.*, 2005
SLC39A4	ZIP4	KO	Die *in utero* during early development		Dufner-Beattie *et al.* 2007
hSLC39A4	hZIP4	AE	Severe zinc deficiency		Wang *et al.*, 2002

SLC39A6/10	ZIP6/10	–	–	FOI; defects in the formation of the embryonic gonad	Van Doren *et al.*, 2003
SLC39A7	ZIP7 (KE4)	–	–	Catsup; semi-dominant lethal	Stathakis *et al.*, 1999
SLC30A1	ZnT1	KO	Embryonic lethal	Cdf-1; died during larval development	Andrews *et al.*, 2004; Bruinsma *et al.*, 2002
hSLC30A2	hZnT2	Mutation	Produce zinc-deficient milk in some women		Chowanadisai *et al.*, 2006
SLC30A3	ZnT3	KO	Prone to seizures elicited by kinic acid treatment		Cole *et al.*, 2000
SLC30A4	ZnT4	lm	Produce zinc-deficient milk		Huang and Gitschier, 1997
SLC30A5	ZnT5	KO	Poor growth; osteopenia; low body fat; muscle weakness; male-specific cardiac death		Inoue *et al.*, 2002

cancer cells (Kagara *et al.*, 2007). The *ZIP7* (*KE4)* gene was discovered while characterizing the major histocompatibility complex on mouse chromosome 17 (Lai *et al.*, 1994), and its human orthologue maps to the HLA class II region on chromosome 6 (Ando *et al.*, 1996). The Drosophila counterpart of *ZIP7*, *Catsup*, is reported to control melanin synthesis (Stathakis *et al.*, 1999), and its plant homolog, *IAR1*, is required to regulate root elongation by controlling auxin conjugate sensitivity (Lasswell *et al.*, 2000), indicating that ZIP7 may have biological significance in higher organisms.

2.2. CDF

CDF stands for ''cation diffusion facilitator,'' a gene that confers metal resistance to many eukaryotic cell types (Palmiter and Findley, 1995), and 10 members of this family have been reported in mammals [named the ZnT (*Zn T*ransporter) family in mammals]. Knockout mice of some ZnT members have been reported (Table 3.1). *ZnT1* knockout mice die as embryos. CDF1, a nematode ZnT1 orthologue, positively regulates Ras-Raf-MEK-ERK signal transduction by promoting zinc efflux, thereby reducing the concentration of cytosolic Zn (Bruinsma *et al.*, 2002). Therefore, ZnT1 might fine-tune the mammalian Ras-mediated signaling pathway. *ZnT3* knockout mice are prone to seizures elicited by kainic acid treatment. *lm* mice, which carry a nonsense mutation in the *ZnT4* gene, produce zinc-deficient milk. *ZnT5* knockout mice show poor growth, osteopenia, low body fat, muscle weakness, and male-specific cardiac death. In humans, a point mutation in the *ZnT2* gene suggests that ZnT2 plays a role in Zn transport into milk, and a recent genome-wide association study identifying novel risk loci for type 2 diabetes (Sladek *et al.*, 2007) included the *ZnT8* gene. In fact, *ZnT8* is expressed exclusively in the β cells of the pancreas (Chimienti *et al.*, 2004), a potential role for ZnT8 in the pathogenesis of diabetes.

2.3. MTs

MTs are small proteins with a high cysteine content that bind Zn, as well as other metal ions, and are thought to be responsible for the intracellular regulation of Zn concentration and the detoxification of nonessential heavy metals. When the concentration of intracellular free Zn reaches a critical value, MTF-1 is activated and induces MTs, which sequester the Zn (Lichtlen and Schaffner, 2001b). Therefore, MTs act as a biochemical device that controls the concentration of free Zn, first by sequestering it and then by releasing it via other biochemical events, such as oxidative signaling. MTs also participate in immune responses. In fact, macrophages from MT-KO (*MT-I* and *MT-II* double knockout) mice are

defective in phagocytic and antigen-presenting activities (Sugiura *et al.*, 2004) (Table 3.1).

As mentioned above, several knockout mice for genes encoding Zn-regulating proteins have been established, but only a few of them show severe phenotypes. The reason of this phenomenon is that Zn transporters have redundant functions. In fact, ZnT5/ZnT6 hetero-oligomers and ZnT7 homo-oligomers both contribute to the activation of zinc-containing enzymes, alkaline phosphatases (Suzuki *et al.*, 2005a, b). In addition, *ZIP1* and *ZIP3* double-knockout mice show a higher rate of abnormal morphogenesis than do knockout mice for the individual genes (Dufner-Beattie *et al.*, 2006).

3. ROLE OF Zn IN THE IMMUNE RESPONSE

Zn is essential for the function of many intracellular proteins, and it is likely that the responses of immune cells are regulated by intracellular Zn homeostasis, although excess Zn is toxic for all cells, including immune cells (Ibs and Rink, 2003; Wellinghausen *et al.*, 1997).

Because Zn deficiency in humans is a frequent dietary problem and accompanies many chronic diseases, several investigations of Zn homeostasis have been performed, using mouse models in which the impact of Zn deficiency on immune function could be assessed at the cellular and molecular levels (Hambidge, 2000). It is important to point out that chronic diseases such as gastrointestinal disorders, chronic diarrhea, renal disease, sickle cell anemia, cirrhosis, some cancers, cystic fibrosis, pancreatic insufficiency, and autoimmune arthritis in humans lead to suboptimal Zn status (Bhutta *et al.*, 2000; Dutta *et al.*, 1998; Fraker *et al.*, 2000; King *et al.*, 1995; Krebs *et al.*, 1998; Prasad, 1985; Tapazoglou *et al.*, 1985; Zemel *et al.*, 2002). These disease states are associated with increased infections of prolonged duration, which is a clear indication of compromised immunity and suggests that Zn plays a role in immune cell homeostasis *in vivo* (Fraker *et al.*, 2000; Prasad, 1985). Indeed, many experiments using animal models have confirmed that Zn deficiency induces thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated responses, resulting in increased rates and longer duration of infection. In this section, we discuss Zn and immunity mainly with regard to adaptive immunity, such as the functions of T cells and dendritic cells. The roles of Zn in mast cells are discussed in Section 4.

There appear to be several ways that Zn deficiency could induce thymic atrophy. One critical explanation is indicated by the observation that glucocorticoids, in particular corticosterone, are chronically elevated in Zn-deficient mice, and adrenalectomy or the removal of these steroids

prevents the thymus from atrophying during Zn deficiency (DePasquale-Jardieu and Fraker, 1979, 1980). In addition, King *et al*. showed that a Zn-deficient diet causes a substantial loss of CD4+CD8+ thymocytes, the thymic population that is most sensitive to the apoptosis caused by glucocorticoids (King *et al*., 2002). Thus, the chronically produced glucocorticoids found in Zn deficiency may accelerate apoptosis in the thymus, followed by a reduction in T cells in the peripheral environment. On the other hand, mature CD4 and CD8 T cells are quite resistant to Zn deficiency and survive well in the otherwise atrophying thymus (King *et al*., 2002). Interestingly, Zn deficiency in an experimental mouse model causes an imbalance between Th1 and Th2 functions in the periphery. The production of IFN-γ and IL-2 (products of Th1) decreases, whereas those of IL-4, IL-6, and IL-10 (products of Th2) are not affected (Prasad, 2000).

Another explanation for the immune deficiencies induced by inadequate Zn, including peripheral lymphopenia and compromised immune responses, is that gene expression levels in some cells, including T cells, are altered. Indeed, microarray analysis showed that a modest Zn deficiency in mice changes the expression of 1,200 genes in T cells (Moore *et al*., 2001). Such alterations in gene expression might change the survival and/or responses of T cells and dendritic cells, which are among the most important cells affecting T-cell homeostasis *in vivo*. In fact, culturing Th0 and Th1 cell lines in low-Zn medium followed by mitogenic stimulation leads to reduced mRNA expression for IL-2 and IFN-γ, which would adversely affect the cells' functional capacity (Bao *et al*., 2003). In addition to T cells, other immune cells also show altered patterns of gene expression with Zn deficiency. The myeloid-like human precursor cell line HL-60 survives in culture at low concentrations of Zn (Bao *et al*., 2003), but when these cells are stimulated, they exhibit enhanced expression of the mRNA for TNFα, IL-1β, and IL-8. These altered gene expression levels are not surprising, because a decrease in intracellular Zn increases the activity of tyrosine phosphatases that are critical regulators of the tyrosine kinase pathways that influence the expression levels of these cytokines. Whichever explanation holds true, intracellular Zn homeostasis clearly plays a role in the responses of immune cells, including T cells and dendritic cells.

We recently showed that the exposure of mouse dendritic cells to the bacterial endotoxin lipopolysaccharide (LPS), a stimulant of TLR4, leads to a decrease in intracellular free Zn concentration; by the same token, the artificial depletion of intracellular Zn by a Zn chelator triggers dendritic cell maturation. On the other hand, the artificial elevation of intracellular Zn levels suppresses the ability of dendritic cells to respond to LPS. In fact, Zn is required for the endocytosis of MHCII molecules expressed on the plasma membrane, and it inhibits trafficking of MHCII vesicles toward the plasma membrane from the perinuclear region, indicating that

Zn suppresses the surface expression of MHCII at multiple steps. LPS affects the expression of a number of Zn import and export transporter molecules, with the net effect being to increase Zn transport out of the cells. Importantly, the overexpression of a Zn import transporter, ZIP6, the expression of which is reduced by LPS stimulation, suppresses the maturation of dendritic cells and inhibits their CD4+ T-cell stimulatory activity. A similar effect of Zn is observed in live animals, in which the injection of LPS induces reduced levels of intracellular free Zn and of ZIP6, in dendritic cells, and treatment with Zn-depleting agents increases dendritic cell maturation (Kitamura *et al.*, 2006). These results clearly show that intracellular Zn homeostasis is critically involved in dendritic cell maturation, which is an important step for T-cell activation (Fig. 3.2). This finding further indicates that Zn acts as a signaling molecule, as discussed in Section 5.

What types of molecules require Zn or are regulated by Zn in immune cells? There are over 300 enzymes and many transcription factors and signaling molecules that have Zn-binding motifs, such as classical Zinc fingers (ZnFs), and RING, LIM, and PHD domains (Gamsjaeger *et al.*, 2007). Here we describe some of these proteins in detail, especially those involved in the responses of T cells and dendritic cells.

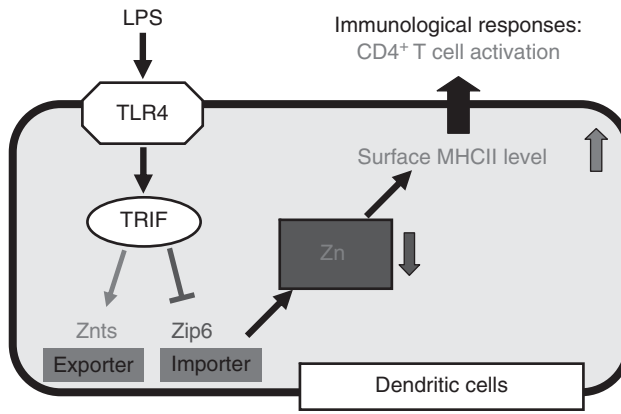

FIGURE 3.2 Decrease in intracellular free Zn is critical for LPS-mediated CD4+ T-cell activation by dendritic cells. LPS, which is a component of the Gram-negative bacterial cell wall, transduces a critical signal for dendritic cell (DC) activation. LPS binds to a receptor, TLR4, on DCs and initiates Myd88- and TRIF-mediated signaling. We showed that TRIF-mediated signaling via TLR4 increased the ZnT family (Zn exporters) mRNA but decreased the ZIP family (Zn importers) mRNA, resulting in a decrease in the intracellular free Zn level in DCs. We also demonstrated that the reduction in intracellular free Zn in DCs is critical for their surface expression of MHC class II molecules, which is important for the activation of CD4+ T cells.

One Zn-binding molecule, Lck, is unique in that its Zn-binding motif is created by two molecules, Lck and its coreceptor, CD4 or CD8, in T cells. Lck is a Src-family tyrosine kinase that transduces signals required for normal T-lymphocyte development and for the antigen-dependent activation of mature T cells. Lck associates noncovalently but with high affinity via its N-terminal region with the cytoplasmic tail of the T-cell coreceptor CD4 or CD8 in a manner dependent on the conserved cysteine motifs within the coreceptor tail (a CxCP motif in CD4 and CD8) and the Lck unique domain (a CxxC motif) (Shaw *et al.*, 1990; Turner *et al.*, 1990). These four conserved cysteine residues coordinate a Zn-binding site that is critical for complex formation (Huse *et al.*, 1998; Kim *et al.*, 2003; Lin *et al.*, 1998). More recently, the homodimerization of Lck molecules via their SH3 domains was also shown to be dependent on two Zn (Romir *et al.*, 2007).

Many molecules with ZnF motifs are reported to be critical for the immune system, including the GATA family, Ikaros, RAG, cytosine deaminases (APOBECs) such as AID, BTB family members such as TH-POK and LRF, TRAF, Aire, ZNAF, Foxp3, Blimp-1, Bcl6, and others. This prevalence of ZnF motif-bearing molecules indicates that Zn is critically involved in the immune system, at least as a constitutive essential component, and may suggest that Zn also functions as a signaling molecule (see Section 5).

A20 is a cytoplasmic protein that contains an N-terminal ovarian tumor domain and seven novel ZnF structures in its C-terminal domain (Makarova *et al.*, 2000; Opipari *et al.*, 1990). Overexpression studies show that A20 inhibits TNF-mediated cell death and NF-κB activation (reviewed in Beyaert *et al.*, 2000). These crucial physiological functions were confirmed by studies in A20-deficient mice, which develop severe multi-organ inflammation and are extremely susceptible to sub-lethal doses of TNF (Lee *et al.*, 2000). This phenotype correlates with an enhanced sensitivity to TNF-induced apoptosis and prolonged NF-κB activation of A20-deficient cells. The importance of the ZnF-containing domain for A20's suppression of NF-κB activation has also been reported (Song *et al.*, 1996).

Ikaros is a nuclear regulator that has four ZnF structures and has been implicated in the control of early hematopoiesis, including that by T cells. Biochemical studies in two different cellular systems, T cells and erythroid progenitors, showed that Ikaros is entirely associated with a higher-order chromatin remodeling complex that predominantly contains components of the nucleosome remodeling and deacetylase (NuRD) complex (Kim *et al.*, 1999; O'Neill *et al.*, 2000). A direct partner of Ikaros in this NuRD-based chromatin remodeling complex is the ATPase Mi-2β, which can alter the association of DNA with histones, potentially modifying the ability of different chromatin and transcriptional regulators to access neighboring genetic loci (Becker and Horz, 2002; Tong *et al.*, 1998; Xue *et al.*, 1998;

Zhang *et al*., 1998). In addition, the Ikaros NuRD complex contains histone deacetylases, which can covalently modify chromatin and facilitate transcriptional repression (Cress and Seto, 2000; Kim *et al*., 1999). Importantly, ZnF-mediated protein interactions are reported to modulate Ikaros activity (Sun *et al*., 1996). Moreover, leukemic cells from infants with acute lymphoblastic leukemia (ALL) express dominant-negative Ikaros isoforms that lack critical N-terminal ZnFs, suggesting that Ikaros's binding of Zn plays a role in its function (Sun *et al*., 1999).

Activation-induced cytidine deaminase, AID, which belongs to a family of Zn-dependent cytosine deaminases (the APOBECs), is an essential enzyme regulating the class-switch recombination, somatic hypermutation, and gene conversion of immunoglobulins (Honjo *et al*., 2005). AID deficiency causes a complete defect in class switching and yields a hyper-IgM phenotype with enlarged germinal centers that contain strongly activated B cells with or without immunization (Muramatsu *et al*., 2000). All the Zn-dependent deaminases including AID form a dimer interface from the helices that both contain the Zn-coordination residues (helices 2 and 3) and form the catalytic pockets for the enzyme activity, suggesting Zn binding by AID is important for its function (Losey *et al*., 2006; Prochnow *et al*., 2007).

TRAF6 has three ZnF structures and acts like a junction, transducing signals from the TNF receptor superfamily, the TLR/IL-1R family, and CD40 to activate the transcription factors NF-κB and AP1. TRAF6-deficient mice produce autoantibodies and show inflammation of multiple organs, including the liver, lung, and pancreas. Akiyama *et al*. showed that TRAF6 is required for the development of a specialized subset of thymic stromal cells known as thymic medullary epithelial cells (mTECs), but not dendritic cells (Akiyama *et al*., 2005). Using thymic grafts depleted of hemopoietic cells, they showed that grafted TRAF6-negative thymic stroma was sufficient to recapitulate the negative selection defect and the development of autoimmunity in athymic nude mice with intact TRAF6.

AIRE has been identified as a responsible gene for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which is characterized by three abnormal features: chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency (Betterle *et al*., 1998). AIRE is a 545-amino acid protein with several domains resembling those found in transcriptional regulators, including two ZnF-containing PHD motifs and a SAND domain (named for Sp100, AIRE1, NucP41/75, and DEAF1/suppressin), which serves as a DNA-binding motif in some transcription factors but is missing the critical DNA-interacting residues in AIRE. AIRE1, which is enriched in mTECs, controls a wide array of tissue-restricted antigens derived from nearly all organs in the body and uses these antigens to regulate the negative selection of T cells in the thymus. Consistent with these roles, AIRE1-deficient mice show a wide

variety of autoimmune diseases induced by a defect in tolerance induction in autoreactive T cells in the thymus (Mathis and Benoist, 2007). Importantly, some APECED patients have mutations in the ZnF domains of AIRE, suggesting that Zn binding plays a role in AIRE's function (Peterson and Peltonen, 2005).

ZFAT (ZnF gene in AITD susceptibility region) has been identified as one of the susceptibility genes of autoimmune thyroid disease, which is caused by an immune response to self-thyroid antigens and has a significant genetic component (Shirasawa *et al.*, 2004). ZFAT localizes to the nucleus and is a large protein of 1104 amino acids that has at least 13 ZnF domains. ZFAT is expressed in CD4+ T cells, CD8+ T cells, CD19+ B cells, and CD14+ monocytes, but not in the thyroid itself. It is hypothesized that ZFAT plays critical roles in B-cell functions that are involved in the development of the autoimmune thyroid diseases.

4. ROLE OF Zn IN MAST CELLS

Mast cells are thought to participate in a variety of immune responses, such as allergic reactions, innate immunity, and autoimmunity (Galli and Nakae, 2003; Galli *et al.*, 2005). Engagement of the FcεRI on mast cells initiates signaling pathways that lead to the exocytosis of granules containing various cytokines and chemical mediators. These released molecules play pivotal roles in the inflammatory reactions observed in patients with allergies and autoimmune diseases.

Mast cells contain many granules, which include various chemical mediators such as histamine, lipids, cytokines, and proteases. Interestingly, electron microscopic studies in the 1960s demonstrated that mast cell granules are rich in heavy metals such as Zn (Gustafson, 1967), although the role of Zn is not known.

We showed that Zn is required for both degranulation and cytokine production in mast cells (Fig. 3.3). The Zn chelator TPEN (*N*,*N*,*N*,*N*-tetrakis (2-pyridylmethyl) ethylenediamine) inhibits histamine release, cytokine production, and lipid mediator secretion (Kabu *et al.*, 2006). The inhibitory effects of TPEN are cancelled by the addition of $ZnSO_4$, and chelators of other metals, such as copper, iron, and manganese, do not affect mast cell function. These results showed that Zn is involved in the mast cell degranulation process and cytokine production.

The detailed mechanism for how Zn chelators inhibit mast cell function remains unclear. We have reported that the degranulation of mast cells can be divided into two processes. First, FcεRI stimulation triggers microtubule polymerization and granule translocation to the plasma membrane in a calcium-independent manner. Fyn/Gab2/RhoA, but not Lyn/SLP-76, signaling plays a critical role in this calcium-independent

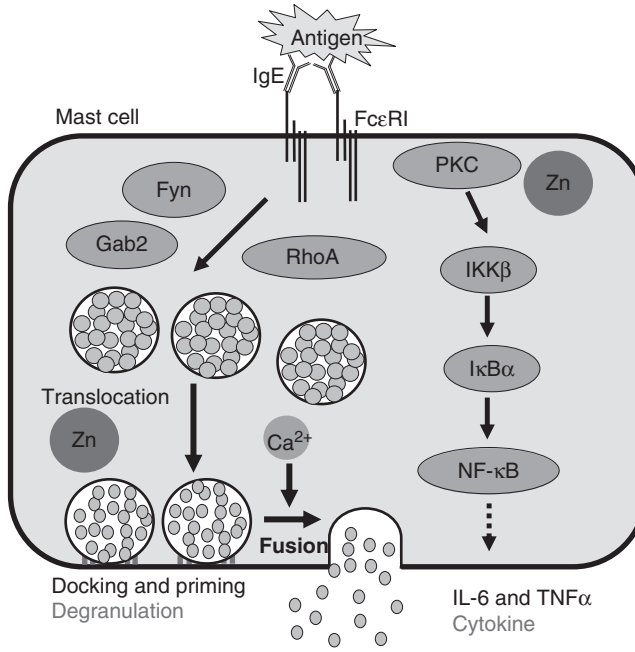

FIGURE 3.3 Zn is involved in FcεRI-mediated mast cell activation. Zn is required for multiple steps of FcεRI-induced mast cell activation, such as degranulation and cytokine production. We demonstrated that the Zn level is dependent on the FcεRI-induced granule translocation process, which is regulated by a Fyn/Gab2/RhoA-mediated calcium-independent pathway. In addition, we showed that Zn is required for the translocation of protein kinase C to the plasma membrane and for the subsequent nuclear translocation of NF-κB, which leads to the production of cytokines such as IL-6 and TNFα.

microtubule-dependent pathway (Nishida *et al.*, 2005). Second, the granules fuse with the plasma membrane in a well-characterized calcium-dependent manner. Therefore, we first assessed whether TPEN might interfere with one of these processes. In fact, TPEN has little effect on the early FcεRI-induced tyrosine phosphorylation of a variety of signaling molecules or on calcium mobilization, but it suppresses the FcεRI-induced granule translocation. Given that transport generally depends on cytoskeletal proteins such as tubulin and actin (Goode *et al.*, 2000), and that microtubules are important for granule translocation and vesicle transport (Nishida *et al.*, 2005; Smith *et al.*, 2003), it was possible that TPEN affected microtubule assembly. However, no such effect by TPEN on FcεRI-induced microtubule formation was found, suggesting the existence of a Zn-regulated molecule that links microtubules and granules. Because recently, kinesin receptors, or linker-cargo proteins, were identified as key molecules for microtubule-dependent vesicle trafficking

(Schnapp, 2003), the target of TPEN might have a kinesin-interacting region and link kinesin to vesicles. Thus, one target of TPEN might be such a linker molecule, with a ZnF or Zn-binding domain.

In addition to granule translocation, Zn might control the process of granule-membrane fusion, through Rab-effector molecules. Several groups have reported that Rab-effector molecules such as Rim, Granulophilin, and Rabphilin regulate the fusion of synaptic vesicles and have a ZnF domain (Chung *et al.*, 1995; Sudhof, 2004).

Finally, TPEN suppresses FcεRI-mediated cytokine production and transcription of IL-6 and TNFα mRNA. PKC is reported to be activated upon FcεRI stimulation and is involved in cytokine production through NF-κB activation (Klemm *et al.*, 2006; Rivera, 2006), and TPEN also inhibits the FcεRI-mediated plasma membrane translocation of PKCβI (Kabu *et al.*, 2006). These results suggest that one of the targets of TPEN involved in cytokine production is PKC. Consistent with this idea, the Zn-binding domain of PKC is required for its plasma membrane translocation after stimulation (Nechushtan *et al.*, 2000; Oancea *et al.*, 1998; Sakai *et al.*, 1997). Thus, Zn might also play a role in the regulation of PKC plasma membrane translocation and thereby regulate cytokine production.

5. Zn SIGNALING

As discussed above, in addition to being an essential structural constituent (Prasad, 1995; Vallee and Auld, 1993), Zn may also act as a signaling molecule.

5.1. Zn as a neurotransmitter

An exchangeable Zn ion in the brain was first discovered by Maske in 1955 (Maske, 1955). Since then, a broad outline of the function of glutamate and Zn-releasing neurons has emerged. It is now thought that Zn may modulate the overall excitability of the brain through its effects on receptors for glutamate, and probably γ-aminobutyric acid (GABA), and therefore Zn might act as a neurotransmitter that functions in synaptic plasticity. In fact, Zn is highly enriched in synaptic vesicles, from which it is co-released with glutamate in an activity-dependent manner (Assaf and Chung, 1984). Microfluorescence imaging of Zn secretion upon exocytotic presynaptic stimulation showed Zn is released from hippocampal Mossy fiber terminal vesicles into the surrounding milieu (Li *et al.*, 2001; Qian and Noebels, 2005; Ueno *et al.*, 2002). It is then taken up into the cytoplasm of neighboring cells through gated Zn channels. The rapid influx of Zn through Ca^{2+}-permeable AMPA/kainate (Ca-A/K) channels triggers the generation of reactive oxygen species (ROS) and is potentially neurotoxic (Weiss and Sensi, 2000).

In the above scenario, the action of Zn is very similar to that of neurotransmitters, which are stored in membrane-enclosed synaptic vesicles and released by exocytosis, activating postsynaptic cells through transmitter-gated ion channels (Colvin *et al*., 2003; Hershfinkel *et al*., 2001; Li *et al*., 2001; Xie and Smart, 1994), although the interpretation of the signals obtained in these studies has been a matter of debate (see Kay, 2006). ZnT3 is involved in the loading of synaptic vesicles with releasable Zn (Cole *et al*., 1999), and ZnT (SLC30) and ZIP (SLC39) family proteins as well as Zn-sequestering proteins, like metallothioneins, control the synaptic Zn concentrations (Baranano *et al*., 2001; Kambe *et al*., 2004; Smart *et al*., 2004). Since Zn modulates both the current responses mediated by excitatory and inhibitory neurotransmitter receptors and the efficacy of transporter-driven neurotransmitter reuptake (Smart *et al*., 2004), synaptically released Zn has been proposed to function as an important regulator of synaptic transmission and plasticity (Lu *et al*., 2000; Vogt *et al*., 2000) and/or as an ''atypical neurotransmitter'' (Baranano *et al*., 2001). This concept has, however, been challenged, because (1) mice deficient in ZnT3 are behaviorally normal despite a loss of synaptic Zn staining (Cole *et al*., 1999) and (2) the available Zn imaging techniques are argued to be particularly sensitive to artifacts (Kay, 2006).

In support of Zn's role in neurotransmission, many groups using different methods have reported that Zn can affect synaptic receptors. Using site-directed mutagenesis, Hosie *et al*. identified a pair of Zn-binding sites and completed the characterization of a third Zn-binding site in the GABA receptor (Hosie *et al*., 2003). Hirzel *et al*. showed that Zn modulates neurotransmission, by producing knock-in mice carrying the mutation D80A (a constructed Zn-binding site) in the glycine receptor (GlyR) alpha1 subunit gene (*Glra1)* (Hirzel *et al*., 2006). The authors showed that the *Glra1*(D80A) mice have a hyperekplexia phenotype due to the loss of Zn potentiation of the alpha1 subunit-containing GlyRs, and thus, that synaptic Zn is essential for the proper functioning of glycinergic neurotransmission *in vivo.* It is also known that Zn inhibits NMDA receptor activity through a dual mechanism: a voltage-dependent channel block and a voltage-independent reduction in the probability of channel opening (Christine and Choi, 1990; Legendre and Westbrook, 1990; Mayer and Vyklicky, 1989). Thus, it is likely that Zn acts as a neurotransmitter and has other roles in the neural system.

5.2. Zn as an intracellular signaling molecule

The action of Zn as a neurotransmitter is separate from its functions as an intracellular second messenger. cAMP was the first intracellular second messenger discovered, by Berthet *et al*. (1957), and calcium was the

second. There are still relatively few second messenger species and modes of signal transduction mechanisms known. These include cAMP, cGMP, calcium, NO, lipid mediators, G-proteins and their related molecules, protein kinase, protein phosphatase, nuclear receptors, and some others (Gomperts *et al.*, 2002).

The accumulated evidence indicates that Zn also appears to have intracellular signaling or second-messenger functions. Fujii *et al.* showed the presence of Zn in the nucleolus of the starfish oocyte, suggesting a possible role for Zn in mitosis, although its function is still unknown (Fujii, 1954). We showed that the Stat3-Liv1 (ZIP6) cascade is critically involved in cell migration and the epithelial–mesenchymal transition of zebrafish cells, and that it is required for the nuclear localization of Snail, a ZnF-containing repressor (Yamashita *et al.*, 2004). Since the nuclear localization of Snail is dependent on both its ZnF domain (Dominguez *et al.*, 2003; Yamashita *et al.*, 2004) and the Zn transporter ZIP6/Liv1, we hypothesized that Zn might act as an intracellular signaling molecule. Zn is known to act as a regulator of conventional second-messenger signaling by cyclic nucleotides. For example, Zn suppresses LPS-induced TNFα and IL-1β release from monocytes, and this inhibitory effect is dependent on Zn's suppression of phosphodiesterases (PDEs), which hydrolyze cyclic nucleotide into 5′-nucleotide monophosphate, resulting in an increased intracellular cGMP level. The NO donor SNOC both increases free Zn and inhibits LPS-induced TNFα and IL-1β release. This SNOC-induced increase in intracellular free Zn may therefore mediate SNOC's inhibitory effect via increased cGMP (von Bulow *et al.*, 2005). Since cytokine stimulation sometimes induces NO production, thereby enhancing the intracellular free Zn level (Spahl *et al.*, 2003), Zn may act as a bridge between the second messengers, NO and cGMP. In addition, Huberman's group showed that the nuclear Zn concentration increases within 15 min of PMA (phorbol 12-myristate 13-acetate) treatment in a manner dependent on PKCβ in macrophage cell lines (Glesne *et al.*, 2006). All this evidence suggests that Zn may act as a signaling molecule.

Regarding this intriguing possibility, we showed that TLR-mediated signaling induces a decrease in the intracellular free Zn in dendritic cells, and that this decrease is required for dendritic cell activation followed by CD4+ T-cell activation (Kitamura *et al.*, 2006). LPS, which binds TLR4, decreases the level of intracellular free Zn via a change in the expression profile of Zn transporters; the expression of ZIP family members is downregulated, while that of ZnT family members is upregulated. In this case, the extracellular stimulus, TLR-ligand, induces a change in intracellular free Zn concentration, and this change is critically involved in the biological expression of the extracellular stimulus. Taken together, these results support the idea that Zn acts as an intracellular signaling molecule.

This idea was further strengthened by our finding that the extracellular stimulation of mast cells induces an increase in intracellular free Zn minutes later, which we call the ''Zn wave'' (Yamasaki *et al.*, 2007). The Zn wave originates from the region of the ER and is dependent on calcium influx and MAPK activation in mast cells. The findings that extracellular Zn is not involved in the Zn wave and that it is induced within several minutes of FcεR1 stimulation led us to conclude that Zn is a novel intracellular second messenger. This conclusion is drawn from the following results: (1) An extracellular stimulus, such as FcεRI cross-linking, directly induces an increase in intracellular free Zn, the Zn wave. (2) The source of Zn is an intracellular compartment, possibly the ER. (3) Free Zn at a level similar to that elicited by the Zn wave affects intracellular signaling molecules, such as tyrosine phosphatase, and therefore could modulate the final output triggered by an extracellular stimulus.

An important difference between the Zn wave in mast cells and the findings from zebrafish cells (Yamashita *et al.*, 2004) and dendritic cells (Kitamura *et al.*, 2006) is that the Zn wave is observed several minutes after stimulation, whereas the latter events are totally dependent on the transcriptional regulation of Zn transporters and are therefore detected several hours after stimulation. Thus, we propose that intracellular Zn signaling can be classified into at least two categories: early Zn signaling, involving the Zn wave, which is directly induced by an extracellular stimulus, and late Zn signaling, which is dependent on a transcriptional change in Zn transporter expression (Fig. 3.4). Under the former condition, Zn acts as a conventional intracellular second messenger, capable of directly transducing an extracellular stimulus into intracellular signaling events. Collectively, these results, and the fact that there are many transcription factors and enzymes containing Zn-binding sites, support the idea that Zn is a novel second messenger/signaling molecule that has the potential to influence many aspects of cellular signaling through its effect on Zn-binding proteins.

6. CONCLUSION AND FUTURE PROSPECTS

There is no question that Zn is involved in the immune system as an essential constitutive component. In fact, Zn deficiency causes severe immunodeficiency. Therefore, most studies on Zn have focused on how its homeostasis is maintained in general. However, recent studies show that Zn's functions are more extensive than implied by its simple definition as an essential molecule. Rather, it also seems to be a signaling molecule. Like calcium, Zn's intracellular status changes dynamically in response to extracellular stimuli, and it is capable of transducing an extracellular stimulus into intracellular signaling events. On the basis of

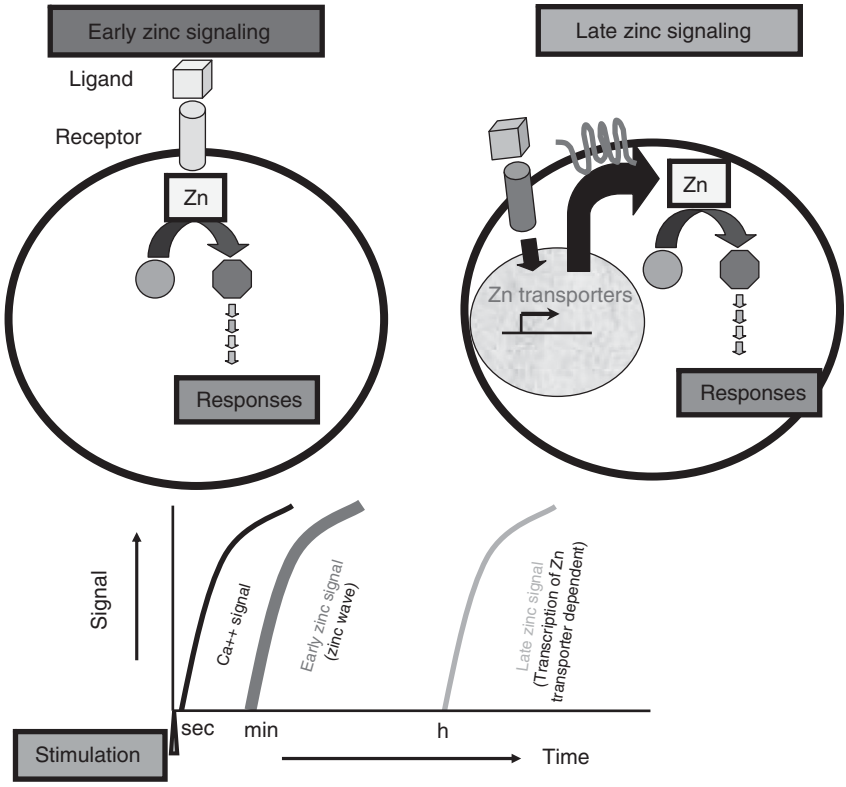

FIGURE 3.4 Early Zn signaling and late Zn signaling. Intracellular Zn signaling can be classified into at least two categories: early Zn signaling, involving the Zn wave, which is directly induced by an extracellular stimulus, and late Zn signaling, which is dependent on a transcriptional change in Zn transporter expression.

the accumulated evidence, then, we propose that Zn acts as a signaling molecule and that there are at least two modes of Zn signaling: ''late Zn signaling'' and ''early Zn signaling,'' which is the ''Zn wave'' type. Of course, many questions remain to be resolved. We do not yet know the real targets, the biological significance, or the mechanisms of Zn signaling, in particular, the mechanisms of the Zn wave. Calcium is not only an essential constitutive component of the body but also a critical signaling molecule in a wide range of biological responses, including immune responses. As in the case of calcium, it is likely that Zn is an important intracellular signaling molecule in systems besides the immune system. Therefore, we expect more researchers will be focusing their attention on Zn signaling and Zn biology in the next decade.

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CHAPTER 4

The SLAM and SAP Gene Families Control Innate and Adaptive Immune Responses

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Abstract

The nine SLAM-family genes, *SLAMF1–9*, a subfamily of the immunoglobulin superfamily, encode differentially expressed cell-surface receptors of hematopoietic cells. Engagement with their ligands, which are predominantly homotypic, leads to distinct signal transduction events, for instance those that occur in the T or NK cell immune synapse. Upon phosphorylation of one or more copies of a unique tyrosine-based signaling motif in their cytoplasmic tails, six of the SLAM receptors recruit the highly specific single SH2-domain adapters SLAM-associated protein (SAP), EAT-2A, and/or EAT-2B. These adapters in turn bind to the tyrosine kinase Fyn and/or other protein tyrosine kinases connecting the receptors to signal transduction networks. Individuals deficient in the SAP gene, *SH2D1A*, develop an immunodeficiency syndrome: X-linked lympho-proliferative disease. In addition to operating in the immune synapse, SLAM receptors initiate or partake in multiple

effector functions of hematopoietic cells, for example, neutrophil and macrophage killing and platelet aggregation. Here we discuss the current understanding of the structure and function of these recently discovered receptors and adapter molecules in the regulation of adaptive and innate immune responses.

1. INTRODUCTION

Adaptive and innate immune responses are orchestrated by the dynamic interactions between cell-surface molecules expressed on various types of leukocytes and cellular effectors. Adaptive immune responses utilize the specific interactions, either between T cell receptors (TCR/CD3) and their cognitive major histocompatibility complex (MHC)/peptide complexes or between B cell receptors (BCRs), that is, surface immunoglobulins and soluble antigens. During the last few decades, a considerable number of co-stimulatory molecules have been reported. Engagement of these co-stimulatory molecules by their ligands (or counter-structures) not only strengthens cell–cell interactions, it also triggers specific intracellular signaling networks that regulate functions such as activation, differentiation, apoptosis, proliferation, and acquisition of effector function and/or tolerance induction (Barclay, 2003; Reichardt *et al.*, 2007). The best-characterized T cell co-stimulatory pathways are initiated by members of the B7 family of receptors, which interact with the CD28/CTLA-4 receptors expressed on T cells. Importantly, while CD28 provides signals that augment and sustain T cell activation in the context of TCR/CD3 signaling, CTLA-4 functions as an inhibitory signal that reduces immune responses and induces tolerance (Greenwald *et al.*, 2005; Teft *et al.*, 2006).

The nine receptors of the SLAM-family (SLAMF1–9: CD150, CD48, CD229, CD244, CD84, SLAMF6, CD319, SLAMF8, and SLAMF9) (Table 4.1) are differentially expressed on the surface of hematopoietic cells. The cytoplasmic tails of six of these receptors carry one or more copies of a unique intracellular tyrosine-based switch motif (ITSM) (T-I/V-Y-x-x-V/I), which has a high affinity for the single SH2-domain signaling molecules SLAM-associated protein (SAP) and EAT-2 (EWS/FLI activated transcript-2). The notion that SLAM-family receptors operate as co-stimulatory molecules was initially based upon the functions in T cell activation ascribed to the cell-surface receptor SLAM (SLAMF1/CD150) and its association with the adapter SAP (Fig. 4.1). However, further studies have revealed that the SLAM-family receptors and their adapters SAP and EAT-2 function in both early phases and in lineage commitment steps of hematopoiesis (Borowski and Bendelac, 2005; Kiel *et al.*, 2005; Wagers, 2005). Furthermore, SLAM-family receptors not only serve as adhesion molecules on the surface of a variety of mature hematopoietic cells, but also control in part certain innate and adaptive

TABLE 4.1 SLAM family receptors

	Expression	Ligands	ITSMs	N-glyco sites	Effectors	Effects of receptor stimulation (Ab or Fc)	Mutant mice
SLAMF1, SLAM, CD150	T, B, DC, Mϕ, plat, HSC	SLAM, measles virus (H)	H: 2; M: 2	H: 8; M: 9	SHIP-1, Dok, Shc, Ras-GAP, PKC-θ, Bcl-10, NF-κB, Akt	T: ↑IFN-γ DC: ↑IL-12, ↑IL-8 Mϕ: ↑IL-12, ↑IL-6	Null (mixed 129-B6): T: ↓IL4, ↓IL-13, ↑IFN-γ Mϕ: ↓IL-12, ↓TNF-α, ↓NO, ↑IL-6 Plat: ↓aggregation
SLAMF2, CD48	Broad	CD244, CD2, FimH	None	H: 5; M: 6	Lck, Fyn	T: ↓IL2, ↓IFN-γ, ↓T cell proliferation (blocking Ab) B: ↓IL-13 by NK; ↓CD40L activation	Null (mixed 129-B6): T: ↓proliferation; ↓IL-2
SLAMF3, CD229, Ly-9	T, B, Mϕ	CD229	H: 2; M: 1	H: 7; M: 8	AP-2, Grb2, ERK	T: ↓IFN-γ	Null (mixed 129-B6): T: ↓IL-4, ↓IL-2, ↓proliferation
SLAMF4, CD244, 2B4	NK, CD8, γδ, baso, eo, mast, MHP	CD48	H: 4; M: 4	H: 8; M: 8	LAT, PI3K, Vav-1, SHIP-1, c-Cbl, ERK, p38, SHP-1, SHP2	NK: ↑killing, ↑IFN-γ CD8: ↑killing, ↑IFN-γ, ↑IL-2 Eo: ↑killing, ↑cytokines, ↑peroxidase	Null (pure B6): NK: ↓killing/IFN-γ $CD48^-$, ↑killing/IFN-γ $CD48^+$, ↓IL-13 induced by B
SLAMF5, CD84	Broad	CD84	H: 2; M: 2	H: 4; M: 3	ND	T: ↑Proliferation; ↑IFN-γ	ND

SLAMF6, NTB-A/ Ly108	T, B, NK(H), neutro	NTB-A	H: 2; M: 2	H: 7; M: 9	PLC-γ, PI3K	NK: ↑killing, ↑IFN-γ T: ↑IFN-γ	$Ly108^{\Delta E2+3}$ (mixed 129-B6): T: ↓IL-4 Neutro: ↓bacterial killing, ↓ROS, ↑cytokines
SLAMF7, CD319, CS1/ CRACC	T, B, NK, DC, Mϕ	CD319	H: 1; M: 0	H:7; M: 5	PLC-γ, Vav-1, PI3K	NK: ↑killing B: ↑Proliferation	ND
SLAMF8, BLAME	DC, Mo	ND	None	H: 3; M: 3	ND	ND	ND
SLAMF9, SF2001	T, B, Mo, DC	ND	None	H: 3; M: 5	ND	ND	ND

Ab, antibody; B, B cells; baso, basophils; CD8, $CD8^+$ T cells; DC, dendritic cells; eo, eosinophils; Fc, Fc fusion protein; γδ, γδT-cells; glyco, glycosylation; H, human; HSC, hematopoietic stem cells; IFN, interferon; IL, interleukin; ITSMs, immunoreceptor tyrosine-based binding motif that bind SAP or EAT-2; M, mouse; Mϕ, macrophages; mast, mast cells; Mo, monocytes; MHP, multipotent hematopoietic progenitors; N-Glyco, N-Glycosylation; neutron, neutrophils; ND, not determined; NK, natural killer cells; NO, nitric oxide; plat, platelets; ROS, reactive oxygen species; T, T cells.

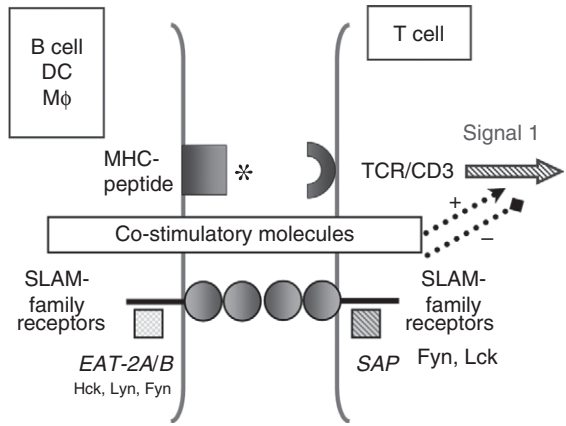

FIGURE 4.1 SLAM receptors in the T cell immune synapse. Receptors of the SLAM-family are widely expressed in hematopoietic cells. Receptor–ligand interactions occur between members of the SLAM-family in a homotypic fashion, except for CD244, which binds to CD48. Binding of SLAM-family members to their ligands induces the phosphorylation of their cytoplasmic tails and the subsequent binding of SLAM-associated protein (SAP) or EAT2 through a tyrosine-containing motif (ITSM). SAP is widely expressed by T cells and EAT2 is expressed by antigen presenting cells (APCs). These two molecules can recruit and activate several Src kinases (including Fyn) that modulate cell activation by signals generated through the T cell receptor (TCR) and co-stimulatory proteins such as CD28. Signals mediated by the SLAM receptors can also affect the function of APCs. SLAM receptors recruit various SH2-domain-containing proteins giving rise to different signals that determine distinct and, in some cases, divergent biological outcomes.

immune responses (Engel *et al.*, 2003; Ma *et al.*, 2007; Veillette, 2006a; Veillette *et al.*, 2006). For instance, SLAM-family receptor–ligand pairs not only regulate proliferation, cytotoxicity, and cytokine production of T lymphocytes, but also modulate lytic activity, cytokine production, and MHC-independent cell inhibition of natural killer (NK) cells; B cell activation and memory generation; and regulation of neutrophil and macrophage killing and platelet aggregation. A considerable degree of genetic polymorphisms as well as usage of isoforms has been observed in the SLAM-family genes. These and other recent advances in the study of this relatively new gene family will be discussed in this chapter.

2. STRUCTURE, LIGANDS, AND EXPRESSION OF THE SLAM-FAMILY RECEPTORS

2.1. The SLAM receptor gene family

The SLAM-family genes are located on chromosome 1 in humans and mice; seven of the genes are located in a contiguous ~400 kb fragment in both mouse (173.39 until 173.8 Mb) and human chromosome 1 (158.7 until

159.1 Mb) (Engel *et al.*, 2003; Veillette *et al.*, 2006) (Fig. 4.2). The human and mouse genes encoding SLAMF8 and SLAMF9 as well as the human adapter EAT-2 (*SH2D1B*) and the mouse adapters Eat-2a/b (*Sh2d1b/Sh2d1c*) are located in close proximity to the ''core'' SLAM locus (Fig. 4.2). Based on the similarities in sequence, genomic organization, gene localization, and ITSMs, SLAM-family genes are thought to have arisen from a common ancestral gene (Fig. 4.3) (Conrad and Antonarakis, 2007; Davis and van der Merwe, 1996; Engel *et al.*, 2003; Sidorenko and Clark, 2003).

Whereas eight SLAM molecules are type I transmembrane glycoproteins with a cytoplasmic domain, CD48 is anchored in the plasma membrane via a glycosyl-phosphatidylinositol (GPI) (Staunton and Thorley-Lawson, 1987; Yokoyama *et al.*, 1991). Ectodomains of most of the SLAM-family members are comprised of an N-terminal V-Ig domain lacking the canonical disulfide bond and a C-terminal C2-Ig domain, characterized by conserved cysteines. The only exception is CD229, which consists of a tandem repeat of two V-Ig/C2 sets of domains (Sandrin *et al.*, 1992). Although the extracellular domain of CD2 and CD58 share some of

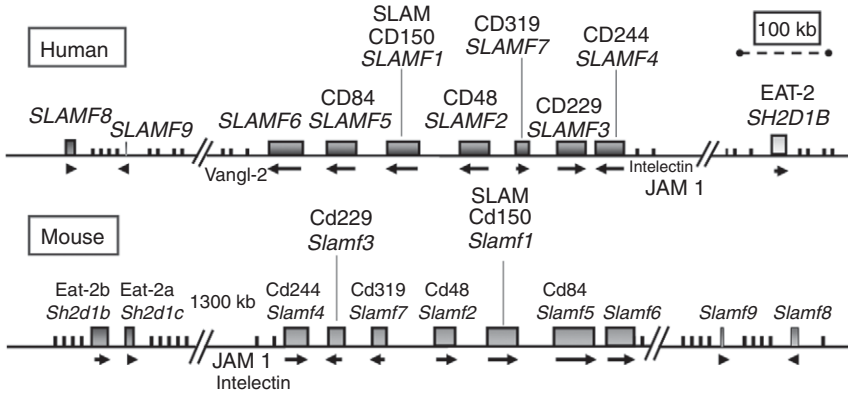

FIGURE 4.2 Genomic organization of the human and mouse SLAM locus. The genes encoding members of the signaling lymphocyte activation molecule (SLAM) family are located on human chromosome 1q23 and mouse chromosome 1 H2. Seven genes belonging to the *SLAM-family* are clustered in a genomic segment of ~400 kb, in both humans and mice. The arrangement of the SLAM-gene family is identical in both genomes. The only difference is their orientation; the genes in humans closer to the centromere are situated in mice closer to the telomere. Three other genes that belong to the SLAM-family (*SLAMF8* and *SLAMF9)* are located in the same chromosome region, but outside of the SLAM locus. Human *EAT-2* and mouse *Eat-2a* and *Eat-2b* are also located close to the SLAM locus. Filled boxes represent blocks of sequence containing a complete set of gene exons. Black arrows denote the transcriptional direction of these genes.

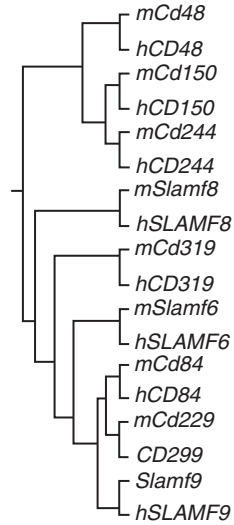

FIGURE 4.3 Dendrogram of the SLAM-family members. A phylogenetic tree of the ectodomain protein sequences of CD150 family members was constructed using the multiple sequence alignment by CLUSTALW. In the case of CD229, the sequences of the two N-terminal domains were used.

these characteristics with the SLAM-family receptors (Ames *et al.*, 2005; Cao *et al.*, 2006; Davis and van der Merwe, 1996; Evans *et al.*, 2006; Tangye *et al.*, 2000b; Yan *et al.*, 2007), the homology between these two leukocyte receptors and members of the SLAM-family is low (less than 15% sequence identity) (Wang *et al.*, 2001) (Fig. 4.4).

The SLAM-family receptors specifically interact with their ligands through their extracellular domains. While CD150, CD84, SLAMF6, CD319, and CD229 participate in homophilic interactions (Falco *et al.*, 2004; Flaig *et al.*, 2004; Kumaresan *et al.*, 2002; Martin, 2001; Punnonen *et al.*, 1997; Romero *et al.*, 2005), CD244 is a receptor for CD48 (Brown *et al.*, 1998; Latchman *et al.*, 1998; Velikovsky *et al.*, 2007).

The structure of NTB-A (SLAMF6) shows that opposing molecules engage with their N-terminal Ig-V domains, resulting in an orthogonal association of two NTB-A molecules (Fig. 4.4) (Cao *et al.*, 2006). The β-sheets at the interface contain multiple residues, which contribute to the stability of the interaction resulting in a Kd of ~2 μM, which is considerably higher than that published for SLAM (~200 μM) (Mavaddat *et al.*, 2000), yet lower than that of CD84 (Yan *et al.*, 2007). As in the case with CD84, the orthogonal association of two NTB-A molecules enables these complexes to localize to the immunological synapse (Cao *et al.*, 2006; Yan *et al.*, 2007). The data obtained in these studies also show that the

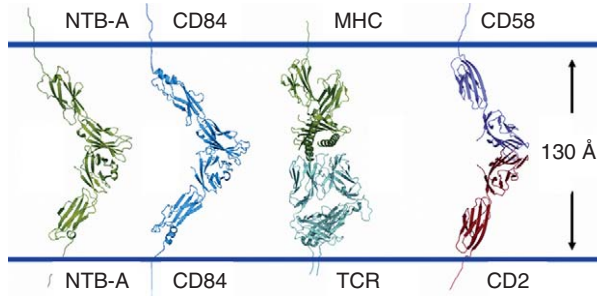

FIGURE 4.4 Homophilic interactions in T cell activation.The ribbon diagrams represent the homophilic interaction of NTB-A and CD84 as well as the heterophilic interaction of cD2 with CD58 between APCs and T cells. The MHC–TCR complex is also shown for a comparison of molecular dimensions.

SLAM-family homophilic affinities span at least three orders of magnitude and suggest that differences in the affinities may contribute to the distinct signaling behavior exhibited by the individual family members.

The cytoplasmic domains of SLAM, CD229, CD84, CD244, NTB-A, and CD319 contain both multiple ITSMs and non-ITSM phosphotyrosine residues. Thus, SAP/EAT2-dependent and -independent recruitment of intracellular molecules leads to the inhibitory and stimulatory signals, which ensue upon engagement of these receptors. Integration of signaling networks determines the outcome of several effector functions depending on the cell type and state of activation (Engel *et al.*, 2003; Ma *et al.*, 2007; Morra *et al.*, 2001a; Veillette, 2006a).

A potential role for SLAM, CD244, and CD48 in the first steps of hematopoietic stem cell differentiation was revealed by the work of the Morrison lab (Kiel *et al.*, 2005). Long-term reconstituting-hematopoietic stem cells (LT-HSC) and multipotent progenitors (MPPs) both give rise to all lineages of blood cell types. However, whereas LT-HSC possess the capacity for extensive self-renewal, as demonstrated by their ability to maintain lifelong production of blood cells, MPP provide only transient contributions to hematopoietic lineages (Fig. 4.5). In transplantation assays, SLAM-positive, but not SLAM-negative, bone marrow cells provided reproducible and robust long-term, multilineage reconstitution of blood cells in irradiated recipient mice, fulfilling the ''gold-standard'' test for HSC function. Remarkably, analysis of additional members of the SLAM-family (CD244 and CD48) demonstrated a strikingly selective expression of different SLAM receptors by LT-HSC, MPP, or oligolineage-restricted progenitor (OP) cells. In contrast to the antibody studies, the transfer of enriched stem cell preparations derived from $SLAM^{-/-}$, $CD244^{-/-}$, or $CD48^{-/-}$ deficient mice was inconclusive (Kiel *et al.*, 2005),

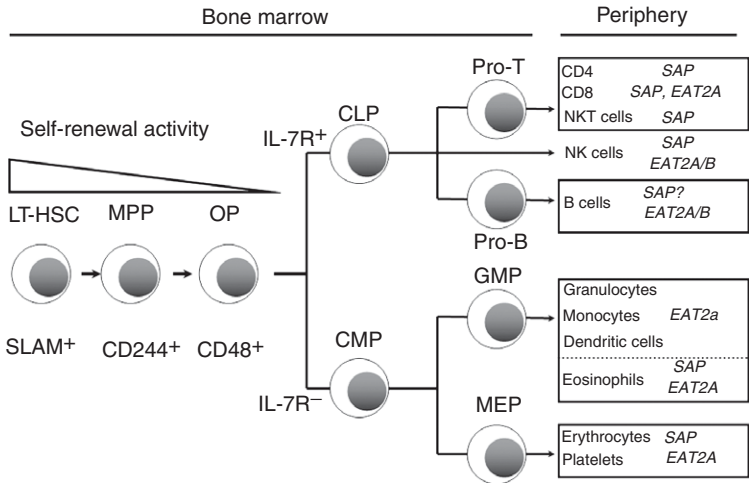

FIGURE 4.5 Differential expression of SLAM-family receptors distinguish hematopoietic and progenitor stem cells.The SLAM-family receptors SLAM, CD244, and CD48 are differentially expressed on hematopoietic stem cells in a way that correlates with progenitor maturity in the bone marrow. Self-renewal potential decreases as SLAM$^+$ long-term reconstituting-hematopoietic stem cells (LT-HSC) differentiate first into CD244$^+$ multipotent progenitors (MPPs) and then into CD48$^+$ oligolineage-restricted progenitors (OPs). This selective expression of SLAM-family members permits a simplified method of isolating distinct stem cell progenitor subsets and allows for the visualization of these cell types in their natural environment. These progenitors further mature into IL-7R$^+$ common lymphoid progenitor (CLP) and IL7R$^-$ common myeloid progenitor (CMP) cells, which give rise to NK cells or to T and B cells precursors in the case of CLPs, or to granulocyte/monocyte progenitors (GMP) or megakaryocyte/erythrocyte progenitors (MEP) in the case of CMPs. These progenitors eventually give rise to all mature immune cells in the periphery. The adaptors (SAP, EAT2A, or EAT2B) that have been identified in each cell type are listed.

suggesting a compensatory role of other genes, possibly belonging to the SLAM-family.

SLAM and Ly108 also appear to cooperate in the development of natural killer T (NKT) cells. Whereas mainstream thymocyte precursors recognize MHC ligands expressed by stromal cells, NKT cell precursors interact with CD1d ligands expressed by cortical thymocytes. Griewank *et al.* (2007) recently found that the cooperative engagement of Ly108 and SLAM and the downstream recruitment of the adapter SAP and the Src kinase Fyn are essential for expansion and differentiation of the NKT cell lineage. The possibility that SLAM receptors, which are often co-expressed on the surface of the same cell, exert overlapping functions remains one of the challenges in the study of this field.

2.2. The individual SLAM receptors

2.2.1. SLAMF1 (SLAM, CD150)

Human SLAM (signaling lymphocyte activation molecule) (*NM_003037)* (Cocks *et al.*, 1995) is a 335-amino-acid type I transmembrane protein with two Ig-domains (N-terminal V- and a C-terminal C2-domain), a 22-amino-acid transmembrane segment, and a 77-amino-acid cytoplasmic tail. The latter contains three tyrosines, two of which are embedded in ITSM sites (Table 4.1) (Cocks *et al.*, 1995; Howie *et al.*, 2002b; Latour *et al.*, 2001; Mikhalap *et al.*, 1999; Sayos *et al.*, 1998; Shlapatska *et al.*, 2001). The predicted amino acid sequences of human and mouse SLAM (*NM_013730)* (57% identity) suggest the presence of eight and nine N-linked glycosylation sites, respectively (Castro *et al.*, 1999; Wang *et al.*, 2001).

Isoforms of both human and mouse SLAM have been detected in activated T and B cells (Table 4.2), including two membrane-bound forms that differ in the length of their cytoplasmic tails, a secreted form that lacks the transmembrane region, and a cytoplasmic form lacking the leader peptide (Castro *et al.*, 1999; Cocks *et al.*, 1995; Isomaki *et al.*, 1999; Punnonen *et al.*, 1997).

Human and mouse SLAM are expressed in thymocytes, memory $CD4^+$ and $CD8^+$ T cells, naïve follicular and marginal zone B cells, dendritic cells (DCs), macrophages, and platelets (Cocks *et al.*, 1995; Henning *et al.*, 2001; Kruse *et al.*, 2001; Munitz *et al.*, 2005; Nanda *et al.*, 2005; Romero *et al.*, 2004; Sayos *et al.*, 2000; Sidorenko and Clark, 1993). Whereas expression of mouse SLAM is the highest on double positive (DP) ($CD4^+$ $CD8^+$) thymocytes, single positive (SP) T cells express low levels of the receptor (Griewank *et al.*, 2007; Jordan *et al.*, 2007). As in the mouse, human SLAM is rapidly up-regulated upon activation of peripheral T and B lymphocytes (Aversa *et al.*, 1997; Cocks *et al.*, 1995; Punnonen *et al.*, 1997; Sidorenko and Clark, 1993), macrophages, DCs, and platelets (Howie *et al.*, 2002a; Kruse *et al.*, 2001; Nanda *et al.*, 2005; Romero *et al.*, 2004; Wang *et al.*, 2001).

2.2.2. SLAMF2 (CD48)

The GPI-anchored protein CD48 (Cabrero *et al.*, 1993; Staunton and Thorley-Lawson, 1987; Staunton *et al.*, 1989; Wong *et al.*, 1990; Yokoyama *et al.*, 1991) is comprised of an ectodomain with an Ig-V-like domain and an IgC2-like domain. The GPI group is attached to a serine (position 195) in the carboxyl terminus (Killeen and Moessner, 1988). The ectodomains of humans (*NM_001778)* and mice (*NM_007649)* share a 51% amino acid identity and carry five or six N-linked glycosylation sites, respectively. CD48 is not only a counter-receptor for CD244 but also for CD2 (Arulanandam *et al.*, 1993; Brown *et al.*, 1998). The affinity of human CD48–CD224 interaction is very similar across species and is much higher

TABLE 4.2 Polymorphisms and isoforms of the SLAM-family members

	Isoforms					Polymorphisms		
							Syn.	
	SP	TYPE	ITSM	Ys	CHANGES	Non Syn.	C	NC
SLAMF1/ SLAM	M	FL 343AA NP_038758	2	3		273: S ⇒P (only NZB)	3	12
		Short#1 329AA AAF22232	1	1	296: ⇒ VRSMPHLAGVSVIFR TGFLIAALHTTMVLQGLLE*			
		Short#2 326AA BAC29870 (B6)	1	0	296: ⇒ VRSMLTLGSVCHIS HRISDSCLAHNHGPAGI*			
	H	FL 335AA NM_003037	2	3		11: F ⇒ L 38: Q ⇒H 290: T ⇒S 315: P ⇒ S 333: P ⇒ T	2	19
		Short 298AA *Q13291–2*	1	1	289:⇒DTHHQTSDLF*		2	32
		Secreted#1 305AA *Q13291–3*	2	3	234–263: MISSING		2	5
		Secreted#2 239AA	0	0	234: ⇒ASSEET*		2	0
SLAMF2/ CD48	M	FL 240AA NP_031675	0	0		4: I⇒R 90: N⇒D	0	14
		mCD48Δ2 151AA BAC40104	0	0	125–214: MISSING			
		Secreted #1 145AA	0	0	125: ⇒DARMFHLKTDVSSSYAEEEA*			
		Secreted#2 164AA	0	0	125: ⇒ DPVPKPSIEINKTEASTD SCHLRLSCEPDLLEYVGLQLG*			
	H	FL 243AA NP_001769	0	0		16: S ⇒ L 50: P ⇒ L 102: E ⇒ Q 241: L ⇒ S	5	9
		Secreted 169AA AAH30224	0	0	128: ⇒ GESGEPKSKSPLQWPQM DHCRASWEAWGTLGEEERKTSGQV*		4	3
SLAMF3/ CD229	M	FL 654AA NP_032560	1	9		10: LP/Extra 5: TM/Cyto	6	15
		Ly9Δ1 544AA BAC40591	1	9	41–151: MISSING			
		Secreted 385AA AK040306	0	0	387: ⇒SEYPSPASSLSQL SSHRGTQEGNIRTSG*			
		Short 636AA AK087345	1	8	633: ⇒VVL*			

	H	FL 655AA NP_002339	2	7		32: L ⇒V 91: R ⇒H 106: R ⇒* 196: P ⇒T 281: V ⇒I 357: Y ⇒Q 602: V ⇒M 622: S ⇒A	3	14
		HLy9ΔCyto 641AA *Q9HBG7–2*	2	7	500–513: MISSING			
		HLy9Δ4 521AA *Q9HBG7–3*	2	7	359–448; 500–513: MISSING			
SLAMF4/ CD244	M	M2B4L 397 AA NP_061199	4	5		37: LP/Extra 10: TM/Cyto	3	27
		M2B4S#1 340AA AAC34859	1	1	309: ⇒MFSSLLAFLLHQFPGSTQRG KEKRERAEKNGK*			
		M2B4S#2 312AA BAE42518	1	2	309: -⇒ IRKEY*			
	H	FL 370AA AAK00233	4	6		89: N⇒D 292: G ⇒A 323: S ⇒F	3	9
		CD244A2 273AA *Q9BZW8–4*	4	6	128–224:MISSING			
		hCD244S 329AA CAC00649	3	4	326:⇒GDRFYSFSG*			
SLAMF5/ CD84	M	FL 329AA NP_038517	2	4		27: M ⇒V 293: E ⇒K	2	4
	H	FL 345 AA Q9UIB8	2	5		288: I⇒T	3	65
		Short#1 272AA AAF21723	0	0	255:⇒ASLQGRASEHSLFRSAVC*			
		Short#2 280AA AAD04232	0	1	261:⇒ KMWKLTFSPPGTEAIYPRFS*			
		Short#3 328AA NP_003865	2	4	254–271: MISSING			
		Short #3 CD84A1 214AA CAI45963	2	4	16–130: MISSING; 254–271: MISSING			
SLAMF6/ NTB-A (Ly108)	M	Ly108–1 351AA AAH30031	2	4			2	19
		Ly108–2 331AA NP_109635	2	3	328:⇒AEYS*		3	16

(*continued*)

TABLE 4.2 (*continued*)

	Isoforms					Polymorphisms		
							Syn.	
	SP	TYPE	ITSM	Ys	CHANGES	Non Syn.	C	NC
	H	FL 331AA NP_443163	2	3		157: A⇒V	5	12
		Short#1 271AA AAH90928	0	0	265:⇒GEHSDS*		5	12
		Short#2 271AA EAW52714	0	1	252:⇒ IPYLCLLSEHRAPQSPQGT*			
		Short#3 317AA EAW52716	0	2	252:⇒ GRFPIFVYSANTGPRRVRK EPRVCFSVSNEQHCVCFSHSFKQGESF QLYMPLLSVLQPLFTNLSP*			
		NTB-AΔ1 221AA CAI15156	2	3	17–128: MISSING		5	8
		Δ1 Short 163AA	0	1	17–128: MISSING; 252:⇒ VQIPYLCLLSEHRAPQSPQGT*		4	8
SLAMF7 CD319	M	FL 333AA NP_653122	0	3		249: M ⇒T 253: G ⇒R	1	23
		FL#2 335AA BAC40914 (B6)	0	3	312: ⇒ RSCPAEHHLTCQPLSLDHAR AQIS*		0	
		Short#1 294AA AAH11154 (FVB)	0	0	254: ⇒EKKTRRRCTKHILFHCAD PQSGKESQLPACKATRAKVIKL*		0	
		Short#2 300AA BAE96340	0	1	254–289: Missing; 312: ⇒ RSCPAEHHLTCQPLSLDHARAQIS*			
	H	CD319-L 335AA NP_067004	1	4		175: H ⇒ Y 249: F ⇒S 269: R ⇒W 276: E ⇒K 302: T ⇒M	3	11
		CD319Δ1 228AA CAD39085	1	4	19–125: MISSING			
		CD319-S#1 296AA AAH27867	0	1	257: ⇒ NNPKGRSSKYGLLHCGNTEK DGKSPLTAHDARHTKAICL*			
		CD319-S#2 328AA CAB76561	0	3	257:⇒NNPKGRSSKYGYSTVEIPKKM ENPTHCSRCQTHQGYLPMRMLSRQQ CTSLSLLLKKKTILGPKKTSEEFLV*			

SLAMF8/ BLAME	M	FL 278AA *(0 ITSM, 0 Y)* NP_083360				107: R ⇒G 116: Y ⇒H 164: R⇒W/Q 175: H ⇒R	6	12
	H	FL 285AA NP_064510	0	0		5: P⇒ T 65: R ⇒* 75: R ⇒C 99: G ⇒S 129: V ⇒M 183: G ⇒R	0	11
		hBLAMEΔ1 176AA *Q9P0V8–2*	0	0	14–122: MISSING		0	14
SLAMF9/ SF2001	M	FL 285AA *(0 ITSM, 1 Y)* NP_083888				29: I ⇒V 74: M ⇒T 204: V ⇒I 210: R ⇒H 224: P ⇒L 263: R ⇒W 280: S ⇒P	3	2
	H	FL 289AA NP_254273	0	0		86: V ⇒M 127: I ⇒L 162: G ⇒S 164: D ⇒H 181: E ⇒K	2	4
		FL#2 358AA EAW52754	0	4	1–15: ⇒MRGATMQSRGQYSCTGQVMYIPQTFTQT SETAMVQVQAPVSHPVLTLHHGPADPAVED VVQLLCEAQRGSPPILYLFYLNGEIL			
		hSLAMF9Δ2 198AA AAQ89161	0	0	132–222: MISSING		0	9

AA, aminoacid; C, coding; Cyto, cytoplasmic tail; Extra, extracellular; FL, full length; H, human; ITSM, immuno tyrosine switch motif; LP, leader peptide; M, mouse; NC, noncoding; *, stop codon; Syn, synonymous; SP, species; TM, transmembrane; underlined polymorphism: specific polymorphisms for the underlined isoform; isoforms in italics correspond to predicted isoforms; accession numbers in italics correspond to Ensembl ID.

than that of CD48 with CD2 (Brown *et al.*, 1998). Because of its low avidity, the physiological significance of the CD2–CD48 interaction in humans is unclear (Arulanandam *et al.*, 1993). However, human CD58, which does not have an ortholog in rodents, is found to interact with the same region of CD2 as CD48, but at a 100-fold higher affinity than CD48 (Arulanandam *et al.*, 1993; Evans *et al.*, 2006) (Fig. 4.4). Structural analyses show that the receptor-binding surface of CD48 is unusually flat and shares a high degree of electrostatic complementarity (Evans *et al.*, 2006). This flat topology may explain why CD48 cross-reacts with both CD2 and CD244 (Ames *et al.*, 2005; Velikovsky *et al.*, 2007; Wang *et al.*, 1999) (Fig. 4.4). Whereas human CD48 was initially identified because of its induction after Epstein–Barr virus (EBV)-induced activation of resting B cells (Thorley-Lawson *et al.*, 1993), both human and mouse CD48 are widely expressed on all human and mouse hematopoietic cells (Kato *et al.*, 1992; Yokoyama *et al.*, 1991).

2.2.3. SLAMF3 (CD229, Ly9)

Among the SLAM-family receptors, the highly glycosylated human and mouse CD229 (NM_002348 and NM_08534) stand apart because their ectodomain comprises four Ig-domains (de la Fuente *et al.*, 2001; Durda *et al.*, 1979; Sandrin *et al.*, 1992). As the first and the third Ig-domains, as well as the second and fourth C2-domains, are very similar (Sandrin *et al.*, 1992), CD229 must have arisen from a two-Ig-domain SLAM-family progenitor. Human and mouse CD229 have eight N-linked glycosylation sites. Among SLAM-family receptors, CD229 has the longest cytoplasmic tail of 179 amino acids and eight tyrosines (Table 4.1). Only two of the eight tyrosines in its cytoplasmic tail are embedded in ITSMs and have been shown to associate with SAP and/or EAT-2 (Morra *et al.*, 2001b; Sayos *et al.*, 2001).

Three different isoforms of human CD229 were isolated from the B cell line Raji by RT-PCR: full length CD229, one isoform lacking the first domain (HLy9Δ1) and a second isoform lacking the fourth domain (HLy9Δ4). Only full length CD229 and HLy9Δ4 were detected by immunoprecipitation in the B cell line Daudi (de la Fuente *et al.*, 2001) (Table 4.2). Mouse CD229 was initially described as an alloantigenic marker of lymphocyte differentiation (Sandrin *et al.*, 1992). One allele, that is, Ly-9.1 is expressed in most mouse strains, whereas Ly-9.2 is only found in C57BL/6 and the related strains (C57BR, C57L, and C58). Sequencing of CD229 cDNAs from Balb/c and C57BL76 mouse strains showed nine differences, four of which are in the first Ig-like domain (Hogarth *et al.*, 1980; Kozak *et al.*, 1984; Tovar *et al.*, 2000). Because mouse CD229 is broadly expressed during lymphoid differentiation, monoclonal antibodies (mAbs) against the Ly-9.1 allotype are used as markers to

examine the contribution of embryonic stem-derived cells to lymphoid development in RAG-1 and RAG-2 deficient blastocyst complementation studies (Tovar *et al.*, 2000).

Domain deletion mutants show that the N-terminal Ig-domain of both human and mouse CD229 mediate homophilic adhesion (Romero *et al.*, 2005). CD229–CD229 binding is severely compromised when the charged amino acids on the predicted BC loop and FG loop of the N-terminal domain are mutated to alanine. Moreover, confocal microscopy image analysis reveals re-localization of CD229 to the contact area of T and B cells during antigen-dependent immune synapse formation (Romero *et al.*, 2005).

All B cell subsets (naive, follicular, and marginal zone) as well as all T cells (naive, effector, memory) express CD229 (de la Fuente *et al.*, 2001; Romero *et al.*, 2004). Using DNA-microarray analysis, CD229 was found to be expressed at higher levels by human follicular helper T cells (T_{FH}) than on other $CD4^+$ T cell subsets (Chtanova *et al.*, 2004; Vinuesa *et al.*, 2005). In contrast to humans (de la Fuente *et al.*, 2001; Romero *et al.*, 2004), murine CD229 is highly expressed in NK cells and monocytes (Romero *et al.*, 2004; Sintes *et al.*, 2007).

2.2.4. SLAMF4 (CD244, 2B4)

Mouse (*NM_018729)* and human (*NM_016382)* CD244 were originally identified as activating NK cell receptors that mediate non-MHC-restricted killing (Garni-Wagner *et al.*, 1993). CD244 is, however, expressed on all NK cells, $\gamma\delta^+$ T cells, a large subset of $CD8^+$ T cells, and monocytes (Boles *et al.*, 2001; Garni-Wagner *et al.*, 1993; Nakajima *et al.*, 1999). CD244 is also expressed on mouse and human mast cells, basophils, and eosinophils (Kubota, 2002; Munitz *et al.*, 2005; Romero *et al.*, 2004).

The Ig-V-like and Ig-C2-like domains of human and mouse CD244 have eight potential N-glycosylation sites, with 40% sequence identity between mice and humans and 15–20% identity with CD150, CD48, and CD84 (Fig. 4.3). In humans, mice, and rats, CD244 is found to be the counter-receptor for CD48 (Brown *et al.*, 1998; Kumaresan *et al.*, 2000; Latchman *et al.*, 1998). NMR analyses show that CD244 has unique structural features that may be important for ligand recognition (Ames *et al.*, 2005). The structure of the CC′ and FG′ loops in CD244 and domain 1 of CD2 differ substantially, which may explain, in part, ligand specificity. The FG loop in CD244 is dynamically disordered, perhaps because ligand binding is needed to stabilize its structure. The dynamics of the FG loop might suggest a model of CD244 employing an induced-fit mechanism for recognition of its ligand CD48 (Ames *et al.*, 2005). Interestingly, two isoforms of human CD244 have been identified, hCD244-A and hCD244-B (Kumaresan and Mathew, 2000), which differ only in a small portion of the extracellular domain because differential splicing results in the

addition of five amino acids at the Ig-V domain. Although the function of the splice variants is yet to be determined, this observation suggests that the two isoforms may have different binding affinities for CD48.

Two isoforms of the cytoplasmic domain of mouse CD244, for example, CD244-L and CD244-S, have been identified, the shorter of which CD244-S contains only one ITSM (Kubin *et al.*, 1999; Schatzle *et al.*, 1999; Stepp and Schatzle, 1999) (Table 4.2). Transfection studies suggest that the differential expression of the short and long isoforms of CD244 may determine distinct CD244 downstream signaling events (Schatzle *et al.*, 1999). Four tyrosine residues in the CD244-L cytoplasmic tail are embedded into ITSM, but recruit, in addition to SAP and EAT-2, several other signaling molecules (Table 4.1) (Chen *et al.*, 2004; Morra *et al.*, 2001b; Parolini *et al.*, 2000; Sayos *et al.*, 2000; Tangye *et al.*, 1999). Not surprisingly, CD244 and SAP co-localize to the immunological synapse between NK cells and target cells (Roda-Navarro *et al.*, 2004). CD244 binds constitutively to the adapter protein LAT via a dicysteine motif (CxC) located in the transmembrane domain (Bottino *et al.*, 2000; Klem *et al.*, 2002). Human, but not mouse, CD244 binds to the adapter 3BP2 that links this receptor to Vav-1 signaling (Saborit-Villarroya *et al.*, 2005).

2.2.5. SLAMF5 (CD84)

Human CD84 (*NM_003874)* is a single chain cell-surface glycoprotein with an extracellular portion of 199 amino acids, which contains four potential N-glycosylation sites (Table 4.1) (de la Fuente *et al.*, 1997). The transmembrane region consists of 25 amino acids and the 83-amino-acid cytoplasmic tail contains four tyrosines, two of which are embedded in an ITSM (de la Fuente *et al.*, 1997, 1999). The human CD84 protein is 57.3% identical to murine CD84 (*NM_013489)* (de la Fuente *et al.*, 1999) and the CD84 amino acid sequence is most homologous to CD229 (de la Fuente *et al.*, 1997) (Fig. 4.3).

CD84 is predominantly expressed by B cells, T cells, platelets, monocytes, DCs, and is also expressed early in hematopoiesis (Table 4.1) (de la Fuente *et al.*, 1997; Kruse *et al.*, 2001; Martin, 2001; Nanda *et al.*, 2005; Romero *et al.*, 2004; Zaiss *et al.*, 2003).

Based on the expression of CD84, B cells can be subdivided into $CD84^{hi}$ and $CD84^{lo}$ populations. The $CD84^{hi}$ population represents a subset of memory B cells, which are characteristic of co-expression of CD27, somatically mutated Ig variable region genes, and vigorous proliferation in response to CD40L and IL-4 compared with $CD84^{lo}$ B cells (Martin, 2001; Tangye *et al.*, 2002). A striking feature of human CD84 is the complex expression of isoforms with several cytoplasmic tails in the T cell tumor line CEM (Palou *et al.*, 2000). CD84 isoforms are generated by several mechanisms, including alternative use of exons, reading frame shift, or absence of splicing. The expression and functional relevance of

these variants is still unknown. Similarly, two CD84 transcripts are detected in murine tissues (Table 4.2).

CD84 strongly self-associates (Martin, 2001; Romero *et al.*, 2005) with a Kd in the submicromolar range, which is driven by the Ig-V domain, forming an orthogonal homophilic dimer (Fig. 4.4) (Yan *et al.*, 2007). These structural data also suggest that, like NTB-A, SLAM-family homophilic dimers adopt a highly kinked organization spanning an end-to-end distance of ~140 Å. Although the CD84 structure is similar to the homophilic dimer of NTB-A, detailed structural and chemical differences in the respective homophilic interfaces provide a mechanism to prevent the formation of undesired heterodimers among the two homophilic receptors.

2.2.6. SLAMF6 (NTB-A, Ly108)

The extracellular region of human SLAMF6 (NTB-A, *NM_052931)* consists of two extracellular Ig-like domains with seven potential N-glycosylation sites (Bottino *et al.*, 2001; Fraser *et al.*, 2002; Peck and Ruley, 2000). The 83-amino-acid cytoplasmic tail contains three tyrosines, two of which are in an ITSM (Table 4.1). Several splice variants of mouse SLAMF6 (Ly108, *NM_030710)* have been identified: In the two isoforms, Ly108–1 and Ly108–2, which differ by expressing alternative terminating exons that result in the substitution of 4aa C-terminal in Ly108–1, with 24 amino acids in Ly108–2 (Table 4.2). The C-terminal 24 amino acids of Ly108–2 contains an additional SH2 domain binding motif TGYNQP (Peck and Ruley, 2000; Wandstrat *et al.*, 2004). Splice variants exist in human NTB-A as well (Table 4.2). In addition to the isoform that is predominately expressed in T, B, and NK cells, Bottino *et al.*, cloned a second cDNA coding for a putative allelic isoform of NTB-A from NK cell clones (Bottino *et al.*, 2001). This variant of NTB-A has an extra codon (CAG) resulting in the insertion of an Ala residue in proximity to the transmembrane region. BLAST analysis of human EST sequences identified a third isoform, characterized by the lack of a V-like domain (Table 4.2). Although alternative splicing is one of several mechanisms by which some SLAM-family members attain variability, the functions of the splicing variants of human NTB-A remain unknown.

SLAMF6 is primarily expressed in human and mouse lymphoid organs (Fraser *et al.*, 2002; Peck and Ruley, 2000). Expression is high in the spleen, thymus, and lymph nodes, and low in the bone marrow, lung, and liver. NTB-A is expressed on the cell surface of all human NK and T and B cells and the receptor is up-regulated on activated T and B cells (Bottino *et al.*, 2001; Valdez *et al.*, 2004).

Homotypic interactions of NTB-A have a relatively high Kd (~2 μM) (Falco *et al.*, 2004; Flaig *et al.*, 2004; Valdez *et al.*, 2004). The 3.0 Å crystal structure of the complete NTB-A ectodomain revealed a rod-like

monomer that self-associates to form a highly kinked dimer spanning an end-to-end distance of ~100 Å. The NTB-A homophilic and CD2–CD58 heterophilic dimers show overall structural similarities but differ in detailed organization and physicochemical properties of their respective interfaces (Cao *et al.*, 2006).

2.2.7. SLAMF7 (CD319, CRACC, CS1)

Human (*NM_021181)* and murine (*NM_144539)* CD319 are comprised of two-Ig-like domains: an Ig-V, which lacks disulfide bonds, and a truncated Ig-C2 with four cysteines enabling two disulfide bonds (Boles and Mathew, 2001; Bouchon *et al.*, 2001; Tovar *et al.*, 2002). Comparison of the predicted amino acid sequences of both human and mouse CD319 shows an identity of 47% and 44% respectively, to SLAM.

The cytoplasmic tail of human CD319 contains one ITSM, while mouse CD319 has none (Lee *et al.*, 2004a; Tassi and Colonna, 2005; Tovar *et al.*, 2002). Interestingly, although human CD319 is able to bind SAP in over-expression systems (Tovar *et al.*, 2002), it recruits EAT-2 and not SAP in human NK cells (Tassi and Colonna, 2005). An association of CD319 with SHP-1, SHP-2, or SHIP has not been detected (Bouchon *et al.*, 2001; Lee *et al.*, 2004a).

A short splice variant form of human CD319 (CD319-S) was identified, which is 39 amino acids shorter than wild-type CD319 (CD319-L). Comparison of the predicted protein encoded by the two isoforms reveals that they share the same extracellular and transmembrane domains, but differ in their cytoplasmic regions. CD319-S lacks the SAP/EAT2 tyrosine-based motif (Table 4.2). The observation that CD319-L transfected RNK16, but not CD319-S transfected cells, is able to mediate redirected cytotoxicity and intracellular calcium increase demonstrated that CD319-L is an activating receptor, while CD319-S may not function because it lacks SAP/EAT2 binding motif in the tail (Lee *et al.*, 2004a). Interestingly, RT-PCR analysis showed the presence of a mouse CD319 isoform (Tovar *et al.*, 2002). This isoform (BAE96340) lacks the first 35 amino acids of the cytoplasmic tail (Table 4.2). The significance of this isoform still remains to be resolved.

The expression of mouse and human CD319 is predominantly restricted to hematopoietic tissues, with the exception of the testis, in which high levels of CD319 mRNA are detected (Tovar *et al.*, 2002). CD319 is expressed on all NK cells, a large subset of $CD8^+$ T cells, and a very small percentage of peripheral $CD4^+$ T cells (Table 4.1). CD319 is detectable on a small population of B cells and not on immature DCs, whereas it becomes strongly expressed on B cells and mature DCs upon activation through CD40 or influenza virus, respectively (Bouchon *et al.*, 2001; Lee *et al.*, 2004a; Tovar *et al.*, 2002). Similar to other members of the SLAM-family, CD319 is a self-ligand and its ligation induces NK

cytotoxicity (Bouchon *et al.*, 2001; Kumaresan *et al.*, 2002; Stark and Watzl, 2006; Tassi and Colonna, 2005) and B cell proliferation (Lee *et al.*, 2007a).

2.2.8. SLAMF8 (BLAME)

BLAME is a type I transmembrane protein with three potential N-glycosylation sites and was originally cloned from a human activated lymphocyte library (Kingsbury *et al.*, 2001). Its two-Ig-like domains, Ig-V and Ig-C2, are most closely related to CD48 (Kingsbury *et al.*, 2001). The sequence of the mouse ortholog (*NM_029084)* is 75% identical to human BLAME (*NM_020125)*. The 31-amino-acid cytoplasmic tail does not contain any distinctive signaling motifs, including the ITSM, characteristic of other members of the SLAM-family. Whereas BLAME is expressed on B cells, DCs, and activated monocytes (Table 4.1), its function remains unknown.

2.2.9. SLAMF9 (CD84-H1, SF2001) (NM_033438)

This gene maps in the proximity of BLAME (Fennelly *et al.*, 2001; Fraser *et al.*, 2002; Zhang *et al.*, 2001).The mature protein consists of an extracellular region of 289 amino acids, a 20-amino-acid transmembrane region, and a short cytoplasmic tail of 30 residues. The extracellular region contains only two potential N-glycosylation sites (Table 4.1), and exhibits greatest homology to CD229 domains 1 and 2, (45% similarity) as well as to CD84 (41% similarity) (Fig. 4.3) (Fennelly *et al.*, 2001). The ligand of this molecule is not known. Since SLAMF9 has a short cytoplasmic tail lacking tyrosine-based motifs or other known signaling motifs, no signal transduction mode has been postulated. SLAMF9 mRNA has been found in human monocytes, T cells, B cells, and DCs (Fennelly *et al.*, 2001; Zhang *et al.*, 2001).

3. THE SLAM-FAMILY GENE ISOFORMS AND POLYMORPHISMS AND THEIR ROLE IN A GENETIC PREDISPOSITION FOR SYSTEMIC LUPUS ERYTHEMATOSUS

3.1. SLAM locus haplotypes

Examination of SLAM-family mouse genomic sequences indicates that two major haplotypes exist in the region containing the seven SLAM-family genes: one is represented by C57BL/6 and the second by 129Sv, NZW (Perlegen Sciences, 2007; Wandstrat *et al.*, 2004). The coding regions of CD244 and CD229 differ the most between the two major haplotypes (C57BL/6 and 129Sv). Less variation is found in the ectodomains of CD48 and none in the mouse SLAM, CD319, CD84, and Ly108 ectodomains

(Fig. 4.6) (Veillette *et al.*, 2006). In addition, increasing numbers of cDNAs lacking one of the extracellular domains, part of the cytoplasmic tail, or the transmembrane segment of a SLAM-family member, have been reported (Ensembl, NCBI, Table 4.2). How many of these isoforms are expressed as proteins is not yet known. Nonetheless, based on analyses of tumor cell lines, the notion that several isoforms can be expressed by a single cell is highly likely (Palou *et al.*, 2000).

There are also alternative splice forms of most of the SLAM-family receptors, which affect the length of the cytoplasmic tail and consequently signal transduction events (Table 4.2). For instance, the short forms of human and mouse CD244 and SLAM (Fig. 4.6) bind only one SAP or EAT-2A/B adapter molecule, while the long form binds two or three of these adapters (Ma *et al.*, 2007). Similarly, the long, but not the short isoform of CD319 can bind SAP or EAT-2 (Lee *et al.*, 2004a; Tassi and Colonna, 2005). Thus, signal transduction events will depend upon which isoform(s) are expressed in a given cell, and the interplay between the isoforms (Rietdijk and Terhorst, unpublished observation). Nucleotide differences between the haplotypes are also found in the intronic regions of all the SLAM-family genes, which could account for differences of expression or alternative splicing forms. Indeed, differences in expression have been found for CD48, Ly108, CD84, and CD319 in splenic B cells and $CD4^+$ T cells between the two haplotypes (Perlegen Sciences, 2007; Wandstrat *et al.*, 2004).

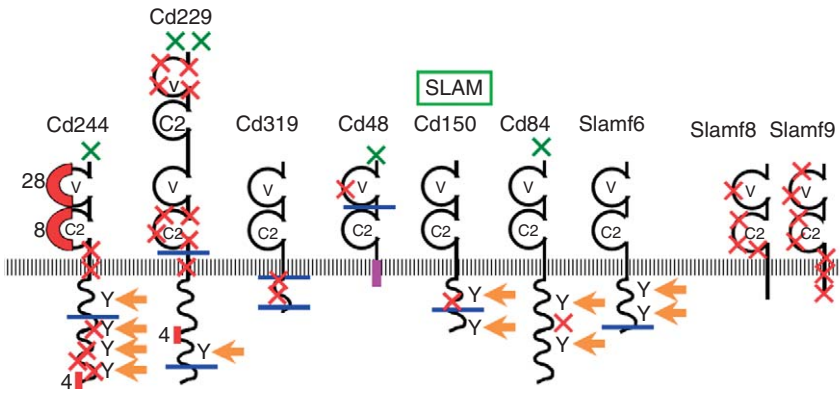

FIGURE 4.6 Variants of mouse SLAM receptors. Amino acid differences of mouse SLAM-family receptors between haplotype 1 and 2 are represented by green crosses (those found in the leader peptide sequence) or red crosses (those found in the rest of the protein). Areas containing multiple amino acid differences are displayed as red boxes. Arrows indicate ITSM motifs. Blue lines correspond to the C-terminal of short isoforms. The purple box represents the GPI-linked domain of CD48.

3.2. SLAM-family genes and SLE

Several studies have revealed that polymorphisms and alternative isoform usage contribute to the role of the human and mouse SLAM-family genes in susceptibility for systemic lupus erythematosis (SLE) as they are part of epistatic pathogenic networks with other genes (Chen and Morel, 2005).

SLE is a multisystem autoimmune disease, marked by a range of auto-antibodies with a long prodromal phase of auto-antibody development and epitope spreading. This pre-diagnosis phase (positive anti-nuclear antibodies and musculoskeletal discomfort) is often marked by elevated serum BLyS/BAFF/MIF levels, which implicates myeloid arm (macrophage, DCs) stimulation and B cell activation. The mechanism underlying the putative loss of tolerance (e.g., a primary B cell, T cell or antigen presenting cell defect, an ''over-stimulation'' effect) remains unknown. Even if initial events are more focused on the B cell arm, overt clinical disease involves a ''network'' of immunological cells (T cells, B cells, DCs, and macrophages) and the repertoire of mechanisms leading to an inflammatory response (Fairhurst *et al.*, 2006).

Genome-wide linkage scans of families with multiple members affected with SLE have consistently demonstrated the presence of a susceptibility locus on chromosome 1q23, which includes the SLAM-family genes (Gray-McGuire *et al.*, 2000; Johanneson *et al.*, 2002; Moser *et al.*, 1999; Shai *et al.*, 1999; Tsao *et al.*, 1997, 2002; Wakeland *et al.*, 2001). In addition, Vyse *et al.* (2005) established a first-generation haplotype map, using 186 single nucleotide polymosphisms (SNPs) across the SLAM region in a set of 98 complete trios (mother–father–child). Using a preliminary haplotype map, 63 SNPs were selected to type in 254 SLE trios and 160 discordant sibling pairs. There were numerous single marker tests that had nominal p values < 0.01 and mostly clustered around the *CD244*, *CD319*, and *CD84* genes. These preliminary results strongly suggest that there is variation within the human SLAM region that is associated with SLE. A more comprehensive examination of the genetic variation over the entire SLAM region in a larger set of families will be necessary to map the true association signal(s), that is, distinguishing true positives from false positives, and determining the independence of signals when there is more than one positive result.

In mice, the region syntenic to human 1q23 has been implicated in three different models of spontaneous lupus: the (NZB $\times$ NZW) F2 intercross, the NZM/Aeg2410 New Zealand mouse, and the BXSB mouse (Hogarth *et al.*, 1998; Kono *et al.*, 1994; Rozzo *et al.*, 1996). The disease phenotype of these mice is similar to that of SLE patients, in terms of production of auto-antibodies as well as severe nephritis and multiorgan involvement. A strong, spontaneous, anti-nuclear antibody

response, reacting primarily with H2A/H2B/DNA subnucleosomes occurred in congenic mice (*B6.Sle1)* derived from the mouse strains NZM2410 (NZB × NZW/F1) and C57BL/6. The presence of the NZW-derived chromosome 1 segment (*Sle1)* on a C57BL/6 background was sufficient to generate these auto-antibody responses and an expanded pool of histone-reactive T cells (Morel *et al.*, 2001; Wandstrat *et al.*, 2004). Consequently, *Sle1* is thought to be a major player in orchestrating the selective loss of B cell and T cell tolerance to chromatin, either directly or by presentation of chromatin in an immunogenic fashion. Fine mapping of the *Sle1* locus determined that three loci within this congenic interval, termed *Sle1a*, *Sle1b*, and *Sle1c* (Fig. 4.7), could independently cause a loss of tolerance to chromatin, a necessary step for full disease induction (Chen *et al.*, 2005b,c; Croker *et al.*, 2003; Morel *et al.*, 2001; Wandstrat *et al.*, 2004). The *Sle1b* locus is the most potent susceptibility locus capable of mediating a breach in tolerance to nuclear antigens and is associated with a high penetrance to anti-chromatin auto-antibodies and a significant gender disparity (Morel *et al.*, 2001; Wandstrat *et al.*, 2004).

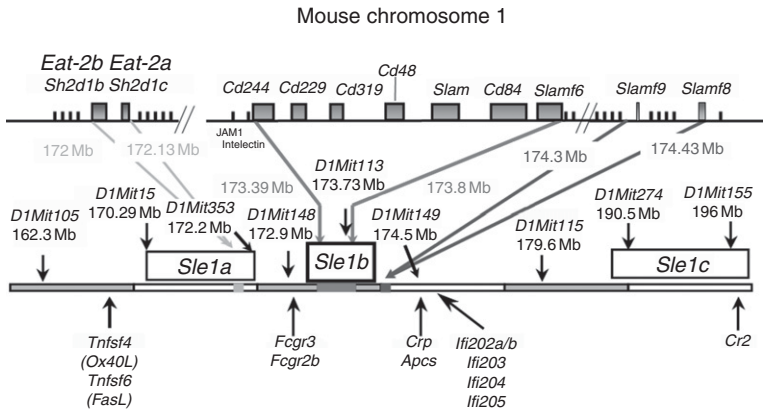

FIGURE 4.7 *Sle* loci in mouse chromosome 1. The upper portion shows the genomic map of mouse chromosome 1 and the position of the SLAM receptors and the EAT-2 loci. Filled boxes represent blocks of sequence containing a complete set of gene exons. The lower portion shows the three defined regions that compose the Sle1 locus (*Sle1a*, *Sle1b*, and *Sle1c*), as well as genomic markers and neighboring genes. *Sle1a* maps to an ~1-cM interval between the genomic markers D1Mit15 and D1Mit353, *Sle1b* is located in an interval of about the same size between D1Mit148 and D1Mit149, and *Sle1c* maps to the extreme telomeric end of chromosome 1, specifically to an ~2-cM interval between D1Mit274 and D1Mit155. The Eat-2 and SLAM receptor loci map to the *Sle1a* and *Sle1b* region, respectively. Genomic markers and their positions in chromosome 1 are noted in the map.

Sle1b has been defined as an ~0.9-Mb segment (0.4 cM) that includes the SLAM-family locus (Wandstrat *et al.*, 2004). Although this locus contains 24 expressed genes, 19 of which are expressed in the spleen, these findings strongly suggest that NZM2410-based variants, the SLAM-family receptor genes, on an otherwise C57BL/6 background contribute to the pathogenesis of SLE in the S*le1b* interval (Fig. 4.7). This notion is supported by studies with congenic strains derived from crosses between 129 and C57BL/6 mice, which develop spontaneous autoimmunity. By creating a C57BL/6 congenic strain carrying a 129-derived chromosome 1 segment, Bygrave *et al.* (2004) found that this 129 interval was sufficient to mediate the loss of tolerance to nuclear antigens (2004). To further dissect the contribution to autoimmunity of this 129 interval, the authors generated six subcongenic strains carrying fractions of the original 129 region and analyzed their serological and cellular phenotypes. At 1 year of age, a congenic strain carrying a 129 interval between the microsatellites D1Mit105 (80 cM) and D1Mit115 (99.7 cM) (*B6.129chr1b*) (Fig. 4.7), had high levels of auto-antibodies. Thus, the NZM2410-derived NZW segments *Sle1b* and the 129-derived *129chr1b* segment when recombined on the C57BL/6 genomic background are sufficient to induce loss of tolerance to nuclear antigens. The NZW and 129 segment each contains the SLAM genes of the same haplotype (Perlegen Sciences, 2007). Mohan, Wakeland, and colleagues postulate that among the SLAM-family members, *Slamf6* in the context of the C57BL/6 background may be the strongest candidate to be causally correlated with the B6.*Sle1b* phenotype. They find that the long isoform of murine Ly108, that is, Ly108–1 is up-regulated, while the short isoform (Ly108–2) is down-regulated in *B6.Sle1b* mice compared with C57BL/6 (Wandstrat *et al.*, 2004). Supporting this hypothesis, transfection of Ly108–2 was able to sensitize the immature B cell line WEHI-231 to undergo apoptosis, suggesting that Ly108 could regulate the stringency with which self-reactive B cells are censored during early development (Kumar *et al.*, 2006).

4. THE SLAM-ASSOCIATED PROTEIN

4.1. SAP is a single SH2-domain protein encoded by *SH2D1A*

SAP was cloned using a yeast two-hybrid system with a human T cell yeast expression library and, as bait, the 77-amino-acid cytoplasmic tail of human SLAM (Sayos *et al.*, 1998). SAP, a single SH2-domain protein with a 28-amino-acid tail could only be cloned in this system because of its unusually high binding affinity to the first nonphosphorylated ITSM (Y281) of the cytoplasmic tail of SLAM, described below. *SH2D1A*, which encodes SAP, was then identified as the gene that is mutated in

the rare primary immunodeficiency X-linked lympho-proliferative disease (XLP) (Coffey *et al.*, 1998; Nichols *et al.*, 1998; Sayos *et al.*, 1998). While the SAP SH2-domain is encoded by the first three exons of *SH2D1A*, exon 4 encodes part of the tail sequence and all of the 3′UTR (Coffey *et al.*, 1998; Wu *et al.*, 2001). The human (*NM_002351.2)* and mouse SAP (*NM_0011364.3)* amino acid sequences are 87% identical, with the highest similarity in the SH2 domain. Two transcripts (0.9 and 2.5 kb) encode human or mouse SAP (Coffey *et al.*, 1998; Sayos *et al.*, 1998; Wu *et al.*, 2001). The non-TATA box promoter of mouse and human *SH2D1A* contains binding sites for several transcription factors, chief among which are the Ets-1 and Ets-2 binding sites. Mutations in the *Ets* consensus sites of the human and mouse gene completely abolish transcriptional activity (Okamoto *et al.*, 2004). Human and mouse SAP mRNA have a short half-life in T cells (Okamoto *et al.*, 2004) and B cells (Al Alem *et al.*, 2005), most likely because RNA-binding proteins, such as AUF1 and HuR, which are known to be involved in mRNA degradation, bind to its 3′UTR (Okamoto *et al.*, 2004).

Thus far, SAP has been detected in all human and mouse T cells, NK cells, NKT cells, a subset of B cells, platelets, and in human eosinophils (Engel *et al.*, 2003; Latour and Veillette, 2004; Ma *et al.*, 2007; Morra *et al.*, 2001a; Sidorenko and Clark, 2003). Mouse SAP is expressed in SP ($CD4^+$ and $CD8^+$), DP ($CD4^+$ $CD8^+$), and double negative (DN) ($CD4^-$ $CD8^-$) thymocytes, with the highest expression occuring in DP cells, and the lowest in DN cells (Wu *et al.*, 2001). Detection of SAP mRNA is complicated, since SAP expression is down-regulated upon anti-CD3 stimulation of peripheral $CD4^+$ and $CD8^+$ T cells. SAP expression is greater in polarized Th1 cells than in Th2 cells, but upon stimulation, its expression decreases in both subsets (Wu *et al.*, 2001). By contrast, SAP expression in NK cells and NK cell lines increases after IL-2 activation (Endt *et al.*, 2007) or upon infection with MCMV or LCMV (Sayos *et al.*, 1998).

Expression of SAP in human or mouse B lineage cells is not supported by all experiments. The SAP protein was initially detected in a few B cell lymphoma lines, for example, EBV + Burkitt's lymphomas, which are phenotypically similar to germinal center or memory B cells (Kis *et al.*, 2003; Shlapatska *et al.*, 2001), but not in EBV-transformed lymphoblastoid cell lines (Sayos *et al.*, 1998). Immuno-fluorescence staining detected SAP in human and mouse germinal center B cells (Morra *et al.*, 2005; Nichols *et al.*, 1998; Shlapatska *et al.*, 2001) and SAP transcripts have been found by some in resting mouse $CD19^+$ B cells (Al Alem *et al.*, 2005). Others, however, have not detected SAP mRNA in mouse B cells (Ma *et al.*, 2006; Nagy *et al.*, 2000), which could be explained by the instability of the SAP mRNA, as suggested by its short (∼1 h) biological half-life (Okamoto *et al.*, 2004) and by down-regulation of the SAP transcripts in response to LPS (Al Alem *et al.*, 2005). As the half-life of the SAP protein is

~20 h (Morra *et al.*, 2001d), antibody detection might be more reliable, although background effects cannot be excluded (Morra *et al.*, 2005). Other factors, which complement these analyses, may include variations in the purity of B cell subsets, transient tightly controlled SAP expression in B cells, dependent upon specific stages of activation, maturation or differentiation, and strain background. Whether or not the functional defects of $SAP^{-/-}$ B cells are cell intrinsic will be discussed in Section 5.3.

4.2. SAP binds to the cytoplasmic tails of six SLAM-family receptors

Although SH2 domains are highly conserved noncatalytic modules, they exhibit considerable flexibility in the way they bind to their respective phosphotyrosine (pY) ligands. Specifically, SH2-domains associate with the pY motif in a ''two-pronged fashion'': a pY-binding pocket and a ''specificity determining'' region that interacts with an amino acid C-terminal of the pY (Leo *et al.*, 2002; Pawson, 2004).

Not only does the SH2-domain of SAP bind with high specificity to the pY-containing ITSMs of SLAM, CD229, CD244, CD84, NTB-A/Ly108, and human CD319 (Bottino *et al.*, 2001; Eissmann *et al.*, 2005; Sayos *et al.*, 1998, 2001; Tangye *et al.*, 1999; Tovar *et al.*, 2002), it also binds with a high affinity to the nonphosphorylated ITSM-based peptide SLAM Y281 (Finerty *et al.*, 2002; Hwang *et al.*, 2002; Poy *et al.*, 1999; Sayos *et al.*, 1998). This unusual ability to bind a nonphosphorylated partner protein is due to a ''three-pronged binding mode'' enabled by the interaction with the following residues of a SLAM-based peptide: (a) pY281, (b) V(+3), and (c) L(−3), T(−2), and I(−1) (Finerty *et al.*, 2002; Poy *et al.*, 1999).

The SH2 domain of SAP adopts a conventional SH2 domain fold characterized by a central β-sheet flanked by two α helices (αA and αB) and a small β-sheet. On one site of the central β-sheet, a number of residues, including R32, form the pocket that interacts with the pY, very similar to other SH2-domains interactions (Cohen *et al.*, 1995; Pawson, 2004). On the other side of the central β-sheet, a hydrophobic cleft, constituted by residues in the EF-loop and the loop between the αB-helix and the βG-strand, interacts with the C-terminal V (+3) (Finerty *et al.*, 2002; Hwang *et al.*, 2002; Poy *et al.*, 1999). The more complex third interaction of SAP involves first and foremost the side-chain of T(−2) in the ITSM, which forms hydrogen bonds with E17 of SAP and a buried water molecule. Furthermore, the phospho- and nonphospho-SLAM peptides make a parallel β-sheet interaction with the βD-strand of the SAP SH2 domain, and the side chains of residues at positions L(−3) and I(−1) intercalate with hydrophobic residues in the βD-strand.

The ''three-pronged binding mechanism'' increases substrate specificity and the affinity of the SAP SH2 domain for its phosphorylated ligand,

the highest affinities among SH2 domains (Finerty *et al.*, 2002; Hwang *et al.*, 2002; Poy *et al.*, 1999). Notably, the binding affinity of SAP with nonphosphopeptide is comparable to other SH2 domains with their ligands. Thus, in principle, SAP should have the capacity to out-compete SH2-domain proteins that bind to the same phosphotyrosine motif with lesser affinity. Although there is a modicum of support for the concept of SAP as an inhibitor of the recruitment of the protein tyrosine phosphatase (SHP-2) to the cytoplasmic tail of SLAM, CD244, CD84, and CD229 (Howie *et al.*, 2002a; Lewis, 2001; Sayos *et al.*, 1998), further experiments are required.

4.3. SAP is an adapter

SH2 domains are typically found in multidomain signaling enzymes or in adapter proteins, where they mediate protein–protein interactions and regulate associated catalytic subunits (Kuriyan and Cowburn, 1997; Machida and Mayer, 2005; Pawson, 2004). The notion that SAP could function as an adapter, coupling protein–tyrosine–kinases to the SLAM-family receptors is based on the finding that Fyn-mediated phosphorylation of the receptors increased markedly with SAP (Latour *et al.*, 2001; Sayos *et al.*, 1998, 2001). More direct experiments with cell lines transfected with SLAM demonstrated that SAP facilitates the selective recruitment and activation of the tyrosine kinase FynT (an isoform of the Src kinase Fyn expressed in T cells) (Latour *et al.*, 2001; Li *et al.*, 2003a). Later experiments demonstrated that SAP recruits Fyn to SLAM in normal mouse T cells (Chan *et al.*, 2003; Latour *et al.*, 2003). *In vitro* biochemical studies and structural analyses unveiled the remarkable mechanism of interaction between the SH2 domain of SAP and the SH3 domain of Fyn that directly couples Fyn to SLAM receptor (Chan *et al.*, 2003; Simarro *et al.*, 2004).The structure of the ternary SLAM–SAP–FynSH3 complex also reveals that SAP binding to Fyn domain does not involve canonical SH3 or SH2 interactions (Fig. 4.8A). Instead, at the point of interaction, the positively charged SAP surface interacts with negatively charged Fyn SH3 surface at a dissociation constant of 3.45 μM for the entire complex. On the SAP SH2-domain, R78 appears to be particularly critical for the interaction, as it forms dual salt-bridge hydrogen bonds with D100 in Fyn, and its guanidinium moiety stacks with the side-chain of W119 in the Fyn SH3 domain (Fig. 4.8B). Indeed, the R78mutation of SAP almost abolishes the interaction with Fyn (Chan *et al.*, 2003; Latour *et al.*, 2003).

This SAP surface, which interacts with Fyn, does not overlap with the SLAM Y281 phosphopeptide-binding groove of the SH2 domain of SAP. SAP mutants unable to bind to the SLAM cytoplasmic tail still bind to Fyn. Similarly, mutations of key residues of Fyn reduce *in vitro* binding to wild-type SAP. Consequently, the SH2 domain of SAP is able to bind

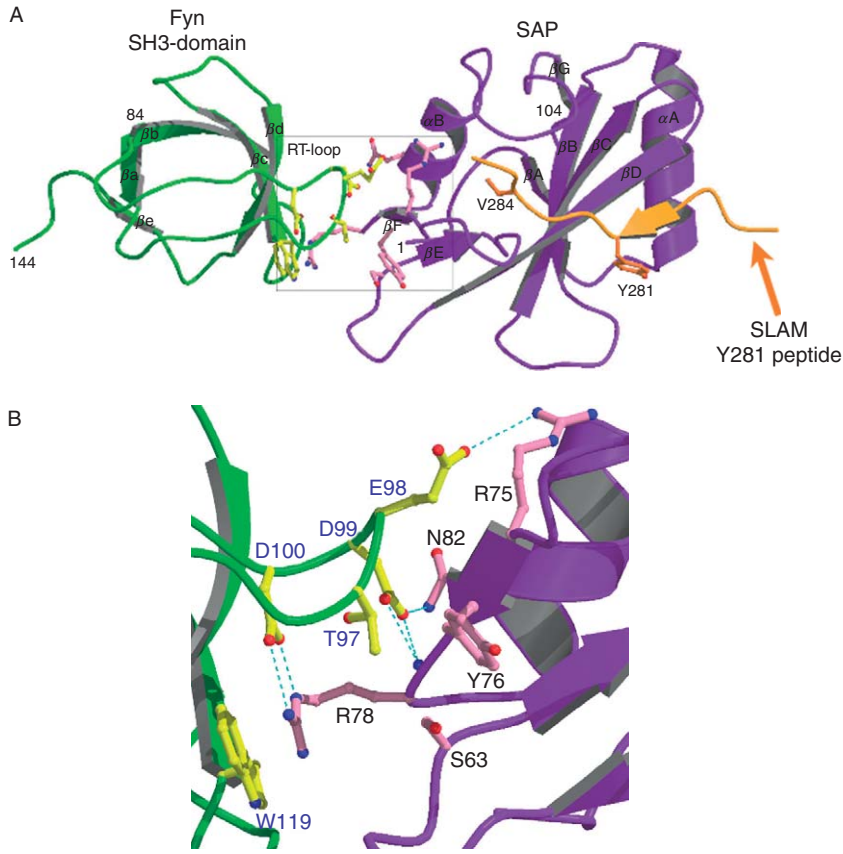

FIGURE 4.8 Structure SAP binding to Fyn. (A) Ribbon diagram showing the overall structure of the ternary SLAM–SAP–Fyn-SH3 complex. The SH2 domain of SAP (purple) binds the Fyn-SH3 domain (green) through a noncanonical surface interaction (boxed area) that does not overlap the SLAM-binding site (orange). (B) Close-up of key amino acids involved in SAP–Fyn interactions. SAP residues are displayed in pink and labeled in black; Fyn-SH3 residues are shown in yellow and labeled in blue. Dashed blue lines indicate critical hydrogen bonds and ionic interactions.

simultaneously to SLAM and Fyn (Chan *et al.*, 2003; Latour *et al.*, 2003). Importantly, superposition of the SAP–FynSH3 complex on the SH3 domain of auto-inhibited Src shows that SAP binding is mutually exclusive with the auto-inhibitory interaction. *In vitro* experiments indicate that SAP can activate the inactive from of Fyn (Chen *et al.*, 2006; Simarro *et al.*, 2004). Thus, the interaction of SAP SH2 with its ligands may control two key events: the tyrosine site location of signaling molecules within the cell and the switch to the inactive or active configuration of Src kinases. These two events allow the activation of src-like kinases at the right time and in

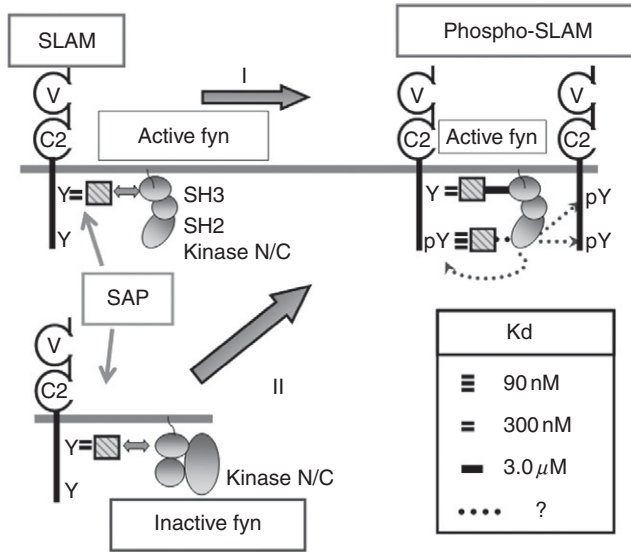

FIGURE 4.9 Fyn recruitment and SLAM activation by SAP. During formation of the immunological synapse between T cells and APCs, clustering of SLAM brings SAP to its cytoplasmic tail. SAP binds to unphosphorylated Y281 (Kd = 300 nM) and consequently mediates Fyn recruitment to SLAM. Interaction of SAP with the Fyn-SH3 domain (Kd = 3.5 μM) stabilizes the open, active conformation of Fyn and directly couples Fyn to SLAM, thereby promoting phosphorylation of Y307 and Y327 in SLAM. This creates docking sites for SH2-domain-containing proteins, including SAP (Kd = 90 nM). SAP can bind to previously active (I) or inactive (II) Fyn molecules. In the inactive form of Fyn, the SAP-binding domain is blocked by intramolecular interactions with the kinase domain. Thus, recruitment by SAP will induce disassembly and activation of the auto-inhibited kinase. Alternatively, SAP can also bind to the kinase domain of Fyn (Kd = undetermined), the implications of which are unknown.

the correct location (Fig. 4.9, option I and II). As indicated in Fig. 4.9, it is conceivable that SAP bound to SLAM could bind to a Fyn molecule, that has been activated by other mechanisms (Fig. 4.9, option I). The association of the negatively charged surface of Fyn with the positively charged surface of SAP is unique because most of the amino acids involved in the SH3 binding surface of Fyn are not conserved in other Src kinases. Nor does the SAP-related molecule EAT-2 contain the positively charged residues that are requisite for the interaction of SAP and Fyn.

Although no triple complex has been detected between SAP–Fyn and CD229, SAP facilitates Fyn-mediated phosphorylation of CD229 (Simarro *et al.*, 2004). Similarly, CD244 phosphorylation is in part dependent on SAP interaction with Fyn, since transfection of SAP–R78A in a T cell line failed to induce CD244 phosphorylation (Chen *et al.*, 2004). Interestingly, mouse and human CD244 are also able to directly associate with the SH2

domain of Fyn, although the precise timing and location of this interaction remains unknown (Clarkson *et al.*, 2007).

SAP may also directly bind to Lck, as suggested by the results from *in vitro* results and yeast two-hybrid analyses (Simarro *et al.*, 2004). Although the putative binding of SAP to the kinase domains of Fyn and Lck remains ill-defined, Lck phosphorylates SLAM, CD84, CD244, and CD229 (Howie *et al.*, 2002b; Martin *et al.*, 2005; Nakajima *et al.*, 1999; Sayos *et al.*, 2000; Tangye *et al.*, 2003).Whether SAP also recruits Lck to the tails of the SLAM-family receptors awaits further clarification. However, the finding that SLAM phosphorylation is reduced, but not eliminated in $Fyn^{-/-}$ T cells suggests that other Src kinases may be involved in SLAM phosphorylation (Simarro *et al.*, 2004).

Recently, an association of SAP with the SH3 domain of the PAK-interacting protein (β-PIX), a guanine nucleotide exchange factor specific for Rac/Cdc42 GTPase, has been demonstrated. Similar to the SAP–Fyn interaction, the binding of SAP to β-PIX is mediated by the C-terminal region of the SAP SH2 domain and the β-PIX SH3 domain (Gu *et al.*, 2006). The mutant SAPR78A, but not SAPR32Q, fails to bind β-PIX, suggesting that β-PIX and Fyn might compete with each other for SAP binding (Gu *et al.*, 2006). Interestingly, SAP recruits β-PIX to CD244, suggesting that β-PIX may mediate some biological functions of the SLAM-family receptors. As judged by *in vitro* experiments using SAP and peptide libraries, it is likely that SAP binds to other signal transduction molecules, for example Lck and LAT (Poy *et al.*, 1999).

5. THE PROTECTIVE ROLE OF SAP IN X-LINKED LYMPHO-PROLIFERATIVE DISEASE

5.1. The primary immunodeficiency XLP

XLP is an extremely rare primary immunodeficiency, which only affects males and was at first indicated by a defective immune response to EBV (Purtilo *et al.*, 1975).

EBV, a human γ-herpes virus, is one of the most successful viruses, which ubiquitously infects humans and persists throughout a person's lifetime (Bornkamm, 2001; Thorley-Lawson *et al.*, 1982). The virus enters the oropharynx and adjacent structures, and preferentially infects B cells via the C3d complement receptor CD21 (Hislop *et al.*, 2007), which proliferate and undergo transformation. Initial responses against the virus are made by NK cells and subsequently by Ag-specific $CD8^+$ T cells, which control proliferation of EBV-transformed B cells. $CD4^+$ T cells provide the necessary help to B cells to become plasma cells and secrete immunoglobulins. Primary EBV infection is usually asymptomatic in children, while at times induces acute

infectious mononucleosis (IM) in susceptible adolescents or adults. During the subsequent lifelong infection, virus carriers do not manifest symptoms as long as they are immunocompetent (Thorley-Lawson *et al.*, 1982).

By contrast, 60% of XLP patients infected with EBV develop fatal or fulminant infectious mononucleosis (FIM) and the majority of them die 1–2 months after contracting the virus (Gilmour and Gaspar, 2003). Patients surviving EBV infection later develop B cell lymphomas (20%) or dysgammaglobulinemia (30%) (Gilmour and Gaspar, 2003; Schuster and Terhorst, 2006). XLP patients often develop more than one phenotype, particularly after exposure to EBV. Consequently, most patients with XLP die by the age of 40 and more than 70% die before they reach 10 years (Moretta *et al.*, 2001).

FIM is caused by a dysregulated immune response, resulting in a large polyclonal proliferation of T cells, B cells, macrophages, and monocytes. This eventually leads to lymphoid infiltration of several organs such as the liver, kidney, thymus, and bone marrow, causing organ failure (Morra *et al.*, 2001a; Nichols *et al.*, 2005b). The extensive destruction of liver and bone marrow often results in fulminant hepatitis and virus-associated hemophagocytic syndrome (VAHS).

XLP patients are usually asymptomatic prior to virus exposure, but after recurrent viral infections they progressively develop dysgammaglobulinemia (Morra *et al.*, 2001a; Schuster and Terhorst, 2006; Sumegi *et al.*, 2000). Patients exhibit low serum levels of IgG and IgG3 and increased levels of IgA and IgM. Median age at hypogammablobulinemia onset is 6–9 years (Grierson and Purtilo, 1987; Seemayer *et al.*, 1995; Sumegi *et al.*, 2000). Some of these males were previously thought to have common variable immunodeficiency (CVID), and eventually have been shown to have mutations in the XLP gene (Eastwood *et al.*, 2004; Gilmour *et al.*, 2000; Morra *et al.*, 2001c).

Malignant and nonmalignant lympho-proliferative disorders occur in XLP patients at rates ~200 times greater than that of the general population (Seemayer *et al.*, 1995). Almost all the lymphomas found on XLP patients are of B cell origin, although tumors with T cell phenotypes have also been described. Those tumors were further classified as Burkitt's lymphomas (53%), immunoblastic lymphomas (18%), large noncleaved lymphomas (12%), small cleaved or mixed cell lymphomas (12%), and unclassifiable lymphomas (5%) (Egeler and Jan De Kraker, 1992; Harrington *et al.*, 1987; Schuster and Terhorst, 2006). Malignant B cell lymphomas and dysgammaglobulinemia are observed in XLP patients with or without serological or molecular evidence of previous EBV infection (Grierson and Purtilo, 1987; Sumegi *et al.*, 2000). However, the prognosis of lymphoma patients who are EBV^+ is much poorer than those that are EBV^-. Thus, although EBV acts as a potent trigger for several clinical manifestations of XLP, other antigenic stimuli are also involved in the pathogenesis of XLP.

5.2. Mutations in the SAP gene of XLP patients

A concerted effort was undertaken in the late 1980s to map the defective gene in XLP patients by restriction fragment length polymorphism (RFLP) analysis. Using linkage studies, Skare *et al.* (1989) first mapped the gene responsible for XLP to the long arm of the X chromosome between Xq24 and Xq25. Subsequently, the critical region was narrowed down considerably (Wang *et al.*, 1993), but attempts to clone the gene failed. It was not until 1998 that three groups independently reported the identification of the gene that is abnormal in XLP, by using (a) a positional cloning approach (Coffey *et al.*, 1998; Nichols *et al.*, 1998) and designating it SH2 domain protein 1A (SH2D1A) or (b) the human SLAM cytoplasmic domain as bait in the yeast two-hybrid system (Sayos *et al.*, 1998), designating it SAP. Because the SAP gene localized to the X chromosome, Sayos *et al.*, tested the possibility that SAP was encoded by the XLP gene using cDNAs from three XLP patients. All three cDNAs were mutated or deleted, in contrast to those from healthy brothers and a number of other control samples (Sayos *et al.*, 1998).

Approximately 100 kindreds (~300 XLP patients) have been found thus far (Hare *et al.*, 2006; Morra *et al.*, 2001d; Seemayer *et al.*, 1995; Sumegi *et al.*, 2000, 2002), with macro- or microdeletions, splice-site mutations, nonsense mutations, or missense mutations of the *SH2D1A* gene (Latour and Veillette, 2003; Morra *et al.*, 2001d; Nichols *et al.*, 2005b; Poy *et al.*, 1999) (Fig. 4.10).

Consequently, signaling through SLAM receptors can be altered either through reducing the half-life of SAP, by impairing the binding to the receptors or by disabling the ability to activate signal transduction downstream of the SLAM–SAP complex (Hare *et al.*, 2006; Hwang *et al.*, 2002; Li *et al.*, 2003a, 2003b; Morra *et al.*, 2001d; Poy *et al.*, 1999).

Missense mutations that disrupt the binding of SAP to SLAM-family receptors interfere (a) with the N-terminal interaction (e.g., T53I), (b) binding in the pY pocket (e.g., R32T, C42W), or (c) interactions with the Y + 3 amino acid in the hydrophobic cleft (E67D, T68) (Morra *et al.*, 2001d; Sumegi *et al.*, 2002). Interestingly, the mutant T53I binds to the phosphorylated SLAM, but fails to bind to CD244 and CD229 (Morra *et al.*, 2001d). A number of the missense mutations that decrease the half-life of SAP are conserved amino acids in SAP isolated from humans, mice, and other species: for example, Y7C, S28R, Q99P, P101L, V102G, Y54C, F87S, and I84T (Hare *et al.*, 2006; Morra *et al.*, 2001d). However, mutations Y54C and F87S also affect SAP binding to some, but not all the SLAM-family receptors, suggesting that the requirement of SAP binding might differ between receptors. No genotype or phenotype correlation has been observed in XLP patients, suggesting an influence of other genetic and environmental factors (Arkwright *et al.*, 1998).

Whereas most XLP patients have mutations in the *SH2D1A* (SAP) gene, more than 20% show normal SAP expression, but have a mutation

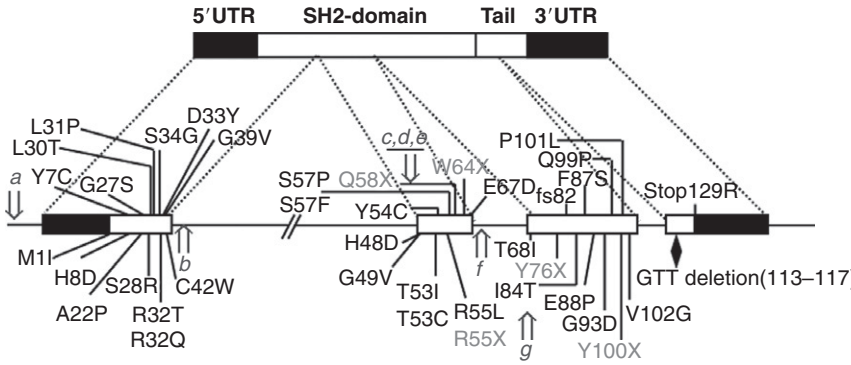

FIGURE 4.10 Mutations in human SH2D1A. Reported mutations of human SH2D1A are shown together with the genomic organization or functional domains. The coding regions and 5′UTR/3′UTR of SH2D1A are shown in white or black bars, respectively. Amino acid positions of missense mutations are represented in black and nonsense mutations in red. An insertion of an adenosine residue at nucleotide position 544 in exon 3 results in a frameshift at amino acid 82 (fs82). This frameshift results in a different amino acid sequence comprising 20 residues, followed by a premature stop codon being introduced at amino acid 103. A point mutation in the termination codon adds (R129Stop) 12-amino-acid residues to the protein (RRKIKHLVLYFL). A natural variant of GTT deletion in the tail of SH2D1A is indicated with a rhombus. The CAAT box and splicing mutations are indicated by an arrow: *a*, ccaat to ctaat; *b*, t(+2)c; *c*, g(−1)t/a; *d*, a (−2)c; *e*, c(−3)g that results in exon 2 skipping; *f*, g(+1)a; g, g(−1) a and results in skipping of exon 3. + indicates a donor splicing site and − a receptor splicing site.

in XIAP (X-linked inhibitor of apoptosis) (Latour, 2007; Rigaud *et al.*, 2006). Hence the SH2D1A-deficiency will now be termed XLP-1 and the XIAP-deficiency XLP-2 (Marodi and Notarangelo, 2007).

5.3. Lessons from XLP and the SAP deficient mouse

XLP is a complex disease syndrome in which $CD4^+$ T cells, $CD8^+$ T cells, NK cells, NKT cells, and B cells are affected. Even though EBV does not infect mouse cells, the study of $SAP^{-/-}$ mice has aided in dissecting cell-specific defects and in elucidating the molecular mechanisms involved in the pathogenesis of XLP.

5.3.1. Altered $CD8^+$ T cell responses

Increased numbers of antigen specific $CD8^+$ T cells and IFN-γ secreting $CD8^+$ T cells and elevated production of IFN-γ per cell occurred upon infection with lymphocytic choriomeningitis virus (LCMV) (Crotty *et al.*, 2006; Czar *et al.*, 2001). Although $SAP^{-/-}$ mice were able to survive acute infection, they failed to resolve chronic infection and died, most likely because of lack of antibody responses (Crotty *et al.*, 2003, 2006; Czar *et al.*, 2001;

Wu *et al.*, 2001) As judged by *in vivo* and *ex vivo* assays, during the first two weeks after infection with the murine gammaherpesvirus-68 (MHV-68), $SAP^{-/-}$ mice have an increased level of virus-specific cytotoxic T lymphocytes (CTL) and resolve the acute infection as effectively as wild-type (*wt)* mice. $SAP^{-/-}$ CTL also control chronic infection with MHV-68 more efficiently than *wt* CTL (Chen *et al.*, 2005a; Yin *et al.*, 2003). $CD8^+$ T cell proliferation defects in $SAP^{-/-}$ mice appear to arise from an intrinsic defect of TCR-mediated activation, which is due to a defect in activation-induced cell death (AICD), resulting in an inhibition of apoptosis (Chen *et al.*, 2007). The notion that $SAP^{-/-}$ $CD8^+$ T cells are more efficient in fighting infections is supported by the increased proliferation and IFN-γ production of $CD8^+$ T cells in response to infection with the parasite *T.gondii* (Czar *et al.*, 2001).

In addition, after infection, mouse $SAP^{-/-}$ $CD8^+$ T cells display greater cytotoxicity than *wt* cells (Chen *et al.*, 2005a; Crotty *et al.*, 2006; Czar *et al.*, 2001; Wu *et al.*, 2001). These results are in contrast to analyses of $CD8^+$ T cell from XLP patients, which exhibit defective lysis of EBV-autologous B cells and a decrease in numbers of IFN-γ producing cells (Harada *et al.*, 1982a,b; Ma *et al.*, 2005; Sharifi *et al.*, 2004). Defective clearance of EBV-transformed B cells could result in the continuous activation of T cells (Ma *et al.*, 2007), thereby leading to the excessive $CD8^+$ T cell proliferation and infiltration seen in XLP patients (Sullivan *et al.*, 1983). The reason for this contradiction might stem from the uniqueness of human $CD8^+$ T cell responses to EBV.

5.3.2. Defective $CD4^+$ T cell responses

One of the most characteristic features of $SAP^{-/-}$ $CD4^+$ T cells is that they display an impaired ability to differentiate into Th2 cells. After *in vitro* activation, they exhibit a slight increase in Th1-like cytokines, like IFN-γ, and diminished production of Th2-cytokines, such as IL-4, IL-10, and IL-13 (Czar *et al.*, 2001; Wu *et al.*, 2001). This Th2 defect is also found *in vivo*, as SAP deficient animals are more resistant to *Leishmania major* infection, a parasite that relies on normal levels of Th2 cytokines to cause disease (Wu *et al.*, 2001). Furthermore, $SAP^{-/-}$ mice display a defective IgE production (Czar *et al.*, 2001; Wu *et al.*, 2001), which in part depends on normal IL-4 secretion. It is important to note that although IL-10 secretion was severely impaired in $CD4^+$ T cells from XLP patients, no differences were observed in IL-4 or IFN-γ secretion (Ma *et al.*, 2005). $SAP^{-/-}$ $CD4^+$ T cells display an impaired up-regulation of the inducible co-stimulator (ICOS) after antigen stimulation, in agreement with data reported for XLP T cells (Cannons *et al.*, 2006; Ma *et al.*, 2005). Furthermore, SAP deficient mice exhibit elevated and prolonged CD40L expression after stimulation (Cannons *et al.*, 2006).

Biochemical experiments using $SAP^{-/-}$ T cells have shed some light on SAP-regulated signaling pathways in $CD4^+$ T cells, namely recruitment of PKCθ to the immune synapse after TCR stimulation and NF-κB

activation (Cannons *et al.*, 2004). Consistent with their reduced IL-4 production, SAP deficient $CD4^+$ T cells also displayed a reduction in TCR-mediated transcription of GATA-3, a transcription factor involved in IL-4 production (Cannons *et al.*, 2004). Experiments with a knock-in mouse, which expresses SAP–R78A (a SAP mutant that fails to bind to Fyn: see Section 4), indicate that SAP-dependent IL-4 production involves Fyn (Davidson *et al.*, 2004). GATA-3 transcription was also impaired in both $SAP^{-/-}$ and SAPR78A $CD4^+$ T cells after TCR stimulation. Restoration of GATA-3 expression reversed this IL-4 defect, thus establishing that the impairment of IL-4 production in $SAP^{-/-}$ and SAPR78A T cells stems from reduced GATA-3 levels (Cannons *et al.*, 2004; Davidson *et al.*, 2004).

SAP also controls the activity of the IL-2 promoter element NFAT (Gu *et al.*, 2006). Overexpression of SAP in Jurkat cells led to an increase in NFAT activity following activation with ionomycin. Interestingly, this activity was inhibited by β-PIX but not by Fyn expression, suggesting that SAP might regulate IL-2 production through its interaction with β-PIX (Gu *et al.*, 2006). This result contrasts with other experiments, in which a marked increase in IL-2 production after TCR stimulation of SAP deficient T cells was observed (Czar *et al.*, 2001).

5.3.3. B cell defects and impaired humoral responses

Defects in humoral responses and lack of germinal centers are observed in XLP patients and in virally infected or immunized $SAP^{-/-}$ mice (Cannons *et al.*, 2006; Crotty *et al.*, 2003; Hron *et al.*, 2004; Kamperschroer *et al.*, 2006; Ma *et al.*, 2006; Morra *et al.*, 2005). This defective germinal reaction could account for the significant decrease in circulating memory B cells and long-lived plasma cells, resulting in a reduced titer of serum IgGs (Crotty *et al.*, 2003; Czar *et al.*, 2001; Ma *et al.*, 2005; Malbran *et al.*, 2004; Morra *et al.*, 2005).

Considerable evidence supports the hypothesis that the defect in germinal center formation in $SAP^{-/-}$ mice stems from a defect in $CD4^+$ T helper cells, in particular follicular B helper T cells (T_{FH}). First, $CD4^+$ T cells from XLP patients failed to provide B cell help, as revealed by a marked reduction in IgM or IgG or IgA secretion of normal B cells when co-cultured with XLP $CD4^+$ T cells (Ma *et al.*, 2005). Second, humoral immune responses to T-dependent antigens were defective in $SAP^{-/-}$ C57BL/6 and Balb/c mice, and were restored after reconstitution with *wt* $CD4^+$ T cells (Cannons *et al.*, 2006; Crotty *et al.*, 2003; Morra *et al.*, 2005). Third, T-independent antibody responses in $SAP^{-/-}$ C57BL/6 and Balb/c mice were identical or slightly elevated compared with *wt* littermates (Cannons *et al.*, 2006; Hron *et al.*, 2004; Morra *et al.*, 2005; Yin *et al.*, 2003). Fourth, $SAP^{+/+}$ but not $SAP^{-/-}$ $CD4^+$ T cells transgenic for an LCMV epitope restored germinal center B cell differentiation in $CD4^{-/-}$ mice after LCMV infection (McCausland *et al.*, 2007).

As defective Th2 cytokine production (5.3.2) cannot be the sole reason for the defective germinal center formation in $SAP^{-/-}$ mice, other mechanisms must play a role (Cannons *et al.*, 2006). Decreased expression of ICOS on $SAP^{-/-}$ $CD4^+$ T cells is likely to be a contributing factor (Cannons *et al.*, 2006), as this receptor assists in the development of germinal centers and memory B cells in both mice and humans (Greenwald *et al.*, 2005; Grimbacher *et al.*, 2003; Ma *et al.*, 2007; McAdam *et al.*, 2001; Vinuesa *et al.*, 2005). Experiments using retroviral re-introduction of mutant SAP (R78A) into SAP deficient mice showed that Fyn is dispensable for SAP-mediated T cell help to B cells and germinal center formation (Cannons *et al.*, 2006; McCausland *et al.*, 2007). This is supported by the observation that $Fyn^{-/-}$ mice do not exhibit defects in germinal center formation (Cannons *et al.*, 2006; McCausland *et al.*, 2007). A defect in IL-10 secretion, which was thought to contribute to defective B cell help in cultures of $CD4^+$ T cells from XLP patients with normal human B cells (Ma *et al.*, 2005), was not confirmed in mice (Cannons *et al.*, 2006; Crotty *et al.*, 2003; Morra *et al.*, 2005).

Although no defects were observed when B cells from XLP patients were stimulated *in vitro* with antigens or with anti-CD40 and IL-4 (Ma *et al.*, 2005; Malbran *et al.*, 2004), B cells from SAP deficient mice stimulated *in vitro* exhibited an impaired IgG class switch recombination, resulting in a reduced IgG and IgA production (Al Alem *et al.*, 2005).

In support of this finding, adoptive transfers of Balb/c $SAP^{-/-}$ B cells and *wt* $CD4^+$ T cells into RAG deficient Balb/c mice indicate that SAP expression in B cells may also be required for intact antibody responses (Morra *et al.*, 2005). However, other investigators using $SAP^{-/-}$ C57BL/6 mice did not obtain these same results (Cannons *et al.*, 2006; Crotty *et al.*, 2003). The reason for such divergent outcomes might lie in background differences of the $SAP^{-/-}$ strains used in the adoptive transfer experiments or in differences in cell purification techniques. The presence of the SAP protein in human and mouse germinal center B cells supports the role of SAP in B cells (Morra *et al.*, 2005; Nichols *et al.*, 1998). Further experiments, for example, the use of conditional SAP deficient mice, are required to ascertain the mechanisms by which SAP controls humoral immunity.

6. THE SAP-RELATED SINGLE SH2-DOMAIN PROTEINS EAT-2A AND EAT-2B

6.1. The EAT-2A and EAT-2B genes

The SAP-like molecule EAT-2 was cloned from NIH3T3 cells transformed with Ewing sarcoma DNA (Thompson *et al.*, 1996). The human and mouse EAT-2 genes encode proteins comprised of a single SH2 domain and a short carboxy-terminal tail (Morra *et al.*, 2001b). Surprisingly, two almost identical mouse EAT-2 genes exist, the original EAT-2A (*Sh2d1b*) and its

duplicate EAT-2B or ERT (*Sh2d1c*); the intergenic distance being ~26 kb (Calpe *et al.*, 2006; Roncagalli *et al.*, 2005; Thompson *et al.*, 1996). The coding regions of mouse *Sh2d1b* and *Sh2d1c*, which span ~6.5 and 20 kb, respectively, are almost identical, as are the flanking intronic sequences (Calpe *et al.*, 2006). Human *Sh2d1b* and mouse *Sh2d1b* and *Sh2d1c*, which are highly conserved, map in proximity (1.3 Mbp) to the SLAM locus on the long arm of chromosome 1 (Fig. 4.2).

The expression pattern of EAT-2 differs from that of SAP. In humans, EAT-2 protein is found in NK cell lines, freshly isolated or in IL-2 cultured NK cells, activated $CD4^+$ and $CD8^+$ T cells, and TCR$\gamma\delta$ T cells (Bouchon *et al.*, 2001; Tangye *et al.*, 2003; Tassi and Colonna, 2005). Mouse EAT-2A and EAT-2B are predominantly expressed in NK cells, although transcripts are also found on B cells, platelets, mast cells, and macrophages (Calpe *et al.*, 2006; Morra *et al.*, 2001b; Nanda *et al.*, 2005; Roncagalli *et al.*, 2005).

6.2. Are EAT-2A and EAT-2B inhibitory molecules or adapters?

Since their SH2 domains are structurally very similar, it came as no surprise that, like SAP, EAT-2A binds with high affinity to the cytoplasmic tails of SLAM, CD244, CD84, human CD319, CD229, and Ly108/NTB-A (Fraser *et al.*, 2002; Morra *et al.*, 2001b; Roncagalli *et al.*, 2005). Studies of a co-crystal of EAT-2A and the phospho-Y281-SLAM-peptide have revealed that EAT-2A interacts with the first ITSM of human SLAM in a ''three-pronged'' fashion. The EAT-2A amino acids, which are requisite for binding to the SLAM receptors, are conserved in humans and mice (Morra *et al.*, 2001b). In over-expression systems, EAT-2A was also shown to block recruitment of SHP-2 to the tail of SLAM, CD84, CD229, and CD244 (Morra *et al.*, 2001b). Although EAT-2B is less well-studied, it has been shown to bind to SLAM and CD244 in 293T cells and NK cells, respectively (Calpe *et al.*, 2006; Roncagalli *et al.*, 2005). Although phosphorylation of SLAM-family members is enhanced by the presence of EAT-2A or EAT-2B, EAT-2 cannot bind directly to the SH3-domain of Fyn and its adapter role remains less well-defined (Calpe *et al.*, 2006; Morra *et al.*, 2001b). Further biochemical analyses have demonstrated that phosphorylation of the tyrosines in the tail of both human EAT-2 and mouse EAT-2A enables them to bind to the SH2 domain of Fyn in NK cells (Clarkson *et al.*, 2007).

7. THE SLAM-FAMILY RECEPTORS IN NK CELL RESPONSES

Upon viral infection, NK cells are the first line effectors, removing virus-infected cells via cytokine production and cytolytic granule release (Lee *et al.*, 2007b). After EBV infection, IFN-γ release by NK cells can inhibit EBV-mediated B cell transformation and might be involved in the control

of infected cells (Hislop *et al.*, 2007). However, the degree to which impairment of NK cell functions contributes to the uncontrolled proliferation of EBV infected B cells in XLP patients is difficult to assess (Bottino *et al.*, 2001; Harada *et al.*, 1982b; Parolini *et al.*, 2000). This is especially problematic because of the interplay between SAP and EAT-2 in human NK cells and due to the fact that EAT-2 has been described as both an inhibitor (Roncagalli *et al.*, 2005) and activator of NK cell functions (Eissmann and Watzl, 2006; Tassi and Colonna, 2005). Human and mouse NK cells express CD244, CD319, and CD84 while the NTB-A equivalent (Ly108, Slamf6) is not expressed in mouse NK cells (Veillette, 2006b). As the role played by SLAM-family receptors in NK cell functions has been described in great detail in a number of recent reviews (Assarsson *et al.*, 2005a,b; Claus *et al.*, 2008; McNerney *et al.*, 2005b; Vaidya and Mathew, 2006; Veillette, 2006b), we will here only highlight the major conclusions reached thus far.

7.1. CD244 inhibits mouse NK cell functions

CD244 was originally identified in mice as an activator of NK cell function that triggered non-MHC-restricted killing. Cross-linking with monoclonal antibodies directed against CD244 was shown to trigger cytotoxicity, granule exocytosis and IFN-γ and IL-2 production (Garni-Wagner *et al.*, 1993; Mathew, 1993). Surprisingly, analysis of CD244 deficient mice showed that CD244 acts as an inhibitor of NK cell function. NK cells from these mice displayed enhanced cytotoxicity against $CD48^+$ target *in vitro* and *in vivo* (Lee *et al.*, 2004b; Vaidya *et al.*, 2005). In support of these results, *in vivo* and *in vitro* killing of $CD48^+$ tumor or target cells is decreased in wt animals compared with $CD48^-$ target cells (Lee *et al.*, 2004b; Mooney *et al.*, 2004; Vaidya *et al.*, 2005).

The studies concerning the role of CD244 in mouse NK cells are complicated by the cooperativity between NK cells with other NK cells, T cells, or B cells. For instance, using *in vitro* and *in vivo* analyses, CD244 deficient NK cells kill $CD48^-$ target cells less efficiently than wt NK cells, possibly due to the absence of ligation of CD244 and CD48 between NK cells (Assarsson *et al.*, 2004; Lee *et al.*, 2004b, 2006). Furthermore, addition of IL-2 activated NK cells to $CD4^+$ and $CD8^+$ T cells induces a dramatic increase in IL-2- and TCR-mediated T cell proliferation, which is inhibited by anti-CD244 or anti-CD48 antibodies (Assarsson *et al.*, 2004). The latter interaction takes place between CD244 expressed on NK cells and CD48 on T cells, as NK cells could not enhance the proliferation of $CD48^{-/-}$ T cells (Assarsson *et al.*, 2004). Similarly, CD244–CD48 interactions between B and NK cells are important for IL-13 production by NK cells (Gao *et al.*, 2006). Thus, CD244–CD48 interactions govern the maintenance and amplification of innate and adaptive immune responses (McNerney

et al., 2005a). Interestingly, recent data suggest that CD244 might function by inhibiting NK–NK cell fratricide, resulting in optimal NK cell function (Taniguchi *et al.*, 2007). These results might explain some of the apparent dual functionality of CD244 and confirm its role as an important inhibitory receptor involved in MHC-I independent NK cell self-tolerance (Kumar and McNerney, 2005). Whether this also plays an important role in NK cell tolerance to lymphocytes is still not known.

It would appear that the murine CD244 initiated inhibitory signal transduction is independent of SAP (Lee *et al.*, 2004b; Mooney *et al.*, 2004; Veillette, 2006b), but is instead EAT-2A and EAT-2B dependent. Although CD244, CD319, and CD84 are expressed on mouse NK cells, EAT-2A and EAT-2B uniquely bind to CD244 (Roncagalli *et al.*, 2005). Indeed, EAT-2A$^{-/-}$ or EAT-2B$^{-/-}$ NK cells exhibit enhanced cytotoxicity and IFN-γ production in response to the engagement of many activating receptors, in particular of CD244 (Roncagalli *et al.*, 2005). Furthermore, NK-cell-mediated cytotoxicity and IFN-γ secretion were reduced in transgenic mice overexpressing EAT-2A or EAT-2B (Roncagalli *et al.*, 2005). The EAT-2-mediated inhibitory function of NK cells is mediated by tyrosine phosphorylation, as mutation of the C-terminal tyrosines eliminates the inhibitory effect of EAT-2. Which signaling molecules are involved in the EAT-2-mediated inhibition remains undetermined. Although EAT-2 can bind Fyn, it seems highly unlikely that Fyn is involved in EAT-2-mediated inhibitory function. It has been postulated that EAT-2 could also bind to inhibitory molecules such as SHP-1, SHP-2, or SHIP and induce their activation. However, as the binding affinity of these molecules to EAT-2 is very weak (Clarkson *et al.*, 2007), a detailed analysis of EAT-2-mediated signaling pathways is needed.

Transfection experiments in the rat NK cell line RNK-16 have demonstrated that CD244-L possesses inhibitory functions, while CD244S plays an activatory role (Schatzle *et al.*, 1999). Both freshly isolated and IL-2 activated C57BL/6 NK cells showed increased CD244L levels over CD244S (Mooney *et al.*, 2004). Thus, differential expression of murine CD244 isoforms could also determine the fate of CD244 signaling.

7.2. Functions of CD244 in human NK cells

In human NK cells, CD48-dependent engagement of CD244 mediates NK cell cytotoxicity as well as lymphocyte proliferation, IFN-γ and IL-8 production, and increased intracellular accumulation of inositol phosphates and calcium (Valiante and Trinchieri, 1993; Watzl *et al.*, 2000). Expression of CD48 on target cells enhances their susceptibility to killing by NK cells while treatment with antibodies against CD48 inhibits this killing by disrupting the CD244–CD48 interaction (Nakajima *et al.*, 1999; Tangye *et al.*, 2000c). Expression of CD48 on target cells is necessary but not

sufficient for CD244-mediated killing, as CD244 functions as a co-receptor, its activity being strictly dependent upon the simultaneous cross-linking of NKp46 and, possibly, of other triggering receptors such as CD16 and NKP44 (Sivori *et al.*, 2000). CD244-mediated NK cell activity is, however, also governed by the inhibitory NK receptors KIR and CD94/NKG2A. Triggering of those receptors decreased CD244 phosphorylation as well as CD244-mediated cytotoxicity, in NK–NK or NK–target-cell contacts (Stark and Watzl, 2006; Watzl *et al.*, 2000). Interestingly, in NK cells from XLP patients, CD244 exerts an inhibitory function (Parolini *et al.*, 2000). Similarly, CD244-mediated killing in immature NK cells, which do not express SAP, is attenuated (Sivori *et al.*, 2002). Because CD244 expression in NK cell development precedes the expression of inhibitory receptors, it is thought to constitute a crucial receptor maintaining NK cell tolerance to self (McNerney *et al.*, 2005b; Moretta *et al.*, 2002; Sivori *et al.*, 2002). Taken together, these results suggest that although CD244 is an activating receptor in human NK cells, it can also act as an inhibitory receptor.

7.2.1. Human activatory pathways of CD244

CD244-mediated activatory pathways in human NK cells appear to be dependent upon recruitment of SAP (Parolini *et al.*, 2000; Tangye *et al.*, 2000c) and CD244 phosphorylation by Fyn and/or Lck (Bloch-Queyrat *et al.*, 2005; Chen *et al.*, 2004; Eissmann *et al.*, 2005; Nakajima *et al.*, 1999; Sayos *et al.*, 2000). However, SAP could also bind to an already phosphorylated CD244 and thus increase its phosphorylation, thereby enabling it to bind and consequently activate other signaling proteins (McNerney *et al.*, 2005b). Indeed, SAP has been found to be crucial for CD244 localization to the NK cell immunological synapse (Roda-Navarro *et al.*, 2004; Watzl and Long, 2003).

Human CD244 is constitutively associated with LAT. Following CD244 engagement, LAT recruits PLC-γ and Grb2 to CD244 and mediates their phosphorylation (Bottino *et al.*, 2000; Chuang *et al.*, 2001). Engagement of human CD244 also leads to phosphorylation of PLC-γ1, PLC-γ2, Akt, Vav-1, and SHIP-1 (Tassi and Colonna, 2005). The LAT–PLC-γ pathway has been linked to CD244-mediated NK cell killing (Tassi and Colonna, 2005), as evidenced by the findings that treatment of NK cells with a specific PLC-γ inhibitor blocked CD244-mediated cytotoxicity (Tassi and Colonna, 2005). Similarly, pharmacological inhibition of PKC-δ decreased CD244-induced cytolytic activity, suggesting that the LAT–PLC-γ pathway might activate PKC-δ through the generation of intracellular calcium (Chuang *et al.*, 2003).

CD244 phosphorylation is required for binding to the p85 subunit of PI3K. In concordance, binding between CD244 and p85 was still present in NK cells derived from XLP patients. However, PI3K activity was

reduced in XLP cells, confirming that PI3K kinase was dependent on SAP binding to CD244 (Aoukaty and Tan, 2002). Thus, these data indicated that phosphorylation of CD244 is followed first by the association with p85 and then by SAP and that this last association is necessary for PI3K activity. Further insights into the pathways controlled by CD244–PI3K interactions were obtained by experiments involving PI3K inhibitors (Chuang *et al.*, 2003). These experiments demonstrated that the CD244–PI3K pathways mediate IFN-γ production. This same group reported that CD244-mediated IFN-γ production was also dependent on the activation of p38 (Chuang *et al.*, 2001). Whether these two pathways are linked is still not known.

A SAP-independent CD244-signaling pathway involves the adapter 3BP2, which binds to the phosphorylated Y337 residue of human CD244 (Saborit-Villarroya *et al.*, 2005). Engagement of CD244 triggers tyrosine phosphorylation of 3BP2 and the subsequent recruitment of Vav-1. This results in ERK activation and NK cell cytotoxicity (Saborit-Villarroya *et al.*, 2005), which is consistent with experiments showing that CD244-mediated killing is inhibited by MAP kinase inhibitors (Chuang *et al.*, 2001). However, the CD244–3BP2 pathway does not affect IFN-γ production by NK cells (Saborit-Villarroya *et al.*, 2005).

7.2.2. Human inhibitory pathways of CD244

CD224 also acts as an inhibitory receptor in human NK cells only when SAP is not present. The exact intracellular effectors of this signaling are not well-defined. The third ITSM has been mapped to be the motif that negatively influences CD244 signaling (Eissmann *et al.*, 2005). It has been proposed that SHP-1, SHP-2, SHIP, and Csk might be involved, as they bind to the third motif of CD244 and their binding is blocked by SAP expression (Eissmann *et al.*, 2005; Tangye *et al.*, 2000c). Thus, it could be postulated that these molecules and SAP compete for the binding of CD244 and that, in the absence of SAP, they might mediate inhibitory signals downstream of CD244. However, SHP-1 but not SHP-2 has been shown to be associated to CD244 in normal human NK cells, and this association was not affected by the presence of SAP (Parolini *et al.*, 2000). In addition, the weak binding affinities of these molecules to CD244 suggest that such interactions might not be relevant (Clarkson *et al.*, 2007). In contrast, human EAT-2 has been shown to have a high affinity to the third ITSM of human CD244, as compared with SAP (Clarkson *et al.*, 2007). Moreover, CD244 preferentially binds to EAT-2 in nonactivated cells, but after pervanadate treatment or CD244 cross-linking SAP displaces EAT-2 and binds to CD244 (Tassi and Colonna, 2005). Furthermore, overexpression of human EAT-2 in human NK cell lines renders the target cell more resistant to killing, though whether this is CD244-mediated remains unknown (Roncagalli *et al.*, 2005). Thus, it is possible

that both EAT-2 and SAP compete for binding to CD244, and the relative expression of these molecules might dictate the functional outcomes of CD244 signaling (Veillette, 2006b).

7.3. NTB-A is an activating co-receptor on human NK cells

Antibody ligation of NTB-A on the surface of human NK cells increases their cytotoxicity, IFN-γ and IL-8 production, as well as accumulation of intracellular inositol phosphates and calcium (Bottino *et al.*, 2001; Falco *et al.*, 2004). Stimulation with the natural ligand also induces cytotoxicity and IFN-γ as well as TNF-α release (Falco *et al.*, 2004). While expression of its ligands on target cells enhances its susceptibility to killing by NK cells, NK cytotoxicity is inhibited when blocking those interactions using antibodies (Falco *et al.*, 2004). However, engagement of NTB-A, among human NK cells does not have any effect in either the expansion or development of their lytic potential (Stark and Watzl, 2006). Killing experiments with human NK cell lines revealed that NTB-A functions as a co-receptor, its activity being strictly dependent upon the simultaneous cross-linking of NKp46 (Bottino *et al.*, 2001).

NTB-A ligation evokes the phosphorylation of its cytoplasmic tail and the subsequent binding to SAP, EAT-2, and SHP-2 (Bottino *et al.*, 2001; Eissmann and Watzl, 2006). Binding to SHIP-1 was also observed, but was shown to be independent of the phosphorylation state of the receptor (Bottino *et al.*, 2001). NTB-A phosphorylation is Src-dependent, since the inhibition of these kinases results in a loss of NTB-A phosphorylation (Eissmann and Watzl, 2006). SAP and EAT-2 can associate with the same NTB-A molecule at the same time and their binding to the receptor is dependent on tyrosine phosphorylation. The first ITSM is essential and sufficient for NTB-A-mediated cytotoxicity and only binds EAT-2. The second ITSM contributes to full NTB-A-mediated NK cell killing and most likely binds SAP, but not EAT-2, suggesting that both adapter molecules are necessary for full NTB-A function (Eissmann and Watzl, 2006). NK cell cytotoxicity via NTB-A is also dependent on actin reorganization as well as the activities of PLC-γ and PI3K and to a lesser extent on the MEK kinase pathway. Interestingly, SAP controls NTB-A-mediated IFN-γ production, suggesting that pathways mediating cytotoxicity and cytokine production depend on the selective recruitment of these adaptors.

7.4. CD319 is an activating receptor on human NK cells

In humans, engagement of CD319 with antibodies activated lysis of P815 target cells (Bouchon *et al.*, 2001). Unlike with CD244 and NTB-A, this pathway was not shown to synergize with those initiated by CD16 or NKp46. Similarly, incubation of YT cells with a CD319–Ig fusion protein

increased their cytotoxic activity (Kumaresan *et al.*, 2002). Recent studies have revealed that CD319 plays a role in NK–NK interactions (Stark and Watzl, 2006). Ligation of CD319 with its ligand triggered NK cytotoxicity but not NK proliferation. Interestingly, this interaction appeared to be dependent on inhibitory receptors.

Human CD319 also expresses isoforms by alternative splicing in the cytoplasmic domain (Boles and Mathew, 2001). Studies in which RNK-16 cells were transfected with either the long (CD319-L) or the short form of CD319 (CD319-S) have shown that CD319-L, but not CD319-S, was capable of mediating redirected lysis of P815 target cells in the presence of anti-CD319 (Lee *et al.*, 2004a). The *in vivo* relevance of these molecules, however, still needs to be refined.

Although CD319-L was found to bind SAP using yeast two-hybrid systems and overexpression experiments (Lee *et al.*, 2004a; Tassi and Colonna, 2005; Tovar *et al.*, 2002), CD319-mediated NK cell lysis was found to be SAP-independent, since NK cells derived from XLP patients lysed target cells as efficiently as NK cells from normal patients (Bouchon *et al.*, 2001). Recent studies have shown that EAT-2, but not SAP, associates with CD319 in human NK cell lines after cross-linking with anti-CD319 antibodies (Tassi and Colonna, 2005). Engagement of CD319 results in its phosphorylation and EAT-2 recruitment. EAT-2 was shown to mediate CD319 phosphorylation in a transfected human NK cell line, possibly by recruiting a Src-family kinase to the cytoplasmic tail of CD319, as pharmacological inhibition of Src kinases inhibits CD319 phosphorylation (Tassi and Colonna, 2005). Engagement of CD319 also results in PLC-γ1, PLC-γ2, Vav-1, SHIP-1, Akt, c-Cbl, and ERK activation.

CD319 function in mouse NK cells still remains unknown. Although mouse NK cells express both SAP and EAT-2, none of these adaptors were found to bind CD319 (Roncagalli *et al.*, 2005; Tovar *et al.*, 2002). Conditional CD319 deficient mice will help elucidate the role of this receptor in NK cells.

8. THE ROLE OF THE SLAM-FAMILY IN LYMPHOID CELLS

8.1. Thymocyte development

During thymic development, conventional T cells undergo positive and negative selection through contact with self-MHC molecules expressed on thymic epithelial cells. The strength of the TCR signal dictates the fate of these T cells, promoting their survival or their death by apoptosis. These processes ensure that mature T cells respond to foreign peptides, but not to self-peptides presented by self-MHC molecules in the periphery. By contrast, development of natural killer T (NKT) cells and of the so-called

T-CD4$^+$ T cells is directed by the recognition of their respective "restriction elements" on the surface of DP thymocytes (Bendelac *et al.*, 1995; Choi *et al.*, 2005; Li *et al.*, 2005). Similarly, development of innate-like CD8$^+$ T cells also depends on interactions with the hematopoietic cells in the thymus (Atherly *et al.*, 2006; Broussard *et al.*, 2006; Dubois *et al.*, 2006). Positive selection and expansion or lineage differentiation of these cells are dependent upon SAP (Chung *et al.*, 2005; Horai *et al.*, 2007; Li *et al.*, 2007; Nichols *et al.*, 2005a; Pasquier *et al.*, 2005).

NKT cells are unconventional T lymphocytes that co-express both TCR- and NK-cell-associated receptors (Bendelac *et al.*, 2007; Kronenberg, 2005). The invariable TCR recognizes CD1d-bound glycolipids followed by a rapid secretion of IFN-γ, IL-4, and IL-17. Although they appear to arise from common T cell precursors, and seem to undergo normal TCR rearrangement, selection of NKT cells depends on thymocyte–thymocyte interactions. Invariant NKT (iNKT) cells are positively selected by the self-ligand iGb3 presented by CD1d on the surface of DP thymocytes (Gapin *et al.*, 2001; Zhou *et al.*, 2004). After this selection step, NKT cell precursors rapidly up-regulate first CD44 and subsequently NK1.1 (Fig. 4.11). Recent studies have established a crucial role for SAP and Fyn in iNKT development as NKT cells were absent in both SAP and Fyn deficient mice, as well as in SAP deficient XLP patients (Chung *et al.*, 2005; Eberl *et al.*, 1999; Gadue *et al.*, 1999; Nichols *et al.*, 2005a;

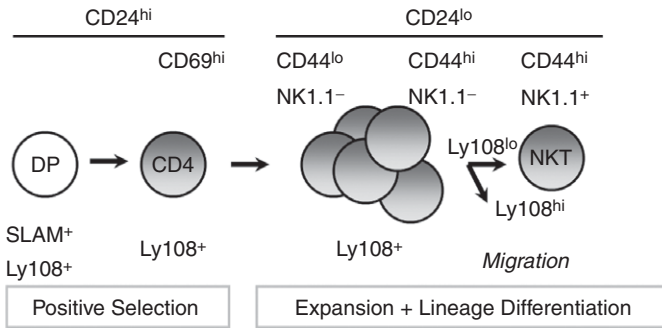

FIGURE 4.11 SLAM and Ly108 expression on double positive thymocytes is required for NKT cells maturation. Immature thymic CD24^{hi} double positive cells express high levels of both Ly108 and SLAM. During selection, these thymocytes further mature into CD24^{hi} CD69^{hi} single positive cells that down-regulate SLAM expression, while maintaining Ly108 expression. These single positive cells further mature into CD24^{lo} CD44^{lo} NK1.1$^-$, which expand and subsequently mature into CD24^{lo} CD44^{hi} NK1.1$^-$ cells, both of which preserve Ly108 expression. At this point, the mature NKT cells either remain in the thymus or migrate to the periphery, where in each case they terminally differentiate into CD24^{lo} CD44^{hi} NK1.1$^+$ cells. Fully mature cells in the thymus are Ly108^{lo}, while those that mature in the spleen are Ly108^{hi}.

Pasquier *et al.*, 2005). The developmental arrest of NKT cells found in $SAP^{-/-}$ mice is similar to that found in $CD1d^{-/-}$ mice, with an incomplete block occurring at a slightly later stage than that found in Fyn deficient mice (Griewank *et al.*, 2007; Pasquier *et al.*, 2005). This suggested that SAP might regulate signaling pathways during NKT selection, partly through Fyn-dependent signaling.

Receptors of the SLAM-family were obvious candidates to mediate NKT development, because their signaling is mediated by SAP and Fyn (Borowski and Bendelac, 2005; Veillette *et al.*, 2007). Furthermore, recent studies using congenic strains derived from C57BL/6 and NOD mice identified SLAM (*slamf1)* as a possible candidate for the control of NKT cell numbers (Jordan *et al.*, 2007). Both Ly108 and SLAM are expressed on DP thymocytes as well as on immature NKT cells, suggesting that the homophilic interactions of these receptors are possibly involved in the selection or propagation of NKT cells (Fig. 4.11). As a modest reduction of NKT numbers was found in $Ly108^{-/-}$ and $SLAM^{-/-}$ mice, and because other experiments suggested an overlap in function between SLAM and Ly108 (Howie *et al.*, 2005; Wang *et al.*, 2004), bone marrow chimeras were generated to investigate the role of SLAM and Ly108 on NKT cell development (Griewank *et al.*, 2007). As predicted, this ''functional double mutant'' (i.e., $Ly108^{-/-}/SLAM^{-/-}$) mouse was severely impaired in its NKT cell development. Together these data demonstrate a complementary role of SLAM and Ly108 in the positive selection of NKT cells, mediated through their homophilic interactions between thymocytes. Importantly, early development of NKT cells prior to the SAP-dependent stage proceeded normally in these bone marrow chimeras (Griewank *et al.*, 2007). It is important to note that while the effects seen on SAP deficient mice are dramatic, the reduction of NKT cells in Fyn or in SLAM/Ly108 double mutant chimeras is not complete, suggesting a possible role of other protein kinases (e.g., Lck), or SLAM-family members such as CD84 and CD229 (Griewank *et al.*, 2007). Although the precise signaling mechanism by which SLAM/Ly108–SAP–Fyn control NKT cell development remains to be elucidated, PKCθ and possibly NF-κB might be involved (Borowski and Bendelac, 2005; Schmidt-Supprian *et al.*, 2004; Sivakumar *et al.*, 2003).

It is conceivable that XLP disease manifestations might be explained, in part, by an absence of NKT cells. For example, NKT cells have been described to play an important role in tumor surveillance by regulating CTL cytotoxicity (Terabe and Berzofsky, 2004; Terabe *et al.*, 2000, 2004), which could explain the hyperproliferation and lymphomas observed in SAP deficient T cells (Nichols *et al.*, 2005b; Sandberg and Ljunggren, 2005). Indeed, CD1d deficient mice also recapitulate the same $CD8^{+}$ T cell hyperproliferation observed in $SAP^{-/-}$ mice after infection (Roberts *et al.*, 2004). Similarly, some SLAM-family members have been implicated

in autoimmune diseases (Chan *et al.*, 2006), which are thought to be regulated by NKT cells (Jiang *et al.*, 2006). Lastly, recent data suggests that NKT cells can provide ''help'' to enhance humoral immunity, which is severely impaired in SAP deficient mice and humans (Cannons *et al.*, 2006; Crotty *et al.*, 2003; Galli *et al.*, 2007; Lang *et al.*, 2006; Morra *et al.*, '2005).

T-CD4$^+$ T cells are another group of lymphocytes that are positively selected by MHCII-expressing thymocytes (Choi *et al.*, 2005; Li *et al.*, 2005). Like NKT cells, TCR-stimulated T-CD4$^+$ T cells can rapidly produce both IFN-γ and IL-4. Recent insights from adoptive transfer experiments revealed a new role for SAP and Fyn in T-CD4$^+$ T cell development (Li *et al.*, 2007). When SAP$^{-/-}$ and Fyn$^{-/-}$ MHCII-expressing T cells were transferred into MHCII Aβ deficient hosts, a dramatic decrease in T-CD4$^+$ T cell numbers in both the thymus and periphery was observed. Like NKT cells, Ly108 deficiency only led to a modest decrease in T-CD4$^+$ T cells, implying that other SLAM receptors might be involved (Li *et al.*, 2007).

The selection and differentiation of innate-like CD8$^+$ T cells also differs from those required for conventional T cell populations (Berg, 2007). They are selected by MHCIb molecules expressed on hematopoietc cells, rather than on thymic epithelial cells. Innate-like CD8$^+$ T cells exhibit properties of innate lymphocyes, such as expression of effector- and memory-like markers (such as CD44, CD122, and NK1.1) and immediate effector functions. Although Itk and RLK, members of the Tec-family of tyrosine kinases, are necessary for the development of conventional T lymphocytes, they are dispensable for the selection of innate-like CD8$^+$ T cells. Indeed, large numbers of these cells are found in mice deficient in Itk or both Itk and RLK (Atherly *et al.*, 2006; Broussard *et al.*, 2006; Dubois *et al.*, 2006), providing an opportunity to study the requirements for their development as well as their function. Studies by Horai *et al.*, have shown that signaling pathways dependent on SAP are indispensable for the thymic selection of innate-like CD8$^+$ T cells (Horai *et al.*, 2007). Which SLAM-family members are participating in the SAP-dependent selection of T-CD4 and innate-like CD8$^+$ T cells requires further investigation.

8.2. CD4$^+$ T cells

The first evidence that SLAM-related receptors regulate immune cells was the finding that anti-SLAM antibodies-mediated TCR-independent proliferation and IFN-g production of previously activated CD4$^+$ T cells (Cocks *et al.*, 1995). However, the engagement of mouse SLAM, human CD84, and NTB-A by specific antibodies increased IFN-γ secretion and

proliferation exclusively in previously activated $CD4^+$ T cells, and only after TCR stimulation (Castro *et al.*, 1999; Howie *et al.*, 2002a; Martin, 2001; Tangye *et al.*, 2003; Valdez *et al.*, 2004). Similar results were obtained when stimulating Ly108 in activated murine $CD4^+$ T cells with a cross-reactive anti-NTB-A antibody (Valdez *et al.*, 2004). Moreover, anti-SLAM antibodies are able to induce Th1 cytokine release by polarized Th_2 cells (Carballido *et al.*, 1997; Howie *et al.*, 2002a). Likewise, when mice were treated with NTB-A-Fc fusion proteins, they displayed a delay in the onset of EAE, indicating a defect of Th_1 responses (Valdez *et al.*, 2004). Based on these observations, it was believed that CD84, NTB-A/Ly108, and SLAM functioned as co-stimulatory receptors that enhanced antigen receptor-induced proliferation and production of Th_1 cytokines by $CD4^+$ T cells.

However, one important issue arising from these experiments was whether the antibodies and fusion proteins mimicked or blocked the effects of physiological engagement of SLAM receptors. Supporting the notion that the SLAM antibodies were blocking, instead of ligating their receptors, was first provided by experiments with BI-141 cells transfected with mouse SLAM. These experiments showed that SLAM signaling was able to selectively inhibit IFN-γ production (Latour *et al.*, 2001). The generation of SLAM deficient mice further supported this hypothesis, since a slight increase in IFN-γ secretion was observed in activated $CD4^+$ T cells from SLAM deficient mice (Wang *et al.*, 2004). Contrary to SLAM and NTB-A antibodies, stimulation with anti-human CD229 antibodies in combination with anti-CD3 antibodies decreased IFN-γ production and ERK activation by human lymphocytes (Martin *et al.*, 2005). Whether these antibodies are agonistic or antagonistic is not known, but as SLAM seems to have a negative effect on IFN-γ, it is possible that this applies to CD229. However, no differences could be detected on IFN-γ production by $CD4^+$ T cells from Ly108 and CD229 deficient mice (Graham *et al.*, 2006; Howie *et al.*, 2005). These surprising results could be explained by functional redundancies between SLAM-family members. Generation of mice lacking combinations of SLAM-family members might help address these issues (Veillette *et al.*, 2006). One of the most perplexing findings was that whereas IL-4 secretion of $CD4^+$ T cells was not affected when engaging SLAM or Ly108/NTB-A with antibodies, it was dramatically decreased in both SLAM and Ly108 deficient mice (Howie *et al.*, 2005; Wang *et al.*, 2004). IL-4 secretion by CD229 deficient mice was only mildly decreased (Graham *et al.*, 2006). SLAM deficient mice did not manifest allergen-induced bronchoalveolar lavage eosinophilia, increased serum IgE, or heightened airway responses compared with *wt* mice (Wang *et al.*, 2006), providing further evidence of the role of SLAM in Th-2 polarization. Similarly, Ly108 deficient mice infected with *L. mexicana* exhibited delayed formation of lesions compared with

wt mice and developed significantly smaller lesions. One major unresolved issue concerns the finding that antibodies directed against the SLAM-family receptors only affect IFN-γ but not IL-4 secretion. One possible explanation for this is that the SLAM signaling pathways involved in IFN-γ and IL-4 secretion might be completely independent from each other and SLAM antibodies only couple the SLAM receptors to signaling pathways involved in IFN-γ secretion.

Three important findings imply that regulation of IL-4 secretion by SLAM (and possibly other SLAM receptors) may be dependent upon SAP–Fyn interactions. First, the reduced IL-4 production by $CD4^+$ T cells found in SAP deficient mice is similar to that of SLAM deficient mice and Ly108 and CD229 deficient mice (Czar *et al.*, 2001; Davidson *et al.*, 2004; Graham *et al.*, 2006; Howie *et al.*, 2005; Wang *et al.*, 2004; Wu *et al.*, 2001). Second, the more dramatic defect observed in $SAP^{-/-}$/SAPR78A $CD4^+$ T cells compared with that of SLAM suggests that the SAP–Fyn pathway may regulate IL-4 production through other SLAM-family members (Davidson *et al.*, 2004). Third, SLAM engagement on $CD4^+$ T cells results in increased PKCθ and Bcl-10 recruitment and subsequent activation of NF-κB in a SAP and Fyn dependent manner following TCR stimulation (Cannons *et al.*, 2004). However, although SLAM engagement affects the PKCθ/NF-κB signaling pathway, it has no effect on IL-4 production, challenging the hypothesis that the SLAM/SAP/Fyn/PKCθ/NF-κB pathway is involved in IL-4 production. Further analyses need to be performed to link the PKCθ/NF-κB and the GATA3-IL-4 pathway. For example, reconstitution of the IL-4 defect by overexpression of NF-κB activity in $SAP^{-/-}$ or SAPR78A T cells would evidence a direct role of this pathway in IL-4 secretion by $CD4^+$ T cells. Similarly, expression of SAPR32Q (in which the binding to the SLAM receptors is impaired) on $SAP^{-/-}$ cells, would ascertain a role of SAP on SLAM-mediated IL-4 production.

Recent studies have demonstrated that co-ligation of CD229 and TCR resulted in CD229 internalization in T lymphocytes (Del Valle *et al.*, 2003). It was later postulated that vesicules containing endocytosed CD229 mainly follow the lysosomal degradation pathway. The molecules implicated in CD229 endocytosis have been recently reported (Del Valle *et al.*, 2003; Martin *et al.*, 2005). The μ2 chain of clathrin-associated adaptor complex 2 (AP-2) and Grb2 have been shown to bind to the tail of CD229 in T cell lines. Mutational analyses demonstrated that while Y470 of human CD229is responsible for its binding to μ2 chain, p-Y606 is indispensable for CD229 binding to Grb2 (Del Valle *et al.*, 2003; Martin *et al.*, 2005). Unlike with μ2, this binding is dependent upon phosphorylation of the receptor. Mutation of one of these tyrosines is enough to completely abrogate CD229 internalization, suggesting that both Grb2 and AP-2 might act synergistically. However, mutation of Y606 had no effect on the CD229-mediated

inhibition of IFN-γ and ERK activation, suggesting that others molecules may be involved in the signaling pathways of CD229 on T cells. The exact consequences of CD229 internalization are not known. CD229 has been shown to localize to the immune synapse between B and T cells (Romero *et al.*, 2005). Thus, it is possible that exclusion of CD229 from the central synapse could prevent CD229 function.

SLAM has been shown to be dispensable for SAP-mediated T–B-cell interactions. Generation of germinal centers as well as T-dependent responses is normal in $SLAM^{-/-}$ mice (McCausland *et al.*, 2007). Whether other SLAM receptors are involved in SAP-dependent T_{FH} function is not known. CD84 and CD229 are attractive candidates as both are expressed in T_{FH} (Chtanova *et al.*, 2004) and B cells (de la Fuente *et al.*, 2001; Martin, 2001; Romero *et al.*, 2004; Tangye *et al.*, 2002) and engage in homophilic interactions.

8.3. $CD8^+$ T cells

Both SLAM and CD244 have been implicated in the regulation of $CD8^+$ T cell effector functions. SLAM cross-linking on activated human $CD8^+$ T cells increases TCR-mediated cytotoxicity and granule release (Henning *et al.*, 2001). Similarly, IFN-γ secretion and cytotoxicity could be augmented following overexpression of human SLAM on $CD8^+$ T cells (Mehrle *et al.*, 2008). These studies also showed that SLAM co-localizes at the lipid raft fraction and that, after peptide activation, migrates to the immunological synapse (Mehrle *et al.*, 2008). However, murine SLAM seems to be dispensable for antigen specific IFN-γ secretion by $CD8^+$ T cells. Infection of SLAM deficient mice with LCMV generated normal numbers of IFN-γ producing $CD8^+$ T cells (McCausland *et al.*, 2007).

Initial reports showed that the cytolytic activity of mouse and human effector $CD8^+$ T cells generated after IL-2 activation for 6 days was restricted to the $CD244^+$ population (Garni-Wagner *et al.*, 1993; Peritt *et al.*, 1999; Valiante and Trinchieri, 1993). A direct involvement of mouse CD244 on $CD8^+$ T cell effector function was revealed when addition of CD244 antibodies or retroviral transduction of CD244 on antigen-specific $CD8^+$ T cells led to an increase of their lytic ability (Garni-Wagner *et al.*, 1993; Lee *et al.*, 2003). However, the involvement of human CD244 on $CD8^+$ T cell-mediated cytotoxicity remains contradictory. Initial analyses demonstrated that cross-linking of human CD244 did not trigger $CD8^+$ T cells or CTL clones cytotoxicity, cytokine production, or proliferation (Nakajima and Colonna, 2000; Peritt *et al.*, 1999; Tangye *et al.*, 2000a). Later studies showed that adding blocking antibodies against CD244 increased the killing ability of $CD8^+$ T cell clones against EBV^+ targets (Dupre *et al.*, 2005). Similarly, another set of experiments using

autologous EBV T cell lines demonstrated that CD3/CD244 or CD244 stimulation resulted in an increase in IFN-γ production (Sharifi *et al.*, 2004). These analyses also proved that defects in CD244, but not in SLAM signaling, might account for the inability of XLP $CD8^+$ T cells to kill target cells. CD244 regulation of effector T cells was shown to be mediated through SAP, since XLP $CD8^+$ T cells exhibited a dramatic decrease in CD244-mediated cytotoxicity and IFN-γ secretion (Dupre *et al.*, 2005; Sharifi *et al.*, 2004). No defects in IFN-γ or proliferation were observed when anti-SLAM and anti-CD3 antibodies were used to activate CD8+ T cells from XLP patients (Sharifi *et al.*, 2004).

Additionally, CD244 and perforin have been shown to localize at the immunological synapse in a SAP-dependent manner (Dupre *et al.*, 2005). The impairment in killing capacity seen in XLP $CD8^+$ T cells was restricted to $CD48^+$ target cells (Dupre *et al.*, 2005), suggesting that CD48–CD244 interactions at the target-$CD8^+$ T cell contact area are relevant and SAP-dependent. Thus, these data seem to indicate that the failure to control EBV infection seen in XLP patients may stem from lytic defects not only on NK cells, but also on $CD8^+$ T cytotoxic cells.

Several lines of evidence also implicate CD244–CD48 interactions in T cell proliferation (Kambayashi *et al.*, 2001; Lee *et al.*, 2003). The findings that expression of CD244 on $CD8^+$ T cells increases the killing of both $CD48^+$ and $CD48^-$ target cells suggests that CD244–CD48 interactions also occur between T cells (Lee *et al.*, 2003). These interactions increase T cell proliferation of neighboring $CD244^-$ cells (Kambayashi *et al.*, 2001), revealing a new role of CD244 as a ligand for CD48 T cells. Indeed, engagement of CD48 induces an enhanced proliferation of CD3-activated $CD8^+$ T cells (Kato *et al.*, 1992). Similar results are observed when these interactions take place between NKT cells, where CD244 acts predominantly on NK cells to increase the proliferation of T cells (Assarsson *et al.*, 2004). These data are in concordance with the findings that both CD244L and CD244S (reported to have contrary functions in NK cells) have similar effects on T cell proliferation, indicating that CD244 downstream signaling might be dispensable for T cell proliferation (Lee *et al.*, 2003).

One major issue that needs to be addressed is the mechanism by which $CD8^+$ T cells from $SAP^{-/-}$ mice and XLP patients display excessive proliferation. Neither CD244 nor SLAM seems to be the receptor that mediates such function. As mentioned above, CD244-mediated control of T cell proliferation might be independent of SAP since CD244 acts as a ligand for CD48 on T cells. Similarly, infection of $SLAM^{-/-}$ mice with LCMV leads to a normal $CD8^+$ T cell response *in vivo* and *ex vivo* (McCausland *et al.*, 2007). Future research will be necessary to unravel the means by which SAP controls T cell proliferation.

8.4. B cells

In addition their effects on T cells, SLAM-specific monoclonal antibodies promoted proliferation and differentiation of human B cells, as well as immunoglobulin synthesis (Punnonen *et al.*, 1997; Sidorenko and Clark, 1993). They have also been reported to increase CD95-mediated apoptosis and calcium mobilization (Mikhalap *et al.*, 1999; Sidorenko and Clark, 1993). As in T cells, SAP binds to SLAM, blocking its association with SHP-2. Interestingly, SHIP binding to SLAM was not abrogated when SAP was present (Shlapatska *et al.*, 2001). Whereas engagement of the BCR results in increased SHIP phosphorylation, SLAM ligation induces a reduction of its phosphorylation, suggesting that the association of SHIP with SLAM might result in dephosphorylation of its tail and probably its activation (Mikhalap *et al.*, 1999). It has been suggested that this phosphatase activity might be due to CD45, as this molecule binds to SLAM in B cell lines (Mikhalap *et al.*, 1999).

The SLAM signaling cascade in B cells seems to be mediated through the ERK and Akt pathways (Mikhalap *et al.*, 2004). Engagement of SLAM results in increased Akt phosphorylation in a SAP-dependent manner. In contrast, cross-linking of SLAM activates the ERK pathway in a SHIP-dependant manner (Mikhalap *et al.*, 2004). The exact molecular mechanisms by which these two independent signaling pathways influence SLAM function in B cells require further investigation. However, it has been proposed that SLAM mediates inhibition of SHIP, which in turn causes an increase in activation-induced cell death. Indeed SHIP deficient B cells are more susceptible to apoptosis and display enhanced calcium mobilization (Ono *et al.*, 1997).

9. THE ROLE OF THE SLAM-FAMILY ON NONLYMPHOID CELLS

9.1. Dendritic cells

DCs are important for the initiation of specific adaptive immune responses (Gogolak *et al.*, 2003; Steinman and Banchereau, 2007). After antigen uptake, maturation, and activation, they become the most potent antigen presenting cells and are involved in many T-cell-dependent immune responses. Co-stimulatory molecules, together with adhesion molecules, are involved in the stabilization of the immune synapse between DCs and T cells. Recent insights from cross-linking experiments indicated a possible role for SLAM as a co-stimulatory molecule in DCs (Bleharski *et al.*, 2001). Engagement of human SLAM with antibodies in CD40L-activated DCs augmented the secretion of pro-inflammatory

cytokines such as IL-12 and IL-8, but not IL-10. These antibodies may have an opposite effect to that of SLAM, as shown by the observation that expression of SLAM and CD40L on human DCs inhibited the production of IL-12, TNF-α, and IL-6 (Rethi *et al.*, 2006). DCs that were previously co-stimulated with CD40L and SLAM-bearing cells inhibited the differentiation of naïve $CD4^+$ T cells into Th1-type effectors, establishing SLAM as a negative regulator of DC inflammatory responses.

IL-10 stimulation of human DCs generates tolerogenic DCs, a DC subset that induces the generation of regulatory T cells (Tregs) *in vitro* (Roncarolo *et al.*, 2006). The mechanism underlying IL-10-mediated inhibition may not stem from a direct regulation of cytokine secretion, but rather from a modulation of surface molecules (McBride *et al.*, 2002). It is interesting to note that SLAM is among those receptors that are up-regulated after IL-10 activation of DCs. Thus, it might be implicated in the mechanisms by which these tolerogenic DCs mediate immunosuppression (McBride *et al.*, 2002; Velten *et al.*, 2004).

9.2. Macrophages

SLAM function in macrophages has been analyzed by using antibodies and by employing cells from SLAM deficient mice. SLAM has been proposed to act as a TLR-4 co-receptor, because after LPS stimulation SLAM deficient macrophages produce less nitric oxide, IL-12, and TNF-α and increased amounts of IL-6 (Wang *et al.*, 2004). In contrast, TNF-α secretion by $SLAM^{-/-}$ macrophages after CpG or peptidoglycan stimulation was similar to *wt* macrophages. These results seem to be in contradiction with the negative role of SLAM on CD40L-mediated cytokine production by human DCs. However, it might be possible that SLAM regulation of cytokine production may differ between macrophages and DCs. Alternatively, it is also plausible that SLAM has two opposing effects, depending on the stimulus (Ma *et al.*, 2007). Indeed, SLAM engagement on human DCs slightly increased LPS-mediated cytokine production (Rethi *et al.*, 2006).

No defects have been detected in macrophages from CD229 and Ly108 deficient mice following TLR4 stimulation (Graham *et al.*, 2006; Howie *et al.*, 2005). Since EAT-2A and not SAP is expressed in DCs and macrophages, it is tempting to suggest that signaling though the SLAM-family of receptors is mediated by EAT-2A in these cells.

9.3. Neutrophils

Although no defects have been observed in macrophages from mice with a targeted deletion in Ly108, a role of Ly108 in neutrophils has been demonstrated (Howie *et al.*, 2005). Neutrophils from $Ly108^{-/-}$ mice

produced 5-fold more IL-12, twice as much TNF-α and had slightly elevated IL-6 production as compared with *wt* neutrophils. Ly108$^{-/-}$ mice were also highly susceptible to *Salmonella typhimurium* and showed a defect in neutrophil-dependent *in vitro* bacterial killing. This defect was found to be due to a defect in reactive oxygen species production and not to an inefficient uptake of bacteria (Howie *et al.*, 2005). These results suggest the involvement of Ly108 in oxidative burst of neutrophils. Whether Ly108 signaling in mouse neutrophils is mediated by SAP, EAT-2A, or EAT-2B remains unknown, since their expression has not been determined. Further analysis will be required to unravel the exact mechanism by which Ly108 controls reactive oxidative species release.

9.4. Platelets

SLAM and CD84 have also been identified as receptors that are engaged when platelets aggregate (Nanda *et al.*, 2005). While CD84 was shown to be important for platelet stimulation, SLAM was found to contribute to the stabilization of platelet aggregates *in vitro* and in thrombus formation *in vivo*. As in lymphocytes, these functions seem to be dependent on receptor phosphorylation, since both CD84 and SLAM are phosphorylated upon platelet aggregation (Nanda *et al.*, 2005). Interestingly, CD84 phosphorylation is not dependent on signals from the integrin, as the receptor also becomes phosphorylated after cross-linking with antibodies or when engaged through homotypic interactions. Unlike lymphocytes, however, SAP seems to be dispensable for SLAM phosphorylation, since SAP deficient platelets exhibit similar levels of SLAM phosphorylation as compared with *wt* platelets. EAT-2 has also been shown to be expressed in platelets. While no binding of EAT-2 and SLAM could be detected in murine platelets, it is possible that EAT-2 mediates SLAM phosphorylation in the absence of SAP (Nanda *et al.*, 2005). Analysis of triple deficient mice (SAP/EAT-2A/EAT-2B) might clarify whether SAP-related adaptors are involved in SLAM function in platelets.

9.5. Eosinophils

CD48 up-regulation in asthmatic patients and in allergen-challenged lungs suggested a potential role for CD48 in asthma pathogenesis. Indeed, treatment with CD48 antibodies, but not CD244, reduced eosinophilic inflammation and cytokine expression in an allergic eosinophilic airway inflammation model (Munitz *et al.*, 2006, 2007). CD48 engagement on eosinophils also caused them to release eosinophil peroxidase (EPO), an enzyme that induces tissue damage. Although CD244 does not seem to participate in pulmonary allergic inflammation, it might have an important function on eosinophil-dependent killing, as engagement of human CD244 also mediated

eosinophil antibody-mediated killing of target cells as well as release of IL-4 and IFN-γ (Munitz *et al.*, 2005). Although SAP protein has been found in human eosinophils, no interaction with CD244 could be detected. While this might reflect some technical limitations, it is also conceivable that other adaptors, such as EAT-2A/B, could be mediating CD244 signaling.

9.6. SLAM as a co-receptor for the measles virus

The measles virus is a member of the Morbillivirus genus in the Paramyxoviridae family. It was first isolated from primary human kidney cells inoculated with the blood and throat washing of a child with measles (Yanagi *et al.*, 2006). The only natural host is humans, where it remains the most transmissible virus known. It is classified as a negative-strand RNA virus with a 16-kb genome that encodes for eight different proteins, including H (hemaglutinin) and F (fusion) proteins, which are essential in viral pathogenesis (Dhiman *et al.*, 2004). Human CD46, a complement regulatory molecule, was identified as the cellular target of measles virus (Dorig *et al.*, 1993; Naniche *et al.*, 1993). Interestingly, only certain strains such as the Edmonston and Halle were able to infect $CD46^+$ cells, whereas B cell isolated strains did not (Lecouturier *et al.*, 1996), suggesting the presence of another receptor for those strains. Further experiments showed that expression of SLAM in those $CD46^+$ -resistant strains rendered them vulnerable to infection and replication (Tatsuo *et al.*, 2000). Another set of experiments demonstrated that the V domain of human SLAM is important for binding with the measles virus hemaglutinin protein and is required to allow measles virus entry into the host cell (Ono *et al.*, 2001).

The generation of transgenic mice expressing human SLAM under the mouse Lck or CD11c promoter provided useful insights into measles virus pathogenesis (Hahm *et al.*, 2003, 2004, 2007). Expression of human SLAM on mouse T and DCs rendered them susceptible to measles virus infection, leading to a decrease in T proliferation and inhibition of DC maturation. After infection, DCs also showed enhanced susceptibility to apoptosis and decreased ability to activate T cells (Hahm *et al.*, 2004). It has been proposed that the mechanisms for MV-induced immunosuppression might stem from changes in the signaling cascade initiated by SLAM. Indeed, recent studies have shown that measles virus binding to SLAM suppresses LPS-mediated IL-12 secretion, implying that measles virus binding to SLAM disrupts its signaling and, consequently, cell activation (Hahm *et al.*, 2007).

9.7. CD48 as the FimH receptor

Mast cells are potent mediators of the host defense response against infectious agents (Marshall, 2004). They strategically reside at sites that are exposed to the external environment, such as the connective and

mucosal tissues. Through direct and indirect receptors that recognize foreign stimuli, they are also able to mediate a rapid antimicrobial response by secreting cytokines (such as TNF-α, IL-4, and IL-13) and granule-associated lipid mediators (histamine) (Marshall, 2004). CD48 is among the mast cell receptors that have been shown to interact directly with bacteria (Shin and Abraham, 2001). FimH, a type I fimbriae present in many Gram-negative bacteria, was found to bind to CD48 on the surface of mast cells (Malaviya *et al.*, 1999). Binding of FimH to CD48 was reported to trigger TNF-α secretion as blocking this interaction with anti-CD48 antibodies inhibited cytokine production. Bacterial entry was also mediated by this interaction (Malaviya *et al.*, 1999). However, internalization of $FimH^+$ pathogens via CD48 differs from that of opsonized bacteria, which follow the classical lysosome–phagosome route (Shin *et al.*, 2000). After interaction with CD48, $FimH^+$ bacteria are phagocyted and encapsulated in caveolae, membrane domains enriched in glycosphingolipids, cholesterol, and caveolin (Shin *et al.*, 2000). These caveolar-like chambers fail to fuse with lysosomes, promoting the survival of bacteria on mast cells (Shin and Abraham, 2001). Uptake of $FimH^+$ bacteria by macrophages has also been shown to be mediated by CD48, and although it enhances bacterial survival inside the cell, it is not known whether it is caveolae dependant (Baorto *et al.*, 1997). Caveolae are involved in the trafficking of macromolecules, but the exact significance of caveolar-like domains in the uptake of bacteria requires further clarification (Shin and Abraham, 2001).

10. CONCLUDING REMARKS

The SLAM-family of cell-surface receptors is quickly emerging as an integral component of both innate and adaptive immunity. Although first recognized as co-receptors on lymphocytes and NK cells, the SLAM-family is clearly involved in development and function of a multitude of hematopoietic cells. These include early stages of hematopiesis, development of NKT, T-$CD4^+$, and innate-like $CD8^+$ T cells and macrophage and platelet functions. As the adapter SAP in conjunction with SLAM receptors controls immune responses to viruses, a further understanding of the regulation of SLAM-family receptor-induced signaling networks should help in designing better vaccination strategies. Furthermore, extensive polymorphisms and co-expression of isoforms of the SLAM-family receptors in epistatic pathogenic networks leads to SLE. The use of conditional disruption of individual genes and groups of the mouse SLAM-family genes will be required to unravel the specific contribution of each individual SLAM-family member as well as the SAP-related adaptors to immune disorders.

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CHAPTER 5

Conformational Plasticity and Navigation of Signaling Proteins in Antigen-Activated B Lymphocytes

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Abstract

Over the past two decades our view of the B cell antigen receptor (BCR) has fundamentally changed. Being initially regarded as a mute antibody orphan of the B cell surface, the BCR turned out to be a complex multimolecular machine monitoring almost all stages of B cell development, selection, and activation through a plethora of ubiquitously and cell-type-specific effector proteins. A comprehensive understanding of the many BCR signaling facets is still out but a few common biochemical principles outlined in this review operate at the level of receptor activation and orchestrate specific wiring of intracellular transducer cascades. First, initiation and processing of antigen-induced signal transduction relies on transient conformational changes in the signaling proteins to trigger their physical interaction with downstream elements. Second, this dynamic assembly of signalosomes occurs at distinct subcellular locations, most prominently the plasma membrane, which requires dynamic relocalization of one or more of the engaged molecules. For both, precise complex formation and efficient subcellular targeting, B cell signaling components are equipped with a variety of protein interaction domains. Here we provide an overview on how these simple rules are applied by a limited number of transmembrane and cytosolic proteins to convert BCR ligation into Ca^{2+} mobilization and Ras activation in an adjustable manner.

Key Words: B cell antigen receptor, tyrosine phosphorylation, protein interaction domains, Ca^{2+} mobilization, Ras/MAPK activation, plasma membrane targeting.

1. INTRODUCTION

During their early development in the bone marrow or the *bursa of fabricius* in case of birds, B lymphocytes rearrange their immunoglobulin (Ig) gene segments to produce clonotypic antigen receptors of almost unlimited variability and with the capacity to recognize all possible classes of biomolecules or even artificial chemicals. The advantage for the immune system is obvious but an *a priori* unpredictable ligand is a challenging task for the proper responsiveness of the mature receptor. In fact, this demand is less dramatic in case of the T-cell antigen receptor (TCR), whose variability is similar to that of the B-cell antigen receptor (BCR) but its binding specificity is restricted to peptides presented in the context of membrane-bound MHC proteins. In light of arbitrary binding partners, it might have been helpful for the functionality of lymphocyte antigen receptors to possess separate polypeptides for recognition of extracellular ligand and signal transduction across the plasma membrane. The antigen-binding subunit of the BCR can be one of five different membrane-bound Ig classes, namely IgM, IgD, IgG, IgA, and IgE (Reth, 1992; Venkitaraman *et al.*, 1991). Each of the Ig molecules is able to noncovalently associate at a 1:1 stoichiometry with the canonical BCR signaling subunit provided by the disulfide-linked Igα/Igβ heterodimer (Schamel and Reth, 2000). Igα (CD79a) and Igβ (CD79b) are type I transmembrane proteins encoded by the *MB1* and *B29* genes, respectively. Only the fully assembled multimeric BCR complex can be deposited on the B cell surface, where it can deliver a tonic, that is, antigen-independent B cell survival signal (Neuberger, 1997; Reth, 1992). This is part of a quality control mechanism, which prevents the appearance of BCR-negative B cells and which is discussed in detail elsewhere (for review see Grande *et al.*, 2007). However, developing B cells have to be checked not only for the presence of a functional BCR but also for nonreactivity against self antigens. Autoreactivity of B cells can be prevented by exchanging the BCR specificity, the induction of BCR nonresponsiveness, or apoptotic elimination of the cell. These key processes of humoral immunity are termed receptor editing, anergy, and negative selection, respectively. Their underlying mechanisms are thought to involve developmental-stage-specific connectivity of the BCR to positive- and negative-regulatory effector molecules. Also immunocompetent B cells of the peripheral lymph organs can mount graduated responses upon antigen encounter. Naïve B cells carrying an IgM-BCR are less potently activated than class-switched IgG-positive B cells even if their affinity for the antigen is identical (Horikawa *et al.*, 2007; Martin and Goodnow, 2002). Hence, the BCR is strikingly different and more complex than conventional receptors such as hormone receptors, which have a defined class of ligands and elicit a unique cellular response.

2. A MINIMAL SIGNALING KIT COUPLES TO PROXIMAL TRANSDUCER ENZYMES

As to the different and in part inverse functions of the BCR, one might expect that it accommodates a number of complex signaling devices. There are in fact at least three different ones. First, the cytoplasmic tail segments of Igα and Igβ both contain one copy of the immunoreceptor tyrosine-based activation motif (ITAM) found in many receptors on hematopoietic cells (Reth, 1989). The ITAM has the consensus amino acid sequence D/ExxD/Ex$_7$YxxI/Lx$_7$YxxI/L (with ''x'' being a nonconserved amino acid). It couples BCR ligation to tyrosine phosphorylation of a diverse array of cellular proteins, including the Igα/Igβ dimer itself. Second, Igα harbors one non-ITAM tyrosine residue that is evolutionary conserved but absent in other ITAM-containing receptors. Following phosphorylation, the non-ITAM tyrosine of Igα acts as signal enhancer that increases the sensitivity of mature B cells to T-cell-independent antigens (Engels *et al.*, 2001; Kabak *et al.*, 2002; Patterson *et al.*, 2006). Third, also the short cytoplasmic segments of membrane-bound IgG molecules, and probably that of IgE as well, participate in the amplification of ITAM-induced signaling (Achatz *et al.*, 1997; Kaisho *et al.*, 1997). Genetically engineered mouse models show that IgG tail signaling is indispensable for the maintenance and improved response of class-switched memory B cells and hence for successful vaccination against pathogens (Kaisho *et al.*, 1997; Martin and Goodnow, 2002). Only recent work from our laboratory revealed that phosphorylation of a single tyrosine residue, which is conserved among the different IgG subclasses and across species, can account for the ability of the IgG-BCR to trigger B cell activation more efficiently than the IgM-BCR (Engels and Wienands, submitted for publication). In conclusion, proximal BCR signaling mechanisms belong to three categories: (i) ITAM-mediated onset of signaling, which is used by many activating receptors of the immune system; (ii) signal amplification in a BCR-specific manner through the non-ITAM tyrosine of Igα; and (iii) further signal enhancement by Ig tail segments that is restricted to certain BCR isotypes of class-switched memory B-cells and mandatory for an effective secondary antibody response. All of the integral BCR signal switches known so far utilize tyrosine phosphorylation as a common mechanism to initiate and modify the reaction to antigen. The responsible phosphotransferases belong to the Src and Syk/ZAP-70 families of cytoplasmic protein tyrosine kinases (PTKs). These enzymes are composed of protein modules termed Src homology (SH) domains. The SH1 domain at the C-terminal end provides catalytic activity. N-terminal to SH1, Src family kinases possess one SH2 and SH3 domain, which bind to tyrosine-phosphorylated and proline-rich peptide

motifs, respectively. A covalently attached myristine and/or palmitine fatty acid anchor at the N-terminus constitutively tethers Src family kinases to the inner leaflet of the plasma membrane. A direct albeit low affinity interaction to the resting BCR has been reported for the Src family members Lyn and Fyn (Pleiman *et al.*, 1994). The spleen tyrosine kinase Syk contains two tandemly arranged SH2 domains in its N-terminal half. In resting B cells, the majority of Syk molecules are located in the cytosol but a weak association of Syk to the unstimulated BCR was instrumental for its initial detection and purification (Hutchcroft *et al.*, 1992).

3. SIGNAL INITIATION: BCR CLUSTERS WITH AN OPEN CONFORMATION

The investigation of ITAM-triggered signal transduction was greatly facilitated by the use of chimeric transmembrane proteins encompassing heterologous ectodomains (e.g., that of CD8) and short cytoplasmic peptide segments derived from either Igα, Igβ, or other immune receptors (Irving and Weiss, 2000). Extracellular cross-linking of these ''minimal receptors'' mimicked the antigen response of the fully assembled BCR complex (Kim *et al.*, 1993; Taddie *et al.*, 1994; Williams *et al.*, 1994). Further, mutational analyses revealed that both ITAM tyrosine residues have to become phosphorylated in order to induce tyrosine phosphorylation of cellular substrates (Williams *et al.*, 1994). This experimental approach demonstrated the awesome power of the ITAM to signal almost independently of its surrounding peptide frame (Cambier, 1995). Yet, it raised a hen-egg problem. What occurs first: activation or action of the PTK? To solve this question, several biochemical events have been proposed as the initial step of BCR signal transduction; that is, BCR cross-linking, BCR translocation into specialized membrane compartments called lipid rafts, the production of reactive oxygen species (ROS) to inhibit protein tyrosine phosphatases (PTP), disruption of BCR oligomers, and conformational changes of the BCR.

3.1. Antigen-induced BCR cross-linking

The *crosslink model* suggests a concerted action of the two BCR-proximal PTK families and is based on the observation that efficient BCR signaling requires multivalent antigen, which most likely induces clustering of ''monomeric'' BCR complexes or the polypeptide chimeras described above. Once clustered from the outside of the cell, the concomitant intracellular aggregation of ITAM-containing subunits allows for transphosphorylation and thereby activation of associated Src family

members. This triggers the phosphorylation of adjacent ITAMs. Phosphorylated ITAMs can stabilize the catalytically active (e.g., open) conformation of Src kinases through binding the enzyme's SH2 domain (Johnson *et al.*, 1995). This is directly supported by a number of genetic and structural analyses revealing that an inactive Src family kinase is in a closed conformation as a result of intramolecular interactions involving the SH2 and SH3 domains (Hubbard, 1999). In fact, Src family kinases represent a paradigm of allosteric enzymes whose activity goes along with conformational changes that can be regulated at multiple levels (Gonfloni *et al.*, 2000). A conformational opening is reported also for Syk upon binding of its tandem SH2 domains to the doubly-phosphorylated ITAM (Kimura *et al.*, 1996). This highly specific interaction not only stimulates Syk activity (Kimura *et al.*, 1996; Rolli *et al.*, 2002; Rowley *et al.*, 1995; Shiue *et al.*, 1995), but more importantly, it alters the subcellular localization of Syk. Only upon translocation from the cytosol to the plasma membrane Syk appears to be able to meet and phosphorylate its substrate proteins (Kurosaki *et al.*, 1995). Interestingly, the ITAM itself provides a Syk substrate as demonstrated by the ability of the BCR to signal albeit with reduced efficiency in the absence of Src family kinases in DT40 B cell mutants (Takata *et al.*, 1994) and reconstituted insect cells (Rolli *et al.*, 2002). Conversely, Syk deficiency abrogates phosphorylation of cellular proteins (Takata *et al.*, 1994). Collectively, the available data show that Src family kinases and Syk can execute ITAM phosphorylation followed by SH2 domain-mediated feedback amplification of kinase activities. Maximal efficiency is achieved in the presence of both proteins, probably reflecting the dependency of Syk on direct phosphorylation by Src kinases for full activation (Kurosaki *et al.*, 1995). Syk appears to be the dominant enzyme for phosphorylation of downstream substrates.

3.2. A role for signaling-competent membrane subcompartments

The *lipid raft model* adds another level of complexity to the sequence of events discussed. Lipid rafts are cholesterol- and glycosphingolipid-enriched plasma membrane microdomains, which function as platforms for the assembly of signaling pathways in many cell types (Simons and Toomre, 2000). Among other signaling proteins, Src family kinases are permanent residents of lipid rafts by virtue of their fatty acid moieties (Cary and Cooper, 2000). By marked contrast, the BCR on resting cells is outside of lipid rafts (Aman and Ravichandran, 2000; Cheng *et al.*, 1999). Together, these observations indicate a spatial separation of the unstimulated BCR and its proximal transducer, suggesting protein translocation as an early and critical step during BCR signal initiation (Cheng *et al.*, 2001). Indeed, a substantial fraction of the BCR is readily found within lipid rafts

of antigen-activated B cells (Aman and Ravichandran, 2000; Cheng *et al.*, 1999). Moreover, dispersion of lipid rafts by cholesterol depletion interferes with BCR signal initiation (Aman and Ravichandran, 2000; Tolar *et al.*, 2005). Nonetheless, allowing the rendezvous of clustered ITAMs with concentrated Src family kinases cannot be the only function of lipid rafts because BCR signaling occurs to some extent in the absence of Src kinases (see earlier).

3.3. Signal onset by inhibition of negative regulators

The *ROS model* appreciates the basic principle that biochemical processes are driven by a dynamic reaction equilibrium. The action of PTKs is counterbalanced by PTPs, which can be inhibited *in vivo* by reversible oxidation of a critical cysteine residue through ROS (Reth, 2002). In B cells, the SH2-domain-containing phosphatase (SHP)-1 is well known to play a pivotal role in the regulation of BCR signaling. SHP-1-negative mice show severe symptoms of autoimmunity owing to hyperreactive B cells (Zhang *et al.*, 2000). SHP-1 directly regulates Syk-mediated substrate phosphorylation (Adachi *et al.*, 2001; Rolli *et al.*, 2002). Exposure of B cells to pervanadate/H_2O_2, which mimics ROS production, induces protein tyrosine phosphorylation and the PTK substrate pattern is identical to that observed upon BCR ligation (Wienands *et al.*, 1996). More recently, BCR-induced production of ROS has been demonstrated (Singh *et al.*, 2005). However, the kinetics of ROS production is rather slow compared with that of PTK activation and moreover was largely dependent on a PTK effector mechanism, the mobilization of Ca^{2+} ions (see later). Hence, further experiments are required to elucidate whether BCR-induced production of ROS is upstream or downstream of PTK activation.

3.4. BCR oligomerization: Is antigen ligation constructive or destructive?

The models of BCR signal initiation discussed so far are not mutually exclusive. The *disruption of preformed BCR oligomers* is a completely different scenario. It takes into account that almost every non-self molecule of biological or chemical nature can act as a *bona fide* antigen. Hence, antigen-induced disruption of a higher-ordered BCR structure might induce signaling more efficient than antigen-induced formation of signaling-competent BCR clusters (Reth, 2001). BCR components purified form resting B cells have been indeed detected in high molecular weight fractions when analyzed under native conditions (Schamel and Reth, 2000). Also the observation that recombinantly expressed cytoplasmic domains of Igα and Igβ are able to homo-oligomerize *in vitro* is in line

with self-assembly of multiple BCR units in the absence of antigen (Sigalov *et al.*, 2004). However, direct evidence for BCR aggregates on the cell surface of resting B cells awaits further experimental analysis.

3.5. Structural flexibility of the BCR

High-resolution fluorescence imaging of living B cells recently revealed *conformational dynamics of the cytoplasmic segments of Igα and Igβ.* In this approach, individual BCR subunits labeled at their C-terminal end with different variants of the green fluorescent protein were expressed in J558L B cells. Subsequently, fluorescence resonance energy transfer (FRET) between the differently tagged BCR components was measured in the absence and presence of antigen by quantitative microscopy (Tolar *et al.*, 2005). This directly confirmed that only the fully assembled BCR complex is transported onto the B cell surface with a stoichiometry of one Ig molecule to one Igα/Igβ heterodimer. Interestingly, during the course of antigen stimulation FRET efficiency between BCR subunits peaked after an initial rise and declined thereafter but never fell below the level that was recorded for unstimulated B cells. Hence, the cytoplasmic domains of the BCR undergo clustering early after antigen stimulation but then spread apart. This transition from a ''closed'' to an ''open'' conformation hampers FRET efficiency. Finally, the sustained basal level of FRET shows that the ligand-binding Ig component and the Igα/Igβ signaling dimer form a stable unit. No evidence was obtained for BCR oligomers in the absence of antigen. The open conformation of the activated BCR was however dependent on ITAM phosphorylation by the continued activity of Lyn but not Syk and occurred within the lipid rafts. Therefore, conformational opening of cytoplasmic BCR domains cannot be the primary trigger of BCR activation. Rather, the open conformation reflects a high energy state in which the BCR is easily susceptible for downstream effector proteins. It is likely that the structural tension of the open BCR conformation is released after its dephosphorylation by PTP, further supporting the idea that a dynamic equilibrium between PTK and PTP activities controls BCR signaling (Wienands *et al.*, 1996).

3.6. Shifting the phosphorylation equilibrium by antigen-mediated substrate provision

Despite their differences, the concepts described above have a common conclusion. Antigen-induced activation of the BCR and concomitant coupling to signal transducers is a highly dynamic process in terms of changes in protein structure and protein motility. Structural plasticity and subcelluar translocation is observed for the BCR and its immediate early effector proteins as well. In fact, circular dichroism (CD) analysis showed

that the isolated cytosolic tail segments of Igα and Igβ are unstructured in solution (Sigalov *et al.*, 2004), suggesting that an ordered domain architecture is fixed only upon binding of the transducer kinase. Thus, the structural impact of the phospho-ITAM/SH2 domain interaction probably works in both directions. It stabilizes the tertiary structure of the activated Src or Syk enzyme and the secondary structure of the Igα/Igβ heterodimer. Even in resting B-cells, the closed, inactive form of PTK exists in equilibrium with the open, active enzyme (Fig. 5.1A). Consequently, a minor pool of active PTK may phosphorylate an individual Igα/Igβ subunit in the absence of antigen stimulation but the reaction chain cannot efficiently continue provided the BCR is not oligomerized on resting cells. Moreover, PTK action is opposed by PTP and the high energy state of the phosphorylated BCR appears to be unstable. Following extracellular cross-linking, the local concentration of ITAMs underneath the plasma membrane increases, which provides a stabilized active kinase with sufficient substrate to continue with phosphorylation, including that of the non-ITAM tyrosine residue in Igα (Fig. 5.1B). This results in recruitment of additional kinases and rapid feedback amplification of PTK activation (Fig. 5.1C), possibly also by ROS production and PTP inhibtion. In this model the antigen acts as extracellular adaptor molecule, which facilitates the interaction of intracellular PTK and their ITAM substrates by abrogating their spatial separation. The net result is activated Syk that is locked at the BCR in a defined membrane topology as the tandem SH2 domains bind the doubly-phosphorylated ITAM in fixed head-to-tail orientation (Futterer *et al.*, 1998).

4. SLP-65: A GATEWAY TO INTRACELLULAR SIGNALING PATHWAYS

Elucidation of the next signaling step was independently accomplished by two groups using two different approaches. Given the prominent role of the adaptor protein Grb2 for signal transduction from many cell surface receptors, the group of Andrew Chan set out to identify Grb2-associated phosphoproteins in activated B cells. Our group monitored the BCR-induced phosphorylation kinetics and early PTK substrate proteins were purified and sequenced. Both approaches led to the identification of the SH2-domain-containing leukocyte adaptor protein of 65 kDa (SLP-65) (Wienands *et al.*, 1998), which is alternatively called BLNK (Fu *et al.*, 1998) or BASH (Goitsuka *et al.*, 1998). SLP-65 is a multidomain adaptor encompassing an N-terminal leucine zipper, several proline-rich recognition motifs for SH3 domains, five consensus tyrosine phosphorylation sites, and a C-terminal SH2 domain. Syk-mediated phosphorylation of SLP-65 turned out to be an indispensible step during antigen-induced

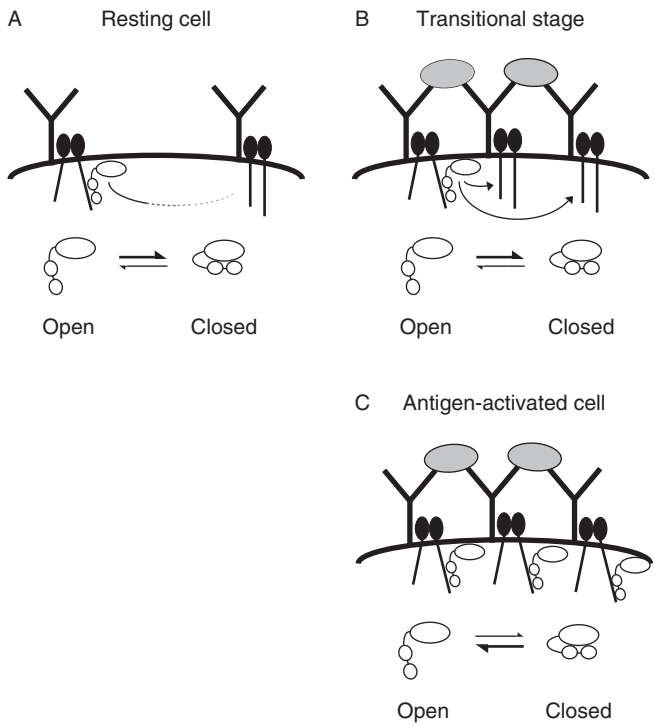

FIGURE 5.1 Antigen binding shifts the equilibrium between closed and open conformations of the BCR and its transducer kinases. (A) In the absence of antigen, BCR-regulated PTKs are in a dynamic equilibrium of closed (inactive) and open (active) conformations. The majority of PTK molecules are in the closed, inactive state but a small pool of active molecules present at a given time point will occasionally phosphorylate and bind to BCR-ITAMs. However, without antigen-induced BCR clustering, the distance to neighboring BCR complexes is too far for a BCR-bound kinase to phosphorylate additional ITAMs. Moreover, the open, active state of the phosphorylated BCR is unstable probably as a result of continued dephosphorylation by phosphatases (not shown). (B) BCR clustering by antigen leads to a high local concentration of ITAMs underneath the plasma membrane, allowing the phosphorylation chain to continue to neighboring ITAMs. (C) Phosphorylation of a large number of ITAMs recruits additional kinases, which are stabilized in their open conformation by binding to phospho-ITAMs. This causes a positive feedback loop which greatly amplifies the BCR signal passing the threshold for a cellular activation signal.

B cell activation (Baba *et al.*, 2001; Chiu *et al.*, 2002a; Ishiai *et al.*, 1999a,b; Su *et al.*, 1999). How exactly Syk ''finds'' its proximal substrate protein is not completely understood but Syk and SLP-65 may exist in a preformed complex prior to BCR ligation (Abudula *et al.*, 2007; Wienands *et al.*, 1996; Zhang *et al.*, 1998). Binding of the SLP-65 SH2 domain to the non-ITAM phosphotyrosine of Igα facilitates SLP-65 phosphorylation and is

responsible for BCR signal amplification in response to T-cell-independent antigens (see earlier).

Once phosphorylated, SLP-65 functions as a master switch for the activation of phospholipase C (PLC)-γ2 whose enzymatic products regulate several of the most prominent signal chains in B cells (Fu *et al.*, 1998; Ishiai *et al.*, 1999a,b; Wollscheid *et al.*, 1999). Activated PLC-γ2 hydrolyzes the membrane lipid phosphatidyl-inositol-4,5-bisphosphate PIP_2 into membrane-bound diacylglycerol (DAG) and soluble inositol-1,4,5-trisphosphate (IP_3) (Kurosaki *et al.*, 2000). DAG recruits and triggers protein kinase C (PKC)-β and Ras guanine nucleotide release protein 3 (RasGRP3), which couple BCR activation to the canonical NF-κB and Ras pathway, respectively (Oh-hora *et al.*, 2003; Saijo *et al.*, 2002; Su *et al.*, 2002). IP_3 production initiates elevation of the cytosolic Ca^{2+} concentration by binding to ubiquitously expressed ligand-gated Ca^{2+} channels of the endoplasmic reticulum (ER) (Kurosaki *et al.*, 2000). Emptying of the ER Ca^{2+} store senses a second phase of Ca^{2+} mobilization from the extracellular space across ion channels of the plasma membrane (Berridge *et al.*, 2000; Engelke *et al.*, 2007). Taken together, the Syk/SLP-65 module is the BCR-specific link to evolutionary well-tried signaling cascades, that is, gene regulation through members of the NF-κB family of transcription factors, cell cycle control and changes in cell morphology downstream of Ras activation, and the plethora of Ca^{2+}-sensitive cell processes. This explains the key role of SLP-65 for B cell activation as manifested by the severe B cell deficits observed in SLP-65-negative mouse mutants (Hayashi *et al.*, 2000; Jumaa *et al.*, 1999; Pappu *et al.*, 1999) and human patients (Minegishi *et al.*, 1999).

5. DYNAMIC REGULATION OF THE BCR-TRIGGERED Ca^{2+} RESPONSE

5.1. SLP-65-controlled activation and navigation of PLC-γ2

Two signaling features of SLP-65 depicted in Fig. 5.2 are of particular importance for the initiation of Ca^{2+} mobilization. First, tyrosine-phosphorylated SLP-65 provides docking sites for the SH2 domains of PLC-γ2 (Fu *et al.*, 1998; Ishiai *et al.*, 1999b) and its upstream activator Bruton's tyrosine kinase (Btk) (Hashimoto *et al.*, 1999; Su *et al.*, 1999). Simultaneous recruitment of both enzymes must occur in ''cis,'' that is, to a given SLP-65 molecule because only trimolecular complex formation enables Btk to phosphorylate and thereby activate its target PLC-γ2 (Chiu *et al.*, 2002a). Second, SLP-65 targets the Ca^{2+} initiation complex to the inner leaflet of the plasma membrane in order to provide cytosolic PLC-γ2 with access to its substrate PIP_2, which represents a minor membrane

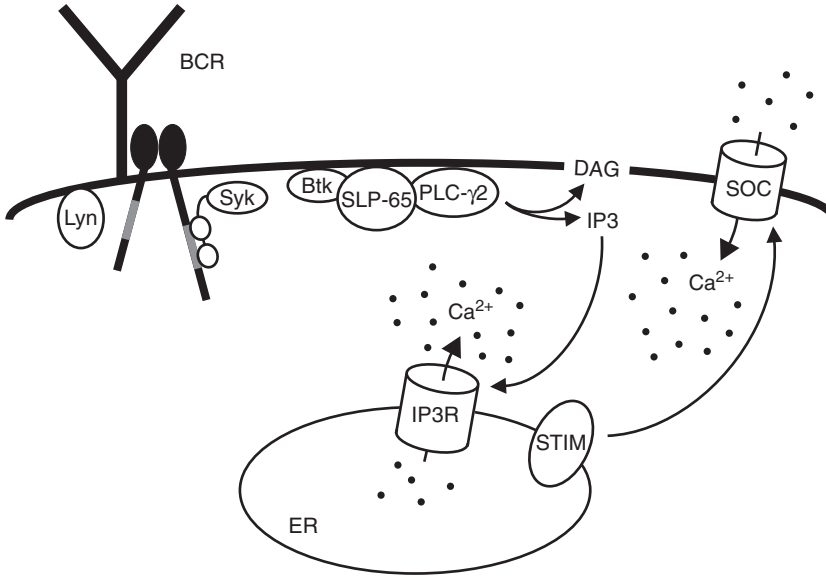

FIGURE 5.2 Mechanism of biphased Ca^{2+} mobilization. BCR-bound, active Syk phosphorylates its proximal substrate SLP-65, which in turn nucleates assembly of the Ca^{2+} initiation complex comprising Btk and PLC-γ2 at the plasma membrane. Btk-activated PLC-γ2 hydrolyzes phosphatidyl-inositol-4,5-bisphosphate (not shown) generating the two messenger molecules diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Whereas the membrane resident DAG recruits C1 domain-containing proteins, IP_3 activates Ca^{2+} channels in the membrane of the ER known as IP_3 receptors (IP_3R) to release Ca^{2+} from internal stores. Ca^{2+} depletion of the ER is detected by the Ca^{2+} sensor STIM1, which couples to opening of Ca^{2+} -permeable ion channels in the plasma membrane (SOCs), resulting in a sustained influx of Ca^{2+} across the plasma membrane.

phospholipid. The molecular details of the localization process are poorly understood but appear to involve constitutive and stimulation-dependent targeting elements. A basic level of permanent membrane tethering is provided by the leucine zipper of SLP-65, which resembles a classical protein–protein interaction domain (Kohler *et al.*, 2005). Interestingly, it even operates upon ectopic expression of SLP-65 in non-lymphoid cells, but the exact membrane anchor remains elusive. The majority of SLP-65 molecules in resting B-cells resides in the cytosol and relocalizes together with its associated binding partners upon BCR ligation to the plasma membrane in an SH2-domain-dependent manner (Abudula *et al.*, 2007). The known ligands for the SH2 domain of SLP-65 are phosphorylated Igα and the hematopoietic serine/threonine kinase HPK1 (Sauer *et al.*, 2001; Tsuji *et al.*, 2001). As discussed above the Igα–SLP-65 interaction is implicated in the amplification rather than the onset of BCR signaling (Patterson *et al.*, 2005). The exact role of HPK1 in B cells is less

clear but appears to be dispensable for BCR-triggered Ca^{2+} flux. Hence, the phosphoprotein ligand for the SH2 domain of SLP-65, which confers reversible membrane anchoring for the Ca^{2+} initiation complex, remains to be identified. Nonetheless, the available data strongly suggest bifurcated membrane targeting for SLP-65. The N-terminal leucine zipper promotes a more general association with plasma membrane residents, while the C-terminal SH2 domain may direct the SLP-65-assembled Ca^{2+} initiation complex into the specialized signaling-competent microdomains such as lipid rafts (Dykstra *et al.*, 2001). Btk and PLC-γ2 contribute to these processes by virtue of their pleckstrin homology (PH) domains (Bolland *et al.*, 1998; Falasca *et al.*, 1998), which specifically bind to differentially phosphorylated derivatives of membrane phosphoinositides (DiNitto and Lambright, 2006). Noteworthy, bifurcated membrane targeting of effector proteins appears to be a common theme in many signaling pathways (McLaughlin *et al.*, 2002). This further demonstrates the importance of precise scaffolding processes at distinct subcellular locations for proper regulation of signaling events.

5.2. Capacitative Ca^{2+} influx by store-operated ion channels

In B cells, an evolutionary highly conserved mechanism monitors the Ca^{2+} concentration in the ER lumen and couples Ca^{2+} loss to opening of store-operated Ca^{2+} channels (SOCs) in the plasma membrane, which produce a distinct Ca^{2+}-release-activated current (I_{CRAC}). The capacitative Ca^{2+} entry is based on a concerted action of stromal interaction molecule 1 (STIM1) (Liou *et al.*, 2005; Roos *et al.*, 2005; Zhang *et al.*, 2005) and ORAI (deduced from the ''keepers of gates of heaven'' in Greek mythology) (Feske *et al.*, 2006; Prakriya *et al.*, 2006). ORAI is also known as CRAC modulator 1 (CRACM1) (Vig *et al.*, 2006b). The basic machinery illustrated in Fig. 5.2 operates already in cells of *Drosophila melanogaster*.

STIM1 represents a type I transmembrane protein that is diffusely dispersed in the ER membrane of resting cells. The ER-luminal part comprises one α-helical EF hand, which is a classical Ca^{2+} binding device and a protein–protein interaction domain called sterile α-motif (SAM), first described for yeast proteins. The cytoplasmic C-terminus of STIM encompasses a coiled-coil domain, several serine/threonine phosphorylation sites and a lysine-rich helix-turn-helix motif. The importance of STIM1 for BCR-induced Ca^{2+} entry was shown by gene targeting in DT40 B cells (Baba *et al.*, 2006). In the absence of STIM1 expression, the long-lasting Ca^{2+} entry across the plasma membrane with electrophysiological properties of I_{CRAC} was almost completely abrogated. Reconstitution experiments showed that STIM1 employs the EF hand to measure the Ca^{2+} load of the ER and requires the SAM, coiled-coil, and the serine/threonine-rich regions for triggering CRAC channels. Confocal imaging techniques revealed that

STIM1 activation is associated with STIM1 oligomerization. STIM1 aggregates redistribute towards the plasma membrane, where they meet and activate ORAI (Huang *et al.*, 2006; Luik *et al.*, 2006; Xu *et al.*, 2006; Zhang *et al.*, 2005). It remains to be seen whether or not STIM1 remains just below the plasma membrane within specialized ER subcompartments or is inserted into it.

Most of our knowledge on the four transmembrane-spanning protein ORAI is deduced from studies with non-B cells, including insect cells, fibroblasts, and T cells. ORAI deficiency accounts for a hereditary form of severe combined immunodeficiency (SCID) in humans, which is caused by the inability of T cells to flux extracellular Ca^{2+} upon antigen receptor ligation (Feske *et al.*, 2005, 2006). Expression of differently tagged versions of ORAI in HEK 293 cells followed by confocal microscopy and reciprocal co-immunoprecipitation studies showed that ORAI homo-oligomerizes (Vig *et al.*, 2006a) and ORAI clusters associate with STIM1 (Luik *et al.*, 2006; Xu *et al.*, 2006). Mutational analysis identified critical glutamate and aspartate residues in ORAI that determine ion selectivity and may contribute to ion pore formation (Gwack *et al.*, 2007; Prakriya *et al.*, 2006; Yeromin *et al.*, 2006). These features resemble those described for the well-characterized voltage-gated Ca^{2+} channels and are consistent with the idea that ORAI is itself the CRAC channel (Feske, 2007).

5.3. Non-I_{CRAC} channels contribute to sustained Ca^{2+} flux in B cells

The selective T-cell defect in ORAI-deficient human patients suggests the existence of additional Ca^{2+} influx pathways in other cell types, including B cells. Indeed, second messenger-operated Ca^{2+} channels, so-called SMOCs, contribute to Ca^{2+} influx following BCR activation (Liu *et al.*, 2005; van Rossum *et al.*, 2004; Vazquez *et al.*, 2002). Likely SMOC candidates are members of the large family of transient receptor potential (TRP) proteins initially described in *Drosophila melanogaster* (Ambudkar *et al.*, 2006; Minke, 2006). They all possess six transmembrane helices with the N- and C-terminus facing the cytosol. B cells express various isoforms of the TRP-C subfamily. DT40 B cell mutants that lack individual or a combination of TRP-C isoforms provided direct evidence for a contribution of some but not all TRP-C to IP_3-independent Ca^{2+} entry (Lievremont *et al.*, 2005; Vazquez *et al.*, 2006; Venkatachalam *et al.*, 2003). Indeed, some TRP-C members are directly activated by the PLC-γ2 product DAG (Hofmann *et al.*, 1999). The analysis of TRP-C and the elucidation of their mode of action during Ca^{2+} mobilization in B cells is hampered by the fact that different TRP-C family members can form hetero-oligomers (Ambudkar *et al.*, 2006), and moreover, some TRP-C isoforms are suspected to play a role in store-operated Ca^{2+} entry via STIM1 and ORAI (Liao *et al.*, 2007;

Smyth *et al.*, 2006). Finally, IP_3 receptors are not exclusively ER-localized but deposited on the plasma membrane of DT40 B cells, where they contribute to Ca^{2+} influx and define another kind of SMOC in B cells (Dellis *et al.*, 2006).

5.4. Fine-tuning of the biphasic Ca^{2+} response

Antigen-induced Ca^{2+} mobilization is not an all-or-nothing response but quantitatively adjusted in individual primary B cell subsets (Engelke *et al.*, 2007; Hoek *et al.*, 2006). Immature splenic B cells of the transitional type 1 (T1), which undergo apoptosis upon antigen encounter, exhibit robust intracellular Ca^{2+} release but only poor extracellular Ca^{2+} influx. As the cells progress to the T2 and follicular B cell stage the extracellular Ca^{2+} fluxes increase dramatically. Marginal zone B cells, which dominate the early antibody response owing to their high antigen sensitivity, mount strongly enhanced intra- and extracellular Ca^{2+} fluxes. Further enhancement of Ca^{2+} mobilization by class-switched memory B cells appears to be requisite for improved secondary immune responses (Horikawa *et al.*, 2007; Waisman *et al.*, 2007). Finally, an unusual high basal level of intracellular Ca^{2+} concentrations is tightly associated with silencing of autoreactive B cells and maintaining their anergic state (Gauld *et al.*, 2005; Merrell *et al.*, 2006). In summary, developing and mature B cell populations are characterized by distinct Ca^{2+} signature profiles, which are likely to play a critical role in the different biological responses of these cells to antigen encounter.

B cells utilize several set screws for shaping the Ca^{2+} profile at multiple levels. BCR-integral elements involve the non-ITAM tyrosine of Igα and the conserved tyrosine residue in IgG tail segments, which upon phosphorylation recruit positive-regulatory elements for signal amplification (see earlier). Down modulation of Ca^{2+} fluxing can be achieved by including inhibitory elements into BCR-proximal and -distal signal circuits. As described above, SHP-1 dephosphorylates early signal effector proteins, including Igα, Igβ, SLP-65, and PLC-γ2 (Adachi *et al.*, 2000, 2001; Nitschke and Tsubata, 2004; Otipoby *et al.*, 2001; Poe *et al.*, 2000; Veillette *et al.*, 2002). The SH2-domain-containing inositol 5′ lipid phosphatase (SHIP) opposes PH-domain-controlled membrane localization, for example, that of Btk, PLC-γ2 (Brauweiler *et al.*, 2000; Helgason *et al.*, 1998; Liu *et al.*, 1998; Okada *et al.*, 1998; Ono *et al.*, 1997), or the Ca^{2+} -promoting B cell adapter molecule of 32 kDa (Bam32) (Marshall *et al.*, 2000; Niiro *et al.*, 2002, 2004). Hence, enzymatic attenuation of Ca^{2+} elevation targets the two basic signaling principles discussed earlier, that is, the physical interaction of effector molecules and their recruitment as part of a larger signalosome to the plasma membrane.

A more indirect mechanism to limit Ca^{2+} mobilization involves the adaptor protein downstream of kinase 3 (Dok-3) (Ng *et al.*, 2007; Stork *et al.*, 2007), which is specifically expressed in hematopoietic lineage cells

(Cong *et al.*, 1999; Lemay *et al.*, 2000). Dok-3 is constitutively tethered to the plasma membrane by virtue of its N-terminal PH domain. Protein–protein interaction motifs of Dok-3 are one phosphotyrosine binding (PTB) domain and three tyrosine phosphorylation sites. In the absence of Dok-3 expression, DT40 B cell mutants mount a sustained biphasic Ca^{2+} response. By contrast, wild-type or Dok-3-reconstituted cells show reduced Ca^{2+} release from the ER and almost no Ca^{2+} influx from the extracellular medium. Mutational analysis revealed that following phosphorylation by Lyn Dok-3 recruits the ubiquitously expressed adaptor protein Grb2, which consists of a central SH2 domain flanked on either side by one SH3 domain. In association with Dok-3, the C-terminal SH3 domain of Grb2 suppresses Btk-mediated PLC-γ2 phosphorylation either by allosteric inhibition of Btk or its incorporation into the SLP-65-assembled Ca^{2+} initiation complex (Stork *et al.*, 2004, 2007). This negative feedback loop may play a prominent role in limiting the Ca^{2+} response of immature B cells like DT40 (see earlier). However, mature B cells need to sequester Grb2 away from its inhibitory binding partner Dok-3 in order to mount a full biphasic Ca^{2+} response for activation. Such a function is provided by the phosphorylated non-T-cell activation linker (NTAL), which is a transmembrane adaptor whose expression is upregulated when B cells mature (Stork *et al.*, 2004; Wang *et al.*, 2005). In summary and similar to phosphatase-mediated Ca^{2+} flux control, also the Ca^{2+} inhibitor Grb2 interferes with the assembly and/or function of signaling complexes. Moreover the phosphorylated adaptor proteins Dok-3 and NTAL provide an additional level of regulation by offering two functionally different ''membrane zip codes'' for cytosolic Grb2. Almost certainly additional and redundant destinations for Grb2 exist at the plasma membrane as indicated by the reported binding of Grb2 to inhibitory BCR co-receptors (Otipoby *et al.*, 2001; Poe *et al.*, 2000; Yohannan *et al.*, 1999) and the mild immunological phenotype of NTAL-deficient mouse mutants (Wang *et al.*, 2005). Although dynamic Ca^{2+} elevation seems to be based on a complicated signaling network, the interplay of B cell-specific and ubiquitously expressed effector proteins is regulated by a limited number of mechanisms aiming at the precise positioning of signaling complexes.

6. MECHANISM AND FUNCTION OF BCR-INDUCED RAS ACTIVATION

The small guanosine-nucleotide-binding (G) proteins of the Ras family provide another paradigm for the concept that subcelluar navigation of signaling proteins controls fundamental cellular processes, including neoplastic transformation (Mitin *et al.*, 2005). Initially identified as

oncoproteins inducing rat sarcomas or neuroblastomas in human, the three isoforms H-, K-, and N-Ras turned out to act as binary switched adaptor proteins that recruit effector proteins to distinct membrane compartments.

6.1. Structure and subcellular compartmentalization of RAS proteins

Ras proteins have a relative molecular mass of about 21 kDa. They all share a highly conserved N-terminal region comprising the core G domain and two short amino acid stretches that account for structural changes between the GDP- and GTP-bound states (Vetter and Wittinghofer, 2001). A C-terminal CAAX amino acid motif is posttranslationally modified by farnesylation and essential but not sufficient for constitutive membrane localization of Ras proteins. H- and N-Ras require additional palmitoylation and K-Ras depends on a unique polybasic region supposed to bind membrane phosphoinositides. Eventually the different targeting devices localize each isoform to distinct membrane compartments (Fig. 5.3A). K-Ras is exclusively located at the plasma membrane. H- and N-Ras shuttle with distinct frequencies between the plasma membrane and the Golgi apparatus by virtue of an isoform-specific de- and re-acylation cycle (Apolloni *et al.*, 2000; Choy *et al.*, 1999; Rocks *et al.*, 2005). There is now growing evidence that the pool of Golgi-associated Ras proteins represents a separate signaling entity (Chiu *et al.*, 2002b; Perez de Castro *et al.*, 2004). Likewise, isoform-specific microlocalization at the plasma membrane is associated with distinct functional properties of Ras proteins despite their lateral mobility. H-Ras is equally distributed in the plasma membrane when bound to GDP but sequestered from lipid rafts when bound to GTP (Prior *et al.*, 2001). K-Ras is predominantly located in the nonlipid raft fractions irrespective of its activation state. Activated H-Ras does however not co-cluster with K-Ras in different cell types, including B cells (Niv *et al.*, 2002; Prior *et al.*, 2003). Consistent with the isoform-specific localization patterns, disintegration of lipid rafts by cyclodextrin treatment of the cells abolishes H-Ras- but not K-Ras-mediated signaling (Carozzi *et al.*, 2002; Roy *et al.*, 1999). These findings demonstrate that proper signaling by Ras isoforms is determined and distinguished by accurate subcellular trafficking and distinct segregation within the plasma membrane. Note that this situation is reminiscent to that of Grb2 during the Ca^{2+} response, where membrane microlocalization to either Dok-3 or NTAL is responsible for negative or positive signaling, respectively.

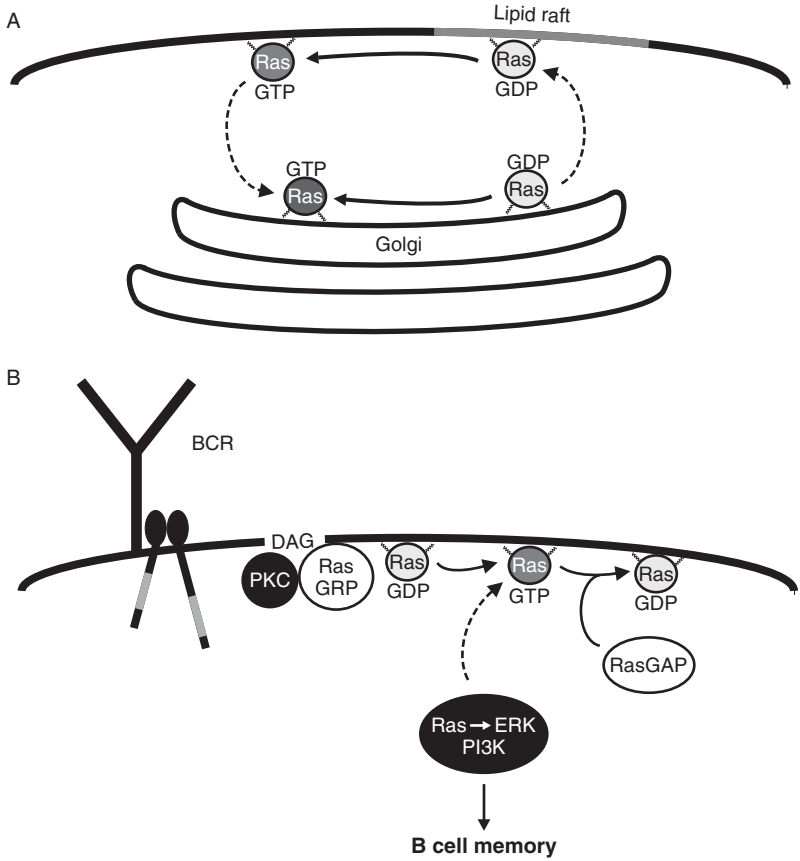

FIGURE 5.3 Navigation and activation of Ras in B cells. (A) B cells express all of the Ras isoforms, H- (Harvey-), K- (Kirsten-), and N- (Neuroblastoma-) Ras, which shuttle between the raft and non-raft fraction of the plasma membrane and the Golgi compartment in an isoform-specific and stimulation-dependent manner and, moreover, regulated by a de- and re-acylation cycle (see text for details). (B) Engagement of the BCR leads to production of DAG and subsequent recruitment of protein kinase C (PKC) and Ras guanosine nucleotide-releasing protein (RasGRP). RasGRP catalyzes the release of GDP, which is then replaced by cellularly abundant GTP. This causes a conformational switch of Ras and allows for membrane recruitment and activation of effector proteins such as Raf (to trigger ERK) and phosphatidyl-inositol-3-kinase (PI3K) to eventually promote establishment and/or maintenance of class-switched memory B cells.

6.2. Linking BCR ligation to RAS activation

Ras signaling is triggered by substitution of GDP for GTP and terminated by hydrolysis of GTP to re-establish the inactive Ras conformation. GDP release cannot be accomplished by Ras itself but requires guanosine

nucleotide exchange factors (GEFs) belonging to three families, Son-of-Sevenless (SOS), Ras guanosine nucleotide-releasing factors (RasGRFs), and Ras guanosine nucleotide-releasing proteins (RasGRPs) (Cherfils and Chardin, 1999). The signature structure of all RasGEFs is a CDC25 homology catalytic domain (Mitin *et al.*, 2005). The GTP-bound state of Ras is not stable owing to a weak intrinsic GTPase activity, which is, accelerated by many orders of magnitude through a variety of GTPase activating proteins (GAPs) (Bernards and Settleman, 2004). Hence, Ras signaling requires the balanced action of positive- and negative-regulatory accessory enzymes, which are precisely recruited from the cytosol into the vicinity of membrane-bound Ras.

All three Ras isoforms are expressed in B lymphocytes and employed for antigen-mediated activation (Ehrhardt *et al.*, 2004). The mechanism that connects the BCR to Ras remained ambiguous for a long period. Several possibilities were discussed based on data obtained for other receptor systems. Most prominently, tyrosine kinase receptors activate Ras by Grb2-mediated membrane recruitment of SOS. However, gene targeting of *SOS* alleles in DT40 B cells strongly compromises receptor tyrosine-kinase- but not BCR-induced Ras activation (Oh-hora *et al.*, 2003). It is now clear that following BCR ligation, the DAG product of PLC-γ2 triggers membrane recruitment of RasGRP family members by virtue of their C1 domain (Beaulieu *et al.*, 2007; Oh-hora *et al.*, 2003; Tognon *et al.*, 1998). Full activation of GEF activity requires additional phosphorylation on threonine residues in a PKC-dependent manner (Aiba *et al.*, 2004; Teixeira *et al.*, 2003). B-cells express the isoforms RasGRP1 and RasGRP3. A prominent role for RasGRP3 is shown by targeted gene disruption in DT40 cells (Oh-hora *et al.*, 2003) and in mice (Coughlin *et al.*, 2005). Ablation of RasGRP3 expression almost abrogates BCR-regulated Ras signaling. The role of RasGRP1 is less well understood. RasGRP1 is recruited to the plasma membrane following BCR engagement (Beaulieu *et al.*, 2007; Caloca *et al.*, 2003) and overexpression of RasGRP1 augments Ras signaling (Beaulieu *et al.*, 2007). Yet, BCR-induced Ras activation appears to be normal in RasGRP1-deficient DT40 B cells (Oh-hora *et al.*, 2003). Hence RasGRP3 seems to be the dominant BCR effector for Ras activation.

Little is known about BCR-mediated regulation of RasGAPs. A member of the membrane-bound family of Dok adaptor proteins, Dok-1, is tyrosine-phosphorylated following BCR ligation and interacts with the SH2 domain of p120RasGAP (Gold *et al.*, 1993; Yamanashi and Baltimore, 1997). Targeted disruption of *dok-1* in mice increases Ras signaling (Yamanashi *et al.*, 2000). Hence, Dok-1 phosphorylation may limit BCR-induced Ras activation by recruiting RasGAP into the membrane microenvironment where Ras resides.

Collectively the available data as summarized in Fig. 5.3B show that BCR signaling to the Ras pathway branches at the level of phospholipid

hydrolysis. The membrane-anchored messenger DAG closes the signaling gap to membrane-bound RAS proteins by recruiting the positive effector RasGRP3 from the cytosol. Following GDP/GTP exchange, Ras undergoes a conformational switch that exposes an interface for binding and activation of downstream signaling partners. Hence Ras activation is just another, yet complex, example for inducible assembly of protein complexes.

6.3. RAS target cascades

The conformation of GTP-bound Ras exhibits affinity for several downstream effector proteins. Among them are activators of other small G proteins such as Ral and Rac that couple to vesicular trafficking and actin reorganization, respectively (Shields *et al.*, 2000). Another hallmark of Ras function is the control of cell survival signals through phosphatidylinositol-3 kinase family members (PI3K) (Shields *et al.*, 2000). The by far best characterized Ras-controlled enzymatic cascade is activation of extracellular signal-regulated kinase (ERK), which together with p38 and C-Jun N-terminal kinase (JNK) build the family of mitogen-activated protein kinases (MAPK) (Gaestel, 2006). B cells express all three MAPKs and their inducible activation requires proper function of the SLP-65/PLC-γ2 signaling module (Grabbe and Wienands, 2006; Hashimoto *et al.*, 1998; Ishiai *et al.*, 1999a; Johmura *et al.*, 2003).

The crucial step for ERK activation downstream of Ras-GTP is recruitment of one of three isoforms of the serine/threonine kinase Raf (Rabbit fibrosarcoma). All Raf isoforms share three conserved regions (CR) (Kolch, 2000; Wellbrock *et al.*, 2004). The N-terminal CR1 encompasses the Ras-binding domain and a cysteine-rich domain, which are both required for Ras-induced conformational changes culminating in full Raf activation (Bondeva *et al.*, 2002; Hu *et al.*, 1997; Luo *et al.*, 1997; Roy *et al.*, 1997). CR2 is rich in serine and threonine residues, which are potential targets for Raf-regulating kinases, whereas CR3 contains the catalytic domain (Chong *et al.*, 2001). Also phosphorylation on tyrosine residues as well as allosteric regulation by scaffolding proteins contribute to Raf activation (Kolch, 2005; Wellbrock *et al.*, 2004). The two B cell isoforms, Raf-1 and B-Raf, have partially redundant functions for BCR-induced phosphorylation of the main Raf substrate, namely the MAPK/ERK-activating kinase (MEK) (Brummer *et al.*, 2002). MEK in turn phosphorylates and thereby activates ERK. More than 70 ERK substrates, including cytosolic proteins and transcription factors, have been reported in different cell types (Murphy and Blenis, 2006). Moreover, Raf kinases appear to execute Erk-independent functions as the lethal phenotype of C-Raf-deficient mice is rescued by

reconstitution with a kinase-inactive variant (Huser *et al.*, 2001). Thus Ras activation is a key step for signal diversification.

6.4. B cell responses to RAS activation

Several transgenic mouse models provide evidence for the importance of Ras signaling during B cell development. For example, B-cell-specific expression of a dominant-negative H-Ras variant perturbs B cell development prior to formation of the pre-BCR (Iritani *et al.*, 1997). The block is partially relieved upon expression of a membrane-targeted Raf chimera (Iritani *et al.*, 1997). Similarly, expression of dominant-negative H-Ras in pre-B cells reduces their life span and hinders progression to the late pre-B and immature B cell stages (Nagaoka *et al.*, 2000). A constitutively active form of H-Ras can bypass the checkpoint control of the pre-BCR in B cells that are deficient for expression of recombination-activating genes (RAGs) (Shaw *et al.*, 1999a, 1999b). Since the signaling mechanics of the pre-BCR is similar to that of the BCR on mature B cells, the data strongly suggest a role for Ras in antigen-mediated B cell activation. Indeed, early biochemical studies have shown that BCR ligation triggers the Ras cascade (Harwood and Cambier, 1993) and its pharmacological inhibition impairs B cell proliferation *in vivo* (Richards *et al.*, 2001).

Recent studies demonstrated a critical role of BCR-regulated Ras activation for the establishment of memory B cells in response to T-cell-dependent antigens. The IgG1 level in the serum of RasGRP3-deficient mice is strikingly reduced compared with wild-type controls (Coughlin *et al.*, 2005). B cells from RasGRP1/3 double-deficient mice completely fail to trigger the Ras pathway after BCR ligation and exhibit markedly reduced IL4- and CD40-mediated cell divisions (Coughlin *et al.*, 2005, 2006). More details were revealed from a transgenic mouse model. In the presence of a dominant-inhibitory H-Ras variant, the generation of IgG-positive germinal center B cells is normal but the recruitment of high-affinity B cells into the memory compartment is strongly impaired. Also the *in vitro* antibody response of IgG-positive memory B cells is compromised upon MEK inhibition (Takahashi *et al.*, 2005). Likewise, ERK is mandatory for efficient IgG production (Sanjo *et al.*, 2007). Overexpression of the anti-apoptotic Bcl-2 protein restores antibody production by Ras- or ERK-defective class-switched memory B cells but not naïve B cells carrying an IgM-BCR (Sanjo *et al.*, 2007; Takahashi *et al.*, 2005). These data suggest the interesting possibility that triggering the Ras/ERK cascade in order to promote cell survival is selectively confined to the IgG-BCR on memory cells (Fig. 5.3B). It remains to be seen whether this is a matter of increased BCR signal strength or qualitative signaling differences brought about by the cytoplasmic tail segment of membrane-bound IgG.

7. CONCLUDING REMARKS

The laborious enumeration of signaling molecules is not always appreciated because of their complex networking in a cell- and receptor-type-specific manner. However, signaling homeostasis has common rules that are also applicable to the BCR and its effector cascades. Two examples are the evolutionary well-tried mobilization of the Ca^{2+} second messenger and activation of the Ras/Raf/ERK pathway. Both processes rely on enzymatically inert adaptor proteins for precise subcellular navigation of individual signaling partners and their physical interaction through common interaction domains. Adaptor proteins can also act as allosteric regulators of catalytic activity. However, even the more specialized components such as the Syk/SLP-65 module in B cells utilize structural building blocks found in many other proteins. This should be considered when designing pharmaceutical agents for clinical trials.

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