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30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
32 Jamestown Road, London, NW1 7BY, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 2009

ISBN: 978-0-12-374801-0
ISSN: 0065-2776 (series)

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Printed and bound in USA
09 10 11 12 10 9 8 7 6 5 4 3 2 1

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CHAPTER 1

Antigen Presentation by CD1: Lipids, T Cells, and NKT Cells in Microbial Immunity

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Advances in Immunology, Volume 102
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)01201-2

Abstract

The discovery of molecules capable of presenting lipid antigens, the CD1 family, and of the T cells that recognize them, has opened a new dimensionin our understanding of cell-mediated immunity against infection. Like MHC Class I molecules, CD1 isoforms (CD1a, b, c and d) are assembled in the ER and sent to the cell surface. However, in contrast to MHC molecules, CD1 complexes are then re-internalized into specific endocytic compartments where they can bind lipid antigens. These include a broad scope of both self and foreign molecules that range from simple fatty acids or phospholipids, to more complex glycolipids, isoprenoids, mycolates and lipopeptides. Lipid-loaded CD1 molecules are then delivered to the cell surface and can be surveyed by CD1-restricted T cells expressing $\alpha\beta$ or $\gamma\delta$ T Cell Receptors (TCR). It has become clear that T cell-mediated lipid antigen recognition plays an important role in detection and clearance of pathogens. CD1a, b and c-restricted T cells have been found to recognize a number of lipid antigens from *M. tuberculosis*. CD1d-restricted T cells are the only CD1-restricted T cell subset present in mice, which lack the genes encoding CD1a, b and c. Evidence from experiments in CD1d-restricted T cell-deficient mice indicates that these cells play an important role in the immune response against awide range of pathogens including several bacteria, viruses and parasites. One subset of CD1d-restricted T cells in particular, invariant Natural Killer T (iNKT) cells, has been extensively studied. iNKT cells are characterized by the expression of a semi-invariant TCR composed of a strictly conserved α chain paired with a limited repertoire of β chains. During infection, iNKT cells are rapidly elicited. Activated iNKT cells can produce a vast array of cytokines that profoundly affect both the innate and the adaptive arms of the immune response. In this review, we describe the pathways and mechanisms of lipid antigen binding and presentation by CD1 in detail, as well as the diverse roles played by CD1-restricted T cells in the context of microbial infection.

1. INTRODUCTION

T cell function is the central basis of adaptive immunity directly providing cytotoxic T cells (CTL)-mediated killing of infected cells and cytokines and surface receptors that activate macrophages, recruit other leukocytes and regulate B cell production of antibody. Prior to the discovery of the CD1 system of antigen presentation, it was assumed that all T cell reactivity was directed against peptides presented by major histocompatibility complex (MHC) Class I and II molecules. Now, CD1-restricted T cells are known to be capable of virtually all of the same effector functions of MHC-restricted T cells as well as unique innate-like functions not found among MHC-restricted T cells. Tremendous excitement has surrounded the discovery that the specificity of invariant natural killer T (iNKT) cells is directed against CD1d-presented lipid antigens. These T cells display a distinctive canonical T cell receptor (TCR) α chain, and are very rapidly activated to provide a T cell component of innate immunity. They also influence the subsequent adaptive T and B cell responses. The nature of the self and microbial lipids that are antigenic is unfolding as are the range of microbes that activate NKT cells as a significant part of the host response to infection. Given their ability to produce stimulatory factors and to modulate the responses of so many other leukocytes, NKT cells are also increasingly implicated in immunopathology. The scope of lipid reactive T cell biology is extensive. It extends, complements, and provides newly appreciated roles compared to peptide reactive T cells. The rich diversity in lipids in microbes has not escaped immune surveillance by sophisticated immune systems. Not surprisingly, newly identified examples of immune evasion of lipid antigen presentation by successful pathogens have emerged. The CD1 and MHC pathways differ from one another and provide independent challenges for microbial pathogens to overcome. In this review, we provide a comprehensive explanation of CD1-based antigen presentation and the functions of CD1 a, b, and c reactive T cells and CD1d reactive NKT cells in infection.

2. THE CD1 ANTIGEN PRESENTATION PATHWAY: CHEMISTRY, STRUCTURE, AND CELL BIOLOGY

2.1. Lipid antigens: Diversity and structure

The mammalian cellular ''lipidome'' is composed of over 1000 different lipid species, with lipids serving functions ranging from roles in energy storage to roles in membrane structure and cellular signaling (van Meer, 2005). Similarly, microbial lipidomes can range from the relatively simple to the wonderfully complex, the diversity and arrangement of lipids in

Mycobacteria cell wall is a dramatic example (Lederer *et al.*, 1975). Group I CD1 molecules can survey a wide variety of microbial antigenic lipid structures including lipopeptides and lipidations of other molecules, fatty acids, mycoketides, phospholipids, glycolipids, and isoprenoids. Through CD1 molecules, the immune system maintains surveillance against this chemical class of microbial compounds (Fig. 1.1).

2.1.1. Microbial lipid antigens for Group I CD1 molecules: CD1a, CD1b, CD1c

The first evidence that CD1 molecules could present lipids as cognate antigens for T cells came from the study of a T cell line specific for a *Mycobacterium tuberculosis* antigen. By extracting *M. tuberculosis* sonicates with a series of organic solvents, Beckman *et al.* (1994) discovered that the CD1b-presented antigens were mycolic acids, lipids in the cell wall of *M. tuberculosis*. Since this seminal study, many additional examples of lipid antigens have been found for CD1a, CD1b, CD1c, and CD1d isoforms.

Mycolic acids (Fig. 1.1) are fatty acids that display a branched acyl chain (α-branch) and a characteristic hydroxylation of the β carbon. Mycolic acids form structural membrane components of several genera of microbes of the actinomycetales order including *Mycobacteria*, *Actinomyces*, *Corynebacteria*, and *Nocardia* species. Mycolic acids are extremely abundant and may make up as much as 40% of the cell wall by dry weight (Brennan and Nikaido, 1995). Mycolic acids derived from these microbes can have various cyclopropane rings, methyl branches, or double bond modifications to their main meromycolate chains and they may have various glycans esterifeid to the carboxylate as mycolates. *Mycobacteria* typically have the longest total lipid chain lengths (C70–C90), while shorter chain mycolic acid-derived GMM (glucose monomycolate) species from *Nocardia* or *Corynebacteria* also bind to CD1b and are recognized by TCRs (Brennan and Nikaido, 1995; Moody *et al.*, 2002). T cells recognizing free mycolic acids or glycosylated mycolates such as glucose monomycolate (GMM) can distinguish the α-branch β-hydroxy motif characteristic of mycolic acids, and also have fine specificity for the esterified glucose headgroup (Grant *et al.*, 2002; Moody *et al.*, 1997, 2000a). In fact, remarkable stereo-specific TCR recognition could discriminate between stereoisomers of glucose (recognized) and mannose (not-recognized) when each was esterified to the same mycolic acid chain (Moody *et al.*, 1997). In some cases, T cells also distinguished mycolic acids based on their lipid tail structures with recognition that is dependent on the ''R'' substitutions of the meromycolate chain such as the presence of oxygen moieties of either keto or methoxy esters (Grant *et al.*, 2002; Moody *et al.*, 1997).

Some of the lipid antigens recognized by T cells may have important functions for the pathogenesis and virulence of the microbes and may not

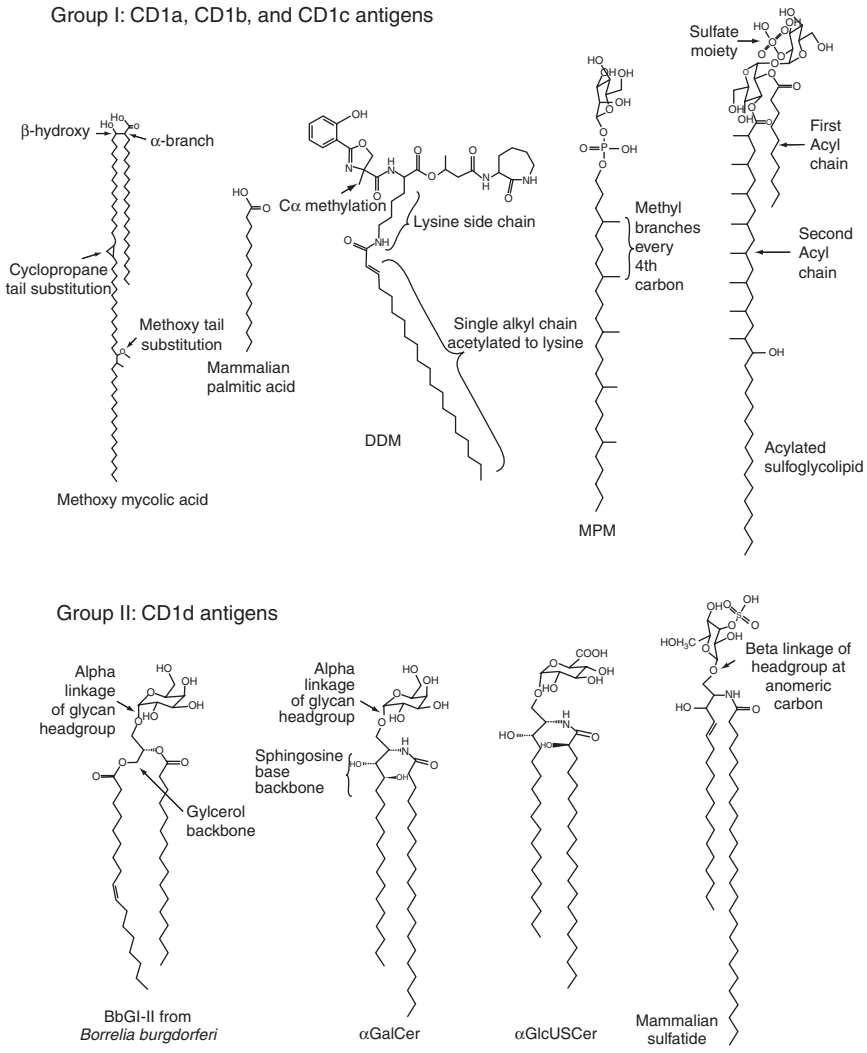

FIGURE 1.1 The structure of lipid antigens. Lipid antigens from a variety of chemical classes are presented for immune recognition by Group I and II CD1 molecules. Group I antigens (top panel) include the fatty acid-derived mycolic acid, the lipopeptide DDM, the isoprenoid-like structure mannosyl phosphomycoketide (MPM), and the acylated sulfoglycolipid Ac_2SGL, all of which were isolated from *M. tuberculosis*. The structure of palmitic acid is shown for comparison. Group II antigens (bottom panel) include the diacylglycerol BbGl-II from *B. burgdorferi*, α-GalCer, from marine sponges, and α-glucuronylceramide from *Sphingomonas*. The structure of mammalian sulfatide is shown for comparison.

be synthesized at high levels until the microbe infects its host. Mycobactin, a lipopeptide synthesized by mycobacteria with iron-scavenging properties, is necessary for bacterial survival *in vivo* (De Voss *et al.*, 2000; Dussurget *et al.*, 1999; LaMarca *et al.*, 2004; Quadri *et al.*, 1998; Wooldridge and Williams, 1993). However, outside the host, where iron is more readily available, mycobactin is not produced at high levels. Recently, a compound related to mycobactin either as a precursor or a metabolite, called didehydroxymycobactin (DDM), was identified as a lipopeptide antigen presented by CD1a that stimulates antigen-specific $CD8^+$ T cells (Moody *et al.*, 2004; Rosat *et al.*, 1999). DDM contains a peptidic headgroup linked through acylation of a lysine residue to a single alkyl chain (Fig. 1.1). The alkyl chain itself can vary in length and saturation, though an alkyl chain length of 20 carbons with 1 unsaturation (C20:1) was found to give maximal stimulation (Moody *et al.*, 2004). DDM differs from mycobactin in its lack of two hydroxyl groups and the presence of a methylation on C_α of the serine moiety of the peptidic headgroup (Willcox *et al.*, 2007). While most siderophores are soluble, both *Mycobacteria* and *Nocardia* species express siderophores with acylated tails (Ratledge and Snow, 1974) that allow their binding to CD1 enabling CD1-dependent recognition by T cells (Moody *et al.*, 2004). CD1-restricted recognition of such lipids produced upon bacterial infection of cells results in killing of the infected cells as well as the microorganism (Stenger *et al.*, 1997).

Lipoarabinomannan (LAM) is a phosphatidylinositol mannoside (PIM) molecule with a complex multiglycosylated headgroup formed using arabinose sugars in addition to mannose sugars (Fig. 1.1). LAM plays many roles in microbial pathogenesis, including both maintaining microbial membrane structure and promoting binding to host macrophage membranes facilitating cell infection (Chatterjee and Khoo, 1998). It is likely that LAM must be processed by the host because its headgroup may be too large to be corecognized with CD1 surface epitopes by TCRs. Alternatively, PIM building blocks themselves can serve as CD1-bound antigens from actinobacteria (Brennan and Nikaido, 1995; de la Salle *et al.*, 2005; Sieling *et al.*, 1995).

In addition to microbial phospholipids, other well-studied glycolipid components of the mycobacterial cell wall can serve as CD1 antigens. A mycobacterial sulfoglycolipid, Ac_2SGL, is a CD1b-restricted antigen (Gilleron *et al.*, 2004). Ac_2SGL consists of a disaccharide trehalose core, containing a 2′ sulfate and two fatty acid acylations (Fig. 1.1). The sulfate moiety on the hydrophilic headgroup was critical for T cell recognition, consistent with the fine specificity of Group I CD1-restricted TCRs for lipid antigens.

Hexoysl-1-phosphoisoprenoid lipids of the glycosyl-1-phosphopolyprenol family (Fig. 1.1) have been identified as CD1c-presented lipid antigens from *Mycobacterium avium* and *M. tuberculosis* (Beckman *et al.*, 1996;

Moody *et al.*, 2000b; Rosat *et al.*, 1999). Semisynthetic versions of the antigenic lipids, made through coupling monosaccharides to synthetic phosphopolyprenol tails, were also shown to stimulate CD1c-restricted T cells, with recognition influenced both by the length of dolichol lipid tails, the presence of an unsaturation at the α-isoprene group, and the structure of the carbohydrate headgroup. High-resolution electrospray ionization mass spectroscopy revealed antigenic lipids from *Mycobacteria* that resembled isoprenoids of the terpene family of molecules in that the alkyl chains contain methyl branches at every fourth carbon. However, in the case of the mycobacterial CD1c lipid antigens, the alkyl chains are saturated and derived by polyketide synthase enzymes rather than from isoprene precursors. These structures are similar to mammalian mannosyl phosphodolichols, which are used as glycan group donors in the synthesis of cell wall components. However, human dolichols contain lipid chains of much greater length (Fig. 1.1; n = up to 16 isoprenoid units, C95 total) as compared to the tail length of microbial dolichols ($n = 3$, C35 total) or the tail lengths of isoprenoid lipids from protozoa such as *Plasmodium falciparum* ($n = 9$, C60 total) (Willcox *et al.*, 2007). This suggests that for such antigens, the hydrocarbon chain lengths of the lipid tail may allow CD1 to distinguish microbial lipids from closely related mammalian lipids.

While the Group I antigenic lipids characterized to date come exclusively from microbes of the actinomycetales order, this likely reflects an ascertainment bias as microbial lipids of related structure are likely to be antigenic as well. *Mycobacteria* have a particularly rich diversity of lipid species in their cell walls, making them a productive source for studies of the Group I CD1 response to the universe of microbial lipid antigens.

2.1.2. Microbial lipid antigens for Group II CD1 molecules: CD1d

CD1d, the Group II antigen-presenting molecule stimulates NKT cells. iNKT cells (or Type I NKT cells) express a TCR utilizing a canonical α chain (Vα24-Jα18 in human, Vα14-Jα18 in mouse) and a limited set of Vβ chain gene segments (Vβ11 in human, Vβ8.2, Vβ7, and β2 in mice). Other CD1d-restricted NKT cells, referred to as diverse (d) NKT cells (or Type II NKT cells), utilize diverse TCRs (Behar *et al.*, 1999b; Cardell *et al.*, 1995; Chiu *et al.*, 1999).

One of the distinguishing characteristics of iNKT cells is their vigorous response to the glycosphingolipid (GSL) α-galactosylceramide (α-GalCer) (Fig. 1.1). Although commonly referred to incorrectly as a galactosylceramide, the compound is instead a galactosylphytosphingosine since it lacks the C3–C4 unsaturation of sphingosine base and is instead fully saturated with two hydroxyl substitutions. The most distinguishing structural feature of α-GalCer is that the galactose sugar is attached to the sphingosine through an α linkage at the anomeric carbon of the sugar headgroup; in contrast, mammalian GSLs typically contain β linkages at

this anomeric carbon. While α-GalCer is a synthetic compound based on structures originally isolated from murine sponges, recent reports demonstrated that other α-linked GSLs occur in microbes that can activate iNKT cells. Alpha-linked sphingolipids from *Sphingomonas*, glycosyl ceramides, α-glucuronosylceramide (α-GlcUCer), and α-galacturonosylceramide (α-GalUCer) were shown to activate iNKT cells in a CD1d-dependent manner in experimental infection in mice (Kinjo *et al.*, 2005; Mattner *et al.*, 2005; Sriram *et al.*, 2005). Thus, α-linked ceramides that resemble the synthetic α-GalCer occur in certain microbes and can stimulate iNKT cells.

An α-linked galactosyl diacylglycerol antigen-binding CD1d that activates iNKT cells was recently isolated from *Borrelia burgdorferi* (Fig. 1.1) (Kinjo *et al.*, 2006b). This lipid, termed BbGL-II by the authors, differs from α-GalCer in that it is not built from a sphingosine base but from an acylglycerol core. Since both of these compounds utilize an α-linked glycan, this α-anomeric linkage may prove to be a structural motif of some potent iNKT cells antigens.

PIMs, in particular, PIM4 was proposed to selectively bind CD1d and stimulate iNKT cells. However, synthetic PIM4 proved to be inactive (Fischer *et al.*, 2004; Kinjo *et al.*, 2006b). Phosphatidylinositol-related lipids from a protozoan, *Leishmania donovani*, activate iNKT cells. *Leishmania* utilize a dense surface glycocalyx, composed in part of the lipid lipophosphoglycan (LPG) and other glycophosphatidyl inositol species, to resist the hydrolytic environment of activated macrophages during infection (Naderer and McConville, 2008). LPG contains a single alkyl chain with a complex glycan headgroup composed of repeating phosphate–galactose–mannose units. *In vitro* assays established that LPG and other *Leishmania*-derived glycophosphatidyl inositol species-bound CD1d could activate iNKT cells without dependence on IL-12, consistent with LPG itself being a cognate antigen for iNKT cells (Amprey *et al.*, 2004a). Further studies on PIM4 and LPG may be necessary to confirm that they are direct cognate antigens recognized by iNKT cell TCRs. Antigen-loaded CD1d tetramer staining of T cells, an example of one such definitive analysis, has not yet been shown for these antigens.

2.1.3. Self-lipid antigens for CD1 molecules

The first CD1-restricted T cells identified were autoreactive for self-lipid antigens as they recognized Group I CD1 molecules in the absence of exogenous ligands (Porcelli *et al.*, 1989). Group II CD1d-autoreactivity was soon recognized as a hallmark of peripheral iNKT cells and a requirement for their thymic selection (Bendelac, 1995; Chen *et al.*, 1997). Thus, both Group I and II CD1-restricted T cells display self- and foreign-reactivity.

Most self-lipid antigens are either phospholipids or β-linked sphingolipids. Sulfatide, a component of myelin, is a GSL with a sulfated β-linked galactose headgroup that can be presented by all four antigen-presenting CD1 isoforms and recognized by T cells (Jahng *et al.*, 2004; Shamshiev *et al.*, 2002). The isogloboside iGb3 can stimulate iNKT cells. Although initially proposed to serve as a dominant selecting ligand for NKT cells in the thymus (Zhou *et al.*, 2004b), subsequent studies have challenged its physiological role (Gadola *et al.*, 2006; Porubsky *et al.*, 2007; Speak *et al.*, 2007). iGb3 remains a reasonably potent stimulatory compound for iNKT cells *in vitro*.

Phospholipid antigens for CD1 molecules include phosphatidylcholine, phosphytidylethanolamine, phosphatidylglycerol, and phosphatidyl inositol (Agea *et al.*, 2005; Gumperz *et al.*, 2000). Phospholipids, which form the bulk of mammalian cell membranes, may serve to act as weak self-antigens and/or as temporary ligands for CD1 molecules prior to exchange for microbial lipids. Studies of lipids eluted from CD1d molecules have identified phospholipids and glycophospholipid species (Joyce and Van Kaer, 2003; Joyce *et al.*, 1998). In general, the self-lipids are weaker agonists than the microbial antigens or α-GalCer (Gumperz *et al.*, 2000; Kinjo *et al.*, 2006b; Vincent *et al.*, 2005; Zhou *et al.*, 2004b). In addition, changes in the self-lipid repertoire in antigen-presenting cells (APC) upon microbial exposure have been found to skew towards more stimulatory self-lipids (De Libero *et al.*, 2005; Paget *et al.*, 2007). Self-lipid antigens may be important in the folding and stable assembly of CD1 molecules and then in some cases be replaced by microbial antigens or other self-antigens while they traffic through the endocytic system. Self-antigens may also play a role in autoimmune diseases such as multiple sclerosis and Guillan–Barre syndrome where self-lipid reactive CD1-restricted T cells may occur (De Libero and Mori, 2007).

2.2. CD1 structure: The binding and presentation of microbial lipids

In order to generate a T cell-based immune response, antigens must bind and be displayed by antigen-presenting molecules. The MHC Class I structure revealed peptide binding in an antigen-binding groove that sits atop an immunoglobulin folded domain, which in turn is anchored to the APC membrane (Bjorkman *et al.*, 1987). The first crystal structure of CD1 revealed an antigen-binding domain composed of two alpha helices (α1 and α2) sitting on a β-pleated sheet floor composed of six strands. Importantly, unlike the MHC peptide-binding groove, the CD1 molecule contains several hydrophobic channels that bind the acyl chains of lipids (Zeng *et al.*, 1997). CD1 molecules bind to the hydrophobic hydrocarbon chains of lipids while the polar moieties attached to the lipid tails are

positioned at the membrane distal end of CD1 for recognition by TCRs. The CD1 antigen-binding heavy chain α1 and α2 domains attach to an immunoglobulin-like α3 domain, a transmembrane domain and a short cytoplasmic tail. The CD1 heavy chain associates with β2 microglobulin. Here, we discuss the principle features of the structural basis for CD1 lipid antigen-binding and T cell recognition of CD1–lipid antigen complexes at the immune synapse and where possible compare this to peptide binding by MHC molecules.

2.2.1. CD1 pockets and portals and the manner of binding lipids

The existing structures of CD1 isoforms show a collective expression of a varied set of pockets for burying the tails of lipid antigens. This binding in turn allows the exposure of the vast array of microbial lipid polar headgroup structures and stereochemistries for recognition by TCRs.

CD1 molecules contain either two or four hydrophobic pockets for binding lipid tails, termed A′, C′, F′, and T′ (Fig. 1.2). The A′, C′, and F′ pockets correspond roughly in position to the similarly named pockets in MHC Class I structure, whereas the T′ pocket forms a tunnel between the A′ and F′ pockets unique to CD1b (Gadola *et al.*, 2002). Contacts between the α1 and α2 helices of CD1 (Phe70 and Leu163 in the first mouse CD1d structure described) form a partial roof over the entrance to these pockets, meaning that instead of the antigen-binding groove being exposed along its entire length to solvent, as is the case for MHC, the main access to the A′, C′, and F′ pockets is from the F′ portal opening (Fig. 1.2). The F′ portal then provides an access point for lipid antigen tails to reach the hydrophobic pockets within. The F′ portal can also play a role in the stabilization and proper placement of lipid headgroups by making hydrogen bonds between portal amino acids and the headgroups (Moody *et al.*, 2005). Additionally, the F′ portal contains a number of ionizable residues in CD1b, CD1c, and CD1d isoforms, which could potentially control pH-dependent access to the pockets through a partial unfolding of alpha helices at lower endosomal pH (Ernst *et al.*, 1998). This notion is also supported by a recent structure in which empty human CD1d was observed to also contain a more open and unfolded conformation of the α helices, perhaps capturing a ''lipid receptive'' state (Koch *et al.*, 2005).

CD1b has a unique additional portal at the distal end of the C′ channel that opens under the α2 helix (Gadola *et al.*, 2002). To date, no structure has been determined that reveals use of this portal by either a lipid tail or the headgroup. The nature of the antigen-binding pockets (A′, C′, T′, and F′) themselves differs among CD1 isoforms, allowing the CD1 system to capture a variety of lipid antigen tail lengths, ranging from the single C20 alkyl chain found in *M. tuberculosis*-derived DDM to the exceptionally long C56 alkyl chain of GMM C80 derived from the same pathogen. In human CD1d, CD1a, CD1b, and mouse CD1d, the A′ pockets are

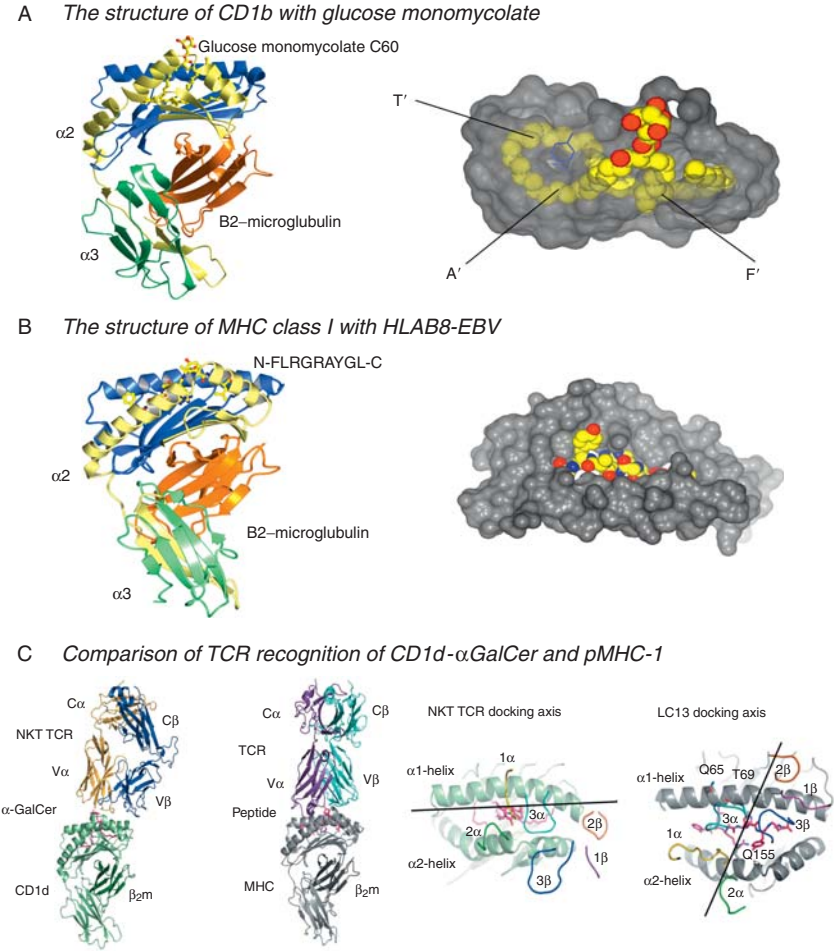

FIGURE 1.2 The structure of antigenic complexes. (A) A ribbon diagram of human CD1b crystal structure loaded with the microbial lipid GMM shown in stick representation. At right is a surface representation of the CD1b–lipid complex. The tails of the lipid antigen bury deep into the hydrophobic antigen-binding groove. (B) In contrast, an analogous view of MHC Class I loaded with peptide antigen reveals a shallower-binding groove exposing a greater length of peptide to solvent. (A, B) Adapted with permission from Barral *et al.*, *Nature Immunology* (2007). (C) *Left*: ribbon diagrams depicting the interaction of NKT TCR with human CD1d–α-GalCer and a typical TCR (LC13) interaction with pMHC-I (HLA-B8-FLR). The orientation of the NKT TCR occurs at an acute angle as compared with TCR-pMHC-I. *Right*: parallel docking mode of NKT TCR onto human CD1d–α-GalCer, and diagonal docking mode of a typical TCR interaction with pMHC-I. **C** Adapted with permission from *Borg et al.*, *Nature* (2007). PDB accession numbers are 1UQS (CD1b), 1MI5 (LC13-pMHC-I), and 2PO6 (NKT–CD1d–α-GalCer).

relatively conserved, containing a structural 'pole' which the *alkyl* chains of lipid antigens generally wrap around. One exception to the rule of alkyl chain binding in the A′ pocket comes from the structure of the self-antigen sulfatide bound to CD1a, in which the *sphingosine* chain binds the A′ pocket, leaving the alkyl chain to bind in the F′ pocket (Zajonc *et al.*, 2003). While the orientation and formative residues of the A′ pocket are relatively conserved among CD1 isoforms, the degree of winding of alkyl lipid antigen tails around the pole itself varies, and along with small differences in size this allows a limited amount of variability in accommodated alkyl chain length (C20 for DDM and CD1a, C26 for α-GalCer and h/mCD1d) (Koch *et al.*, 2005; Zajonc *et al.*, 2005). The A′ pocket of human CD1b, however, can accommodate exceptionally long alkyl chains via its unique continuous connection in series through the T′ tunnel to the F′ pocket on the other side of the binding groove (Gadola *et al.*, 2002). This provides one large continuous A′T′F′ superchannel for accommodating a long alkyl chain such as the C56 alkyl chain of GMM C80 or mycolic acid (Batuwangala *et al.*, 2004).

In comparison to the A′ pocket, the F′-binding pocket of CD1 molecules shows more variability between isoforms. CD1a contains a laterally oriented, partially solvent accessible F′ pocket which can allow the binding of polar headgroup structures such as the β-hydroxybutyryl-lysine portion of the lipopeptide DDM (Moody *et al.*, 2004; Zajonc *et al.*, 2005). The partial solvent accessibility of the F′ pocket in CD1a may help facilitate TCR recognition through allowing direct contacts to residues of the DDM peptide moiety. Additionally, the F′ pocket of CD1a is relatively shallow, giving CD1a the smallest total antigen-binding groove volume of the CD1 isoforms at 1200 $Å^3$, meaning CD1a may be specialized to bind smaller, shorter lipid antigen structures. In contrast, CD1b contains a fourth antigen-binding pocket (C′) in addition to the A′T′F′ superchannel, yielding a total antigen-binding groove volume of 2200 $Å^3$. This large cavity size allows CD1b to bind larger, longer lipid tails (Gadola *et al.*, 2002; Zajonc and Wilson, 2007). The F′ pocket and total antigen-binding groove sizes of mCD1d and hCD1d are intermediate to those of CD1a and CD1b, at 1650 and 1400 $Å^3$, respectively. The F′ pocket of hCD1d is oriented and positioned similarly to the C′ pocket of CD1b despite its nearly identical amino acid composition to the F′ pocket of mCD1d (Koch *et al.*, 2005). It is important to note, however, that despite having relatively large antigen-binding grooves, both CD1b and CD1d can bind lipids of shorter tail length using ''spacer'' lipids to occupy the remainder of the pockets (Garcia-Alles *et al.*, 2006; Giabbai *et al.*, 2005; Wu *et al.*, 2006). An important addition to these data must await a crystal structure of CD1c, which will shed light into how the branched alkyl chains of isoprenoid-like mycoketide antigens are accommodated by its pockets.

While CD1 and MHC both contain antigen-binding grooves in which microbial products are retained for display to T cells, the nature of the chemical interactions that hold the microbial products in place are quite distinct. MHC Class I molecules stabilize peptide binding through a semiconserved network of hydrogen bonds in the A and F pockets between MHC and peptide amino and carboxy terminus atoms (Matsumura *et al.*, 1992). Peptide-binding specificity is determined by the interaction of peptide side chains with pockets in the MHC-binding groove – for example, the P2 pocket of the MHC Class I HLA-A0201 allele which normally accommodates the amino acid leucine cannot bind peptides that contain a substitution of the bulkier, charged side chain of arginine (Colbert *et al.*, 1993). In order to form a productive, stable complex, the side chains of amino acids in the microbial peptide must be compatible with the chemical nature of the MHC Class I pocket in size, shape, and electrostatics (Garrett *et al.*, 1989).

In contrast, the binding of microbial lipid tails in the pockets of CD1 molecules depends upon hydrophobicity, size, and shape. The microbial lipids bound by CD1 molecules have long, aliphatic tails composed of hydrophobic methylene units. While MHC molecules use many types of electrostatic interactions between pockets and amino acid side chains: polar interactions, London dispersion forces (induced dipoles in hydrophobic contacts), and even salt-bridges, the binding of antigen by CD1 molecules is limited only to the London dispersion forces that the hydrophobic tails of lipid antigens can make with the hydrophobic pockets of CD1 (Moody *et al.*, 2005). Consequently, an extensive network of hydrogen bonds stabilizes peptide binding to MHC, while the use of hydrogen bonds in CD1–lipid complexes is far more limited. Lipid antigen tails must still be of acceptable size in the number of methylene units, suggesting that the pockets of CD1 antigen-binding grooves can in some cases serve as a 'molecular ruler' (Zajonc *et al.*, 2003). The shape of lipid antigen tails is constrained as well, as *trans*-double bonds which introduce kinks into the hydrophobic tails can in some cases interfere with binding to CD1, presumably through disallowing the flexibility needed for lipid antigens to properly load and bind into CD1 pockets (Rauch *et al.*, 2003).

Lipid antigens for CD1 have tail lengths requiring CD1 molecules to contain deeper hydrophobic pockets than those found in MHC. This is accomplished in part through the α1 and α2 helices riding 4–6 Å higher above the β-pleated sheet floor than in MHC Class I molecules, due to the presence of bulky amino acids (residues 18, 40, 49) acting as a scaffold on the α1 helix (Moody *et al.*, 2005). In addition to changes in the pocket residues, the elevation of the α helices gives the effect of forming a deeper antigen-binding groove for lipids than the relatively shallow groove observed for peptide binding by MHC. The deep penetration of the acyl chains under the CD1 surface also orients the antigenic lipid more

perpendicular to the α helices of the antigen-binding groove. This orientation exposes the lipid headgroup to solvent for TCR recognition. In contrast, MHC molecules bind and orient antigenic peptide along the length of the binding groove.

MHC polymorphism allows for the presentation of diverse peptides each with slightly a different antigen-binding groove, accommodating a distinct set of microbial peptides. In contrast, the organization of CD1 alleles in achieving microbial defense against lipid antigens is quite different. Humans express the same four relatively nonpolymorphic CD1 isoforms (CD1a, CD1b, CD1c, and CD1d). Each has a different antigen-binding groove which allows for appropriately sized lipid tails to bind. Although the diversity of lipid tails is large, it may be considered small compared to the diversity of amino acid sequences of peptides. Yet, the bound lipids can display an extensive diversity of headgroup structures that can be recognized by TCRs. Thus, it is the combined structure and stereochemistry of the headgroups, the lipid tails and their attachment together that determine CD1 binding and antigenic diversity.

2.2.2. T cell receptors: Structural interactions with CD1 and MHC molecules

iNKT cells are a group of CD1d-restricted lymphocytes expressing a canonical TCRα utilizing the same Vα segment (Vα24 in human, Vα14 in mouse) rearranged to Jα18. The iNKT TCR uses a limited set of β chains (Vβ11 in human, Vβ8.2, Vβ7, and Vβ2 in mice) but they generally contain hypervariable CDR3β regions (Godfrey *et al.*, 2004). The trimolecular CD1d–αGalCer–iNKT TCR structure solved by Borg *et al.* (2007) confirms that, like MHC-restricted peptide recognition, the iNKT-cell TCR makes contacts both to the CD1d molecule and the lipid antigen. However, this trimolecular complex revealed that the iNKT TCR docked on one side of the CD1d antigen-binding groove, with both Vα and Vβ chains positioning themselves over the F′ pocket at a relatively acute angle (Fig. 1.2). This is in contrast to most MHC–peptide TCR structures, in which the Vα segment of the TCR contacts the α2 helix and N-terminal end of peptide, with the Vβ segment contacting the α1 helix and C-terminal end of peptide (Rudolph *et al.*, 2006). If one defines an axis between the center of mass of TCR Vα and Vβ domains and compares this to the axis defined by the length of the α helices of the antigen-binding groove, the MHC Class I–peptide TCR structure identified a diagonal docking orientation for TCR (Garboczi *et al.*, 1996; Garcia *et al.*, 1998) and the MHC Class II–peptide TCR structure identified an orthogonal docking orientation (Reinherz *et al.*, 1999). In contrast to this, the iNKT TCR structure identified a parallel orientation to the length of the α helices on the CD1d–α-GalCer complex (Fig. 1.2).

In addition to the differences in the general positioning of the TCR over CD1 compared to most MHC–peptide complexes, the specific contacts made in the two cases differ in significant ways as well. Three particular MHC Class I positions (65, 69, 155) are contacted by TCR in most trimolecular structures elucidated to date and are proposed to represent a docking motif for MHC restriction (Tynan *et al.*, 2005). None of the equivalent residues are contacted in the CD1d–α-GalCer–iNKT TCR structure. Nonetheless, the iNKT TCR makes extensive contacts to the CD1d molecule, particularly to the α1 helix of CD1d through CDR2β and to both α1 and α2 helices via the Jα18 region of CDR3α (Borg *et al.*, 2007). The Jα18 region of CDR3α additionally makes critical electrostatic contacts to the α-GalCer antigen. The number of critical contacts made to CD1d–α-GalCer by CDR3α through its Ja18 region likely explains the lack of positive selection of NKT cells in Jα18-deficient mice (Cui *et al.*, 1997). Additional contacts to the headgroup of the antigenic moiety come in the form of hydrogen bonds provided by residues contained in CDR1α. Together, the extensive contacts made by the Vα CDR1α and CDR3α segments to the CD1d–α-GalCer complex provide a structural explanation for the use of an invariant Vα segment (Vα24/Vα14) in forming the canonical TCR of iNKT cells.

Despite the CD1d–α-GalCer TCR having a relatively small contact area as compared to MHC–peptide TCR complex, the CD1d–α-GalCer TCR complex exhibits particularly tight binding (K_d for CD1d–α-GalCer–NKT TCR 100 nM–10 μM, compared to 1–50 μM for MHC–peptide TCR complexes) (Gadola *et al.*, 2006; Rudolph *et al.*, 2006). MHC-restricted TCRs often exhibit a great deal of plasticity in making contacts at the immune synapse. However, the conformation of the Vα24/Vβ11 iNKT TCR in the trimolecular CD1d–α-GalCer TCR complex was not appreciably different from the previously determined free Vα24/Vβ11 iNKT TCR structure (Kjer-Nielsen *et al.*, 2006) (also see Gadola *et al.*, 2006). This enables a tighter ''lock-and-key'' interaction, as opposed to the induced fit observed for MHC–peptide TCR complexes, and explains the unusually tight binding of CD1d–αGalCer to the iNKT TCR (Borg *et al.*, 2007). Additional trimolecular CD1d–lipid TCR structures will be necessary to determine if this reorientation of TCR with respect to antigen-presenting molecule is a hallmark of lipid recognition or a unique quirk of the particular binding interactions between the iNKT TCR and CD1d–α-GalCer.

Current hypotheses regarding the interaction of Group I CD1a, b, and c with TCRs are based on modeling or mutagenesis studies aimed at identifying key residues of interaction. These models predict a more MHC-like diagonal orientation of the TCR across the CD1-antigen surface (Grant *et al.*, 1999, 2002; Melian *et al.*, 2000). Thus, it is possible that the CD1a, b, and c interactions with TCR may be more analogous to

MHC interactions given the diversity of the TCRs that recognize them. Nevertheless, determined structures are needed to answer this question.

2.3. Antigen acquisition and uptake

In order to form stimulatory CD1 complexes, microbial lipid antigens must first be taken up by APC. The APC must deliver the lipid antigens to CD1 lipid antigen-loading endocytic compartments. Lipid acquisition and uptake utilized by $CD1^+$ APC is likely to co-opt the same pathways developed by cells to serve metabolic and cellular maintenance functions; lipoproteins, endocytosis, and membrane lipid transporters may play roles in CD1 immune defense in addition to their classical functions. Pathways and molecules developed for innate immune defense such as phagocytosis, scavenger receptors, and/or lectins may also be co-opted for the acquisition of lipid antigens by CD1. The pathways of lipid antigen acquisition detailed below are summarized in Fig. 1.3.

2.3.1. Phagocytosis and pinocytosis

Phagocytosis and the formation of phagolysosomes, especially in dendritic cells (DCs) and macrophages, allows uptake of pathogenic organisms and fragments into the endocytic system. Using immunogold labeling

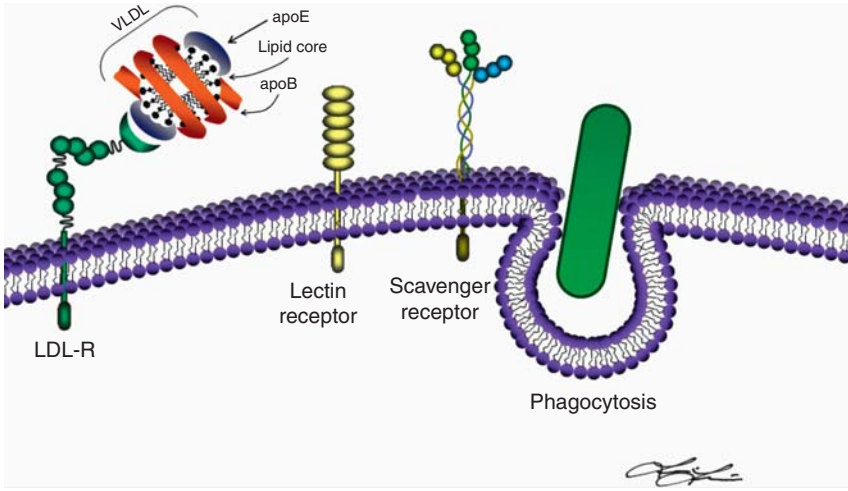

FIGURE 1.3 Pathways of lipid antigen acquisition. Lipids bound in complexes to lipoproteins may be taken up through receptor-mediated pathways utilizing cell surface lipoprotein receptors (e.g., LDL-R), Scavenger receptors and others. Lectin-like molecules (e.g., mannose receptor, DC-SIGN, Langerin) bind to the glycans of glycolipids and mediate their internalization. Phagocytosis mediates the uptake of particles and microbes such as bacteria. Each of these pathways delivers exogenous lipids to intracellular endocytic compartments where CD1 molecules may bind them.

and electron microscopy, Hava *et al.* showed that phagolysosomes formed following infection with *M. tuberculosis* in DC rapidly contain CD1b molecules, making this compartment a candidate location for CD1b loading of *M. tuberculosis*-derived lipids (Hava *et al.*, 2008; Sugita *et al.*, 2000a). Following phagocytosis of live *Mycobacteria*, infected DCs were able to be recognized directly by CD1b-restricted lipid antigen-specific T cells. Apoptotic bodies from dying cells infected with pathogens can also be taken up by uninfected cells through an internalization process closely resembling phagocytosis. This process also enables CD1 antigen presentation by APC (Schaible *et al.*, 2003). Phagocytosis is likely to contribute to the acquisition of lipid antigens from many types of microorganisms.

Pinocytosis and macropinocytosis or ''cell drinking'' may lead to the uptake of fluid phase antigens. Macropinocytosis is an important mechanism for the uptake of extracellular proteins for delivery to MHC Class II molecules. While the solubility of single lipids in fluid phase is low, lipids can bind serum proteins such as albumin and gamma globulins to allow uptake via pinocytosis (Hamilton, 2002). A number of other lipid-binding proteins, such as lipocalins and plasma lipid transfer proteins, are present at lower levels in serum than albumin and might mediate binding and uptake of some lipid antigens.

2.3.2. Lipoprotein particle-mediated uptake

The majority of lipids are carried through blood plasma and the extracellular milieu in the form of serum lipoprotein particles. Lipoproteins are complexes of different apolipoproteins with triglycerides, cholesterols, cholesterol esters, and some hydrophobic vitamins in varying amounts.

While this system is utilized primarily to deliver lipids for metabolic needs, recent work has highlighted its ability to efficiently deliver exogenous lipid antigens to CD1-bearing APC. Apolipoprotein E (ApoE), the primary apolipoprotein found in VLDL, markedly increased the rate of uptake of the model lipid antigen α(1–2)GalactosylαGalactosylceramide (GalαGalCer) into DCs. When added to human serum, the GalαGalCer antigen was primarily distributed in VLDL particles bound to ApoE. When it was added to ApoE-depleted media, ApoE markedly enhanced the ability of GalαGalCer to elicit an iNKT cell response via CD1d-bearing DCs (van den Elzen *et al.*, 2005). The presentation of mycobacterial antigens, such as GMM and mannosyl β-1-phosphomycoketide, were also markedly enhanced in the presence of ApoE, indicating the importance of this pathway for exogenous delivery of a broad array of antigenic lipid structures. ApoE binding to the LDL-R not only provides rapid, receptor-mediated uptake of lipid antigens but also specifically delivers them to the endocytic compartments. The secretion of ApoE and its uptake from cells provides an attractive mechanism by which DC, macrophages, and other cells might sample their milieu for lipid antigens (van den Elzen

et al., 2005). Given that lipoprotein receptors and lipoprotein particles have at least partially overlapping specificities for one another, the serum lipoprotein distribution and internalization pathways followed by any particular antigen are likely to be determined by its biophysical interactions with these components of serum.

2.3.3. Lectin receptors and other pathways

As many lipid antigen structures contain glycan headgroups, carbohydrate-binding lectin molecules have been implicated in the uptake of CD1-presented glycolipid antigens. This was shown most clearly in the case of LAM uptake by the C-type lectin mannose receptor (MR). Anti-mannose receptor antisera or excess free mannan sugars blocked uptake of LAM by human DCs (Prigozy *et al.*, 1997). Other lectins have been implicated in the uptake of mycobacterial lipid antigens as well. Using surface plasmon resonance and flow cytometry, recent work identified Galectin-3, a molecule known to bind β-galactoside lipids, as a lectin present on the surface of DCs capable of binding mycolic acid antigens from *M. tuberculosis* (Barboni *et al.*, 2005). Aside from LAM and mycolic acids presented by CD1b, C-type lectins can play a role in the delivery of glycolipid antigens to CD1a molecules as well. Langerhans cells are a subset of DCs expressing the C-type lectin langerin, and the expression of both CD1a and langerin was necessary to activate CD1a-restricted T cell clones isolated from the skin of patients with leprosy (Hunger *et al.*, 2004). It is likely that other C-type lectins such as DC-SIGN, Dectin 1 and 2, and BDCA-2 among others may also play roles in lipid antigen uptake (Figdor *et al.*, 2002).

While no specific scavenger receptor has yet been identified which mediates the uptake of a CD1-presented lipid antigen, their expression on DCs and macrophages, and the breadth of their lipid-binding capacities, makes them attractive candidates for the acquisition of lipid antigens by CD1 positive APC. Other mechanisms of lipid uptake are also likely to occur for lipid antigens that bind to plasma membranes and that are part of the various internalization routes of plasma membranes that are independent of receptor-mediated uptake. Lipid rafts may play a role in the uptake of some lipids. Interestingly, flippases known for roles in generating the asymmetry in lipid distribution between inner and outer leaflets of the plasma membrane, were recently implicated in generating glycoconjugates of lipids such as GSLs, glycophosphatidylinositol (GPI)-anchored lipids, or even lipopolysaccharide (LPS) in bacteria (Pomorski and Menon, 2006). Given that these proteins already bind, transport, and modify lipids, they are excellent candidates to form lipid acquisition or lipid-loading molecules for CD1 microbial defense pathways. The ABC family of transporters, frequently utilized for their ability to take up pharmacologic drug compounds, are also known to flux hydrophobic

lipidic molecules across membranes (Raggers *et al.*, 2000). Another group that may participate in transporter-mediated acquisition of lipid antigens are the compounds involved in nonvesicular transport of sterol molecules: StAR and START proteins, along with others, mediate the transfer of hydrophobic sterol lipids between cells and between cellular compartments (Prinz, 2007). Any of these molecules or others with lipid binding and transport capacity could potentially be co-opted by APC for the acquisition and loading of CD1 molecules.

2.3.4. Cell surface loading

Considerable evidence suggests that some lipid antigens may be directly loaded into CD1 on the cell surface without need for acquisition and uptake into the endocytic system. $CD1^+$ APC treated with fixative agents such as paraformaldehyde or gluteraldehyde retain the ability to bind and present lipids such as short chain (C32) GMM, α-GalCer, and the self-antigen sulfatide (Moody *et al.*, 2002; Shamshiev *et al.*, 2002). The presence of exogenous lipids in serum was shown to stabilize CD1a in the absence of newly synthesized or recycling CD1a molecules, suggesting that CD1a can undergo lipid exchange by loading exogenous lipids directly at the cell surface (Manolova *et al.*, 2006). In addition, multiple studies have utilized *in vitro* systems in which plate-bound recombinant CD1 molecules can capture and present lipid antigens directly from a fluid phase buffer, suggesting that in some cases the presence of CD1 and an appropriate lipid is sufficient to form complexes stimulatory for CD1-restricted T cells. While it is unknown what role direct surface loading of microbial lipids may play in the setting of *in vivo* infection, where other more efficient pathways of lipid acquisition such as lipoprotein particles or surface receptors are operative, cell surface loading remains a potential means of acquiring or exchanging certain self or microbial lipids.

2.4. The intersection of CD1 molecules and lipid antigens: Trafficking

CD1 molecules must intersect with lipid antigens and bind them in an appropriate intracellular compartment of the APC. MHC Class I molecules selectively bind antigens delivered from the cytosol into the endoplasmic reticulum (ER), while MHC Class II molecules bind antigens delivered to lysosomes. CD1 molecules must intersect with microbial lipid antigens. Here, we show how the routes taken by CD1 molecules, from their synthesis in the ER (Fig. 1.4) to their passage through endosomes, provide opportunities for them to intersect with and bind lipid antigens.

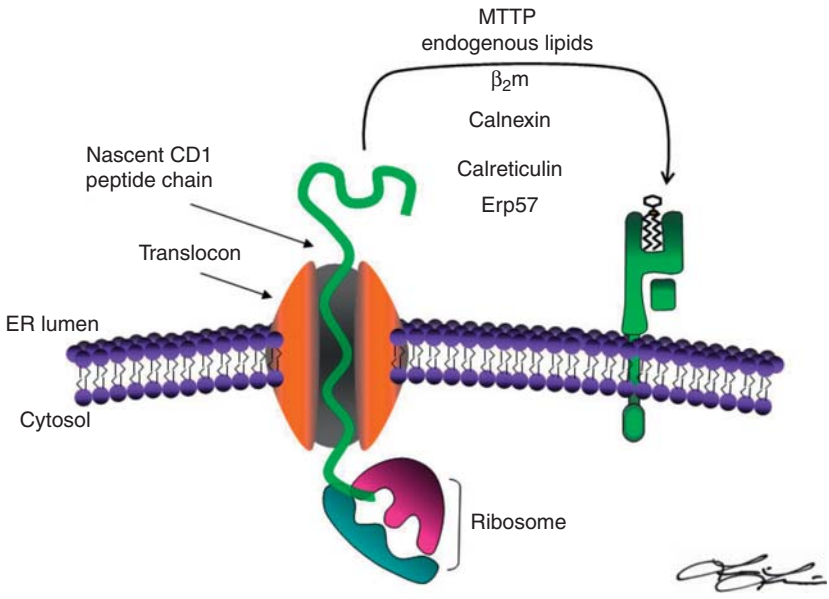

FIGURE 1.4 CD1 assembly in the ER. CD1 heavy chains (HC) are translocated from ribosomes in the cytosol into the ER lumen where N-glycan addition occurs. Mono-glucosylated N-glycan moieties of the CD1 HC bind calnexin and calreticulin. The oxidoreductase Erp57 also associates with HC during its folding and assembly to β2m. Endogenous lipids are loaded into the CD1 HC-binding grooves in the ER and MTTP plays a role in the loading process.

2.4.1. The assembly of CD1 molecules

The mRNA transcripts of all CD1 isoforms contain sequences directing translocation of CD1 molecules into the ER lumen. Like the MHC Class I molecules, they resemble in domain organization, newly synthesized CD1 molecules bind the ER folding chaperones calnexin and calreticulin that recognize CD1 precursor N-linked glycans (Huttinger *et al.*, 1999; Kang and Cresswell, 2002a; Paduraru *et al.*, 2006; Sugita *et al.*, 1997). Consequent to its interactions with calnexin and calreticulin, CD1 molecules bind the thiol oxidoreductase ERp57 which promotes formation of disulfide bonds in the heavy chains (Fig. 1.4) (Hughes and Cresswell, 1998; Kang and Cresswell, 2002a). While both CD1 and MHC heavy chains bind β2m, they differ markedly in the timing of binding and the stringency of the β2m-binding requirement for ER exit. MHC Class I molecules bind β2m as calreticulin replaces calnexin in the Class I assembly complex (Sadasivan *et al.*, 1996). CD1 heavy chains appear to preferentially associate with both calnexin and calreticulin, with β2m binding occurring after full oxidation of CD1 heavy chains by ERp57 (Kang and Cresswell, 2002a). While the β2m association is

required for most MHC Class I alleles to exit from the ER, a fraction of CD1d heavy chains are able to exit the ER without binding β2m and remain competent for antigen presentation (Amano *et al.*, 1998; Brossay *et al.*, 1997; Kim *et al.*, 1999). CD1b heavy chains appear more stringent in their requirement of β2m for ER exit (Huttinger *et al.*, 1999; Sugita *et al.*, 1997).

Current evidence suggests that CD1 molecules are also likely to be loaded with endogenous lipid ligands in the ER. Although calnexin and calreticulin are in complex with CD1, a complex of lipid transporting and loading molecules, analogous to TAP 1, TAP 2, and tapasin (Ortmann *et al.*, 1994; Sadasivan *et al.*, 1996), have not been described. Microsomal triglyceride transfer protein (MTTP) may play a role in the process of loading lipids into CD1 in the ER, although its exact role is not known.

2.4.2. The fate of CD1 after assembly

Following assembly in the ER, most CD1 molecules traffic along the secretory route through the Golgi to the cell surface. Pulse-chase experiments studying the timing of CD1b appearance at the cell surface in addition to examination of the time course of acquired endoglycosidase H resistance, suggest direct traffic from the Golgi to the plasma membrane for CD1 molecules (Briken *et al.*, 2002). Although direct trafficking to the plasma membrane appears to be the dominant route followed by newly synthesized CD1 molecules, several potential alternative trafficking routes may be utilized by a fraction of CD1 molecules. For example, two reports have suggested that CD1d molecules can associate with invariant chain or MHC Class II, which could mediate the trafficking of a portion of CD1 molecules directly to the endocytic system in heteromultimeric complexes with MHC Class II (Jayawardena-Wolf *et al.*, 2001; Kang and Cresswell, 2002b). Furthermore, human CD1d molecules have been suggested to have partially defined motifs for sorting to the basolateral surface of polarized epithelial cells (Rodionov *et al.*, 1999, 2000).

2.4.3. CD1 isoform trafficking in the endocytic system

2.4.3.1. Internalization from the plasma membrane Once newly synthesized CD1 molecules are delivered to the plasma membrane, they are internalized into the endocytic system where binding of microbial lipids or self-lipid exchange occurs. The tails of CD1b, CD1c, and CD1d isoforms all contain tyrosine-based sorting motifs of the form YXXϕ (Y = tyrosone, X = any amino acid, ϕ = bulky hydrophobic amino acid) in their cytoplasmic tails (Brigl and Brenner, 2004). This motif is a hallmark for recognition and binding by adaptor protein 2 (AP-2) which sorts cargo into clathirin-coated pits for internalization from the plasma membrane into the endocytic system (Bonifacino and Traub, 2003). Using surface plasmon resonance and yeast two-hybrid assays, the cytoplasmic tails of CD1b, CD1c, and CD1d were all shown to bind AP-2, and these isoforms

were detected by immunostaining and transmission electron microscopy in clathirin-coated pits (Briken *et al.*, 2002; Sugita *et al.*, 1996, 2000a,b). Consistent with the idea that the cytoplasmic tail drives internalization of CD1 as the dominant mechanism, cytoplasmic tail-deleted forms of CD1b and CD1d molecules show accumulation at the cell surface and reduced internalization kinetics (Briken *et al.*, 2002; Jayawardena-Wolf *et al.*, 2001; Lawton *et al.*, 2005; Sugita *et al.*, 1996). Furthermore, cytoplasmic tail-deleted mCD1d is functionally deficient for NKT cell development and antigen presentation, presumably due to lack of trafficking through the endocytic system (Chiu *et al.*, 2002). Striking functional defects in antigen presentation are also observed for tail-deleted CD1b molecules (Jackman *et al.*, 1998).

While CD1a lacks a tyrosine-based sorting motif, it too is found in the endocytic system. Some CD1a molecules appear in clathirin-coated pits and vesicles in DCs and Langerhans cells (Salamero *et al.*, 2001; Sugita *et al.*, 1999). However, a majority of CD1a appear to follow clathrin and dynamin-independent pathways of internalization (Barral *et al.*, 2008a). The molecular mediators of this internalization pathway remain unknown.

2.4.3.2. Trafficking through the endocytic system and back to the plasma membrane Once internalized, CD1 trafficking broadly covers the range of endocytic compartments. CD1a and CD1c are found in early endosomes and to the endocytic recycling compartment (ERC). In contrast, CD1b and CD1d isoforms are sorted to traffic more deeply into the endocytic system. Yeast two-hybrid analysis established that the cytoplasmic tail of human CD1b, bound strongly to the adaptor protein AP-3 (Sugita *et al.*, 2002). AP-3 is known to direct the trafficking of lysosomal resident proteins such as LAMP-1 and LAMP-2 from the tubulations of sorting endosomes to lysosomes (Bonifacino, 2004; Peden *et al.*, 2004). Murine CD1d also binds AP-3 through its cytoplasmic tail tyrosine-based sorting motif. Thus, both CD1b and mCD1d primarily localize at steady state in late endosomal/lysosomal compartments (Cernadas *et al.*, 2003; Elewaut *et al.*, 2003; Lawton *et al.*, 2005). AP-3 deficiency in cells results in the accumulation of CD1b at the cell surface and in early endosomes, but a near absence in lysosomes. These mutant cells are also deficient in presenting lysosomally loaded lipid antigens such as GMM (Briken *et al.*, 2002; Sugita *et al.*, 2002). Defects in the development of CD1d-restricted iNKT cells were also found in the absence of AP-3, supporting the idea that AP-3 acts to deliver mCD1d molecules to lysosomes (Cernadas *et al.*, 2003; Chiu *et al.*, 2002; Elewaut *et al.*, 2003). The cytoplasmic tail of CD1c and human CD1d do not bind AP-3 with high enough affinity to be detected by yeast two-hybrid interaction. This low or absent AP-3-binding by CD1c and hCD1d results in more limited colocalization with lysosomal markers. Instead, CD1c and hCD1d are

distributed more broadly throughout the endocytic system at steady state (Briken *et al.*, 2000; Sugita *et al.*, 2002).

Following lipid binding or exchange in the endocytic system, CD1–lipid complexes must recycle to reach the plasma membrane to become accessible to TCRs. For example, Arf6 mediates the recycling of CD1a and CD1c back to the cell surface, as they colocalize in vesicles with a dominant negative form of the small GTPase Arf6, suggesting passage through the slow recycling compartment (Briken *et al.*, 2000; Sugita *et al.*, 1999, 2000a,b). CD1a has further been shown to colocalize with both Rab11 and Rab22a, additional markers of the ERC (Barral *et al.*, 2008a; Salamero *et al.*, 2001; Sugita *et al.*, 1999, 2000a,b). Interestingly, CD1a recycling was found to follow a recycling route that was very similar to MHC Class I but somewhat distinct from the classical recycling pathway followed by the transferrin receptor (Barral *et al.*, 2008a).

During DC maturation, MHC Class II molecules traffick along dramatic tabulations that formed from lysosomes to the cell surface (Boes *et al.*, 2002; Chow *et al.*, 2002; Kleijmeer *et al.*, 2001). However, studies on CD1 molecules during DC maturation suggest they do not follow the same route as MHC Class II. CD1 and MHC Class II segregate from one another during DC maturation, and CD1 molecules do not show a net change in steady state distribution form lysosomes to the plasma membrane as do MHC Class II molecules (Cao *et al.*, 2002; van der Wel *et al.*, 2003). Nevertheless, CD1b and CD1d molecules must recycle from lysosomes to reach the cell surface for T cell accessibility following acquisition of lipid antigens. This is especially important since some lipid antigens are known to require lysosoml processing or loading. Furthermore, the observation that herpes simplex virus I (HSV-1) infection downregulates CD1d cell surface levels and leads to CD1d accumulation in lysosomes may result from blockade of a lysosomal recycling pathway by this microbe (Yuan *et al.*, 2006).

The differential trafficking of the various CD1 isoforms may have evolved to allow CD1 molecules to broadly survey the endocytic system, as each subcompartment could represent a potential hiding place for microbial lipid antigens (Dascher, 2007; Dascher and Brenner, 2003).

2.4.4. The trafficking of lipid antigens

A complete picture of where CD1 molecules intersect with antigenic microbial lipids in the endocytic system can only emerge with an understanding of both the trafficking pathways of CD1 molecules and of lipid molecules themselves. While proteins often contain motifs or sequences which direct trafficking along particular pathways, much less is known about the factors that direct lipid trafficking. The original Singer and Nicholson fluid-mosaic model pictured biological membranes as essentially homogenous, but we now appreciate that membranes are heterogeneous and may have subregions such as lipid rafts (Vereb *et al.*, 2003).

Two recent reports have demonstrated that CD1a distributes into lipid raft membrane microdomains on the cell surface (Barral *et al.*, 2008a; Sloma *et al.*, 2008). This distribution is important for efficient CD1a presentation of sulfatide or the microbial lipid antigenDDM, as monocyte-derived DC treated with a cholesterol depleting agent that disrupts lipid rafts displayed reduced stimulation of lipid-specific T cells (Barral *et al.*, 2008a; Sloma *et al.*, 2008). In addition, CD1d localizes into lipid rafts at the cell surface (Lang *et al.*, 2004; Park *et al.*, 2005) and this can influence the nature of the T cell response elicited (S. Porcelli, personal communication).

The lipid composition of endosomal compartments varies, for example, early endosomes are enriched for PtdIns(3)P and cholesterol lipids, whereas late endosomes are enriched for PtdIns(3,5)P2 and lipids such as lysobiphoshpatidic acid (LBPA) (Di Paolo and De Camilli, 2006; Kobayashi *et al.*, 1998). The length of lipid tails and their degree of unsaturation can influence to which endocytic compartment they localize (Mukherjee and Maxfield, 2004; Mukherjee *et al.*, 1999). In fact, GMM with a shorter chain (32 C) loads into CD1b at the plasma membrane or in the early endocytic system, while GMM with longer lipid tails (80 C) loads in the late endocytic system (Moody *et al.*, 2002). Furthermore, some microbial lipids such as LAM largely localize in lysosomes following uptake (Prigozy *et al.*, 1997) (M. Brenner and M. Cernadas, personal communication). However, many factors including the headgroup structure, lipid stereochemistry (Singh *et al.*, 2006) and tail length can all influence the uptake, trafficking and distribution of lipid antigens.

2.5. Antigen processing and loading

The biophysical nature of lipids and their localization in membranes or hydrophobic particles creates a challenge for loading them into CD1 proteins. Here, we outline the available data on endogenous lipid loading during CD1 synthesis in the ER, the processing of microbial lipid antigens, and the exchange of endogenous lipids for exogenous microbial lipids in the endocytic system.

2.5.1. Antigen loading in the ER

The elution of common ER phospholipids from CD1 isoforms suggests self-lipids load in the ER (De Silva *et al.*, 2002; Garcia-Alles *et al.*, 2006; Joyce *et al.*, 1998; Park *et al.*, 2004). Since unfilled hydrophotic cavities are generally energetically unfavorable in protein folding (Matthews, 1995), lipid loading into CD1 may be necessary for the proper folding or stabilization of CD1 heavy chains during their assembly in the ER.

The microsomal triglyceride transfer protein MTTP (or MTP) has been described to play a role in lipid loading of CD1. MTP is well characterized as lipidating apolipoprotein B (ApoB) molecules, promoting ApoB secretion

and in lipid homeostasis and lipid-related diseases such as abetalipoproteinemia (Berriot-Varoqueaux *et al.*, 2000; Hussain *et al.*, 2003). $CD1d^{+}$ hepatocytes and intestinal epithelial cells derived from mice with conditional-deletion of the MTTP gene in these tissues were unable to generate iNKT cell-dependent autoimmune responses in colitis and hepatitis models, establishing a role for MTP in the biology of NKT cell activation by CD1d. Subsequent studies in an *in vitro* model system showed that MTP could transfer phosphosphatidylethanolamine and phosphatidylcholine to recombinant CD1d molecules, and cells treated with MTP chemical inhibitors or siRNA-mediated silencing of MTP expression showed diminished presentation of α-GalCer to NKT cells (Dougan *et al.*, 2005, 2007). More recent work has established the importance of MTP in the presentation of lipid antigens by Group I CD1a, b, and c molecules (Kaser *et al.*, 2008). In primary human DC, blockade of MTP resulted in profound inhibition of CD1a, b, and c expression at the cell surface. Interestingly, even under conditions in which CD1 expression was still present at the cell surface in CD1 transfectant cells, MTP blockade markedly abrogated the presentation of exogenously added microbial lipid antigens (Kaser *et al.*, 2008). Together, these studies on CD1a, b, c, and d implicate MTP both in the presentation of self-lipid antigens and in the presentation of endosomally acquired exogenous lipid antigens. Since MTP is resident in the ER and not reported to be found in late endosomes, the mechanism by which it might alter exogenous lipid antigen presentation or recycling from the lysosome to the plasma membrane is not known (Kaser *et al.*, 2008; Sagiv *et al.*, 2007).

2.5.2. Antigen processing in the endocytic system

Evidence suggests that some lipid antigens are processed most likely in late endosomes or lysosomes. Acidic pH such as in lysosomes can activate cellular glycosidases involved in the processing of complex glycolipid headgroups. To date, three lipid glycosidase enzymes have been implicated in CD1 lipid processing: α-D-mannnosidase, α-galactosidase, and β-hexosaminidase, and others are likely to participate. These enzymes were first identified for their roles in cellular glycolipid metabolism and the absence of any of these enzymes leads to lysosomal storage diseases characterized by inappropriate intracellular accumulation of glycolipid species that cannot be metabolized. α-D-Mannosidase was first appreciated to play a role in the trimming of LAM (Sieling *et al.*, 1995) and the headgroup of PIM series molecules (de la Salle *et al.*, 2005). Processing of a synthetic lipid substrate, GalαGalCer, by α-galactosidase enzyme was necessary to reveal the α-GalCer moiety stimulatory to NKT cells (Prigozy *et al.*, 2001). A role for β-hexosaminidase is suggested by the finding that mice deficient in β-hexosaminidase fail to develop NKT-cells (Zhou *et al.*, 2004b). A number of lysosomal storage diseases broadly affecting the lipid content, structure, and function of lysosomes all lead

to defects in human CD1d presentation (Gadola *et al.*, 2006). These enzymes may alter the processing of antigenic self-lipids or the effects may be nonspecific or they may so alter lysosomal function that they nonspecifically interfere with CD1 loading and presentation.

Recently, CD1e was shown to play a role in facilitating the processing of PIM6 to PIM2 by mannosidase enzyme through promoting accessibility and solubility of the microbial lipid to the processing enzyme (de la Salle *et al.*, 2005). CD1e molecules themselves are found in soluble forms in the late endocytic system, and are targeted there by a series of unique ubiquitinations of the cytoplasmic tail without first reaching the plasma membrane (Angenieux *et al.*, 2005; Maitre *et al.*, 2008). Although no CD1e atomic structure has been determined, CD1e is predicted to form one large hydrophobic cavity with which it might bind and transport lipids for enzymatic accessibility. The CD1e gene is relatively more polymorphic when compared to the other CD1 isoforms, with at least a subset of these polymorphisms affecting its function in solubilizing microbial lipids (Tourne *et al.*, 2008). Thus, CD1e may influence processing or loading onto CD1d isoforms but its precise function is not yet clear.

Many lipid antigens may not require processing. For example, one lipid with a complex headgroup structure, the self-lipid antigen GM1 which binds CD1b, does not require processing (Shamshiev *et al.*, 2000). Similarly, other lipids with less complex headgroup structures, such as sulfatide or cellular phospholipids, also lack intracellular processing requirements as evidenced by their ability to bind CD1 isoforms both on the cell surface and in cell free *in vitro* plate-binding assays (Gumperz *et al.*, 2000; Shamshiev *et al.*, 2002). CD1a contains a solvent accessible F′ pocket which may allow significant surface loading of unprocessed lipids, and evidence exists that CD1c loading may follow pathways independent of lysosomal acidification (Briken *et al.*, 2000). Thus, lipid antigens can be expected to exhibit differential processing requirements, making them distinct from protein antigenic moieties for MHC molecules, which must always be processed through hydrolysis into peptides. Additionally, to date no examples of cellular processing enzymes covalently modifying lipid tail structures to reveal an antigenic moiety from a microbial lipid have been reported. Since $CD1^+$ APC are known to possess desaturases and other fatty acid metabolic enzymes capable of modifying aliphatic hydrocarbon chains, such processing is at least feasible.

2.5.3. Antigen loading in the endocytic system

Lipid antigen loading into CD1 in liposomes is facilitated by low pH, conformational changes in CD1, and saposin-related accessory proteins. The requirement for lysosomal acidification for T cell antigenicity were demonstrated in early studies by showing that chloroquine treatment abrogated antigen presentation by CD1b (Porcelli *et al.*, 1992). Subsequent experiments

further established this principle for CD1b recognition of LAM molecules and demonstrated that subsets of CD1d-restricted T cells also depend on endocytic trafficking and lysosomal acidification in order to acquire stimulatory capacity for T cells (Roberts *et al.*, 2002; Sieling *et al.*, 1995).

Several studies have shown more efficient lipid antigen loading into CD1b and CD1d in the lysosomal pH range (Batuwangala *et al.*, 2004; Gilleron *et al.*, 2004; Gumperz *et al.*, 2000; Moody *et al.*, 2002; Roberts *et al.*, 2002). Endosomal pH not only promotes the activity of lysosomal hydrolytic enzymes but also can affect the conformation of CD1 molecules themselves. Using surface plasmon resonance, Ernst *et al.* (1998) demonstrated that CD1b molecules bind LAM antigens at a pH optimum of 4.0, with binding completely abrogated by pH values greater than 6.0. Circular dichroism measurements demonstrated that CD1b molecules become partially unfolded at low pH, potentially rendering the antigen-binding groove into a more lipid-accessible state (Ernst *et al.*, 1998).

Moving lipids from membranes to load into CD1 molecules may involve the help of lipid-loading accessory proteins. Saposins were originally described for their roles in GSL metabolism and the storage diseases that result when they are defective (Sandhoff and Kolter, 2003). However, saposins are now known to play a role in lipid loading into CD1. Saposins consist of four isoforms that can either directly bind GSLs in membrane bilayers or disrupt membrane bilayers, rendering individual lipids more accessible to other accessory lipid-binding proteins. Saposins were shown to mediate loading of exogenous lipids onto human CD1b and CD1d, as well as murine CD1d molecules (Fig. 1.5) (Kang and Cresswell, 2004;

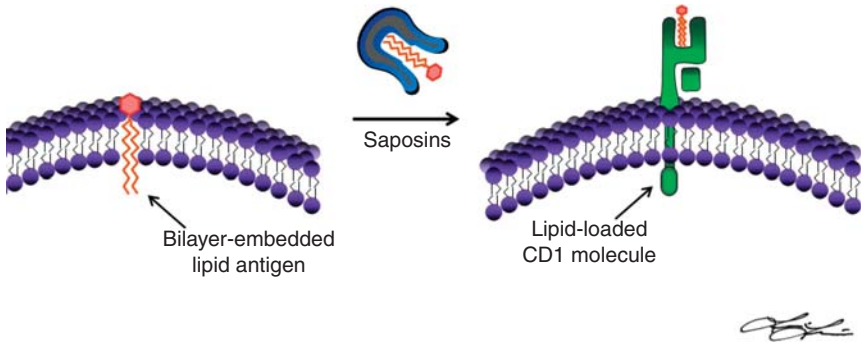

FIGURE 1.5 The role of saposins in lipid antigen loading. Lipid antigens typically localize in cellular membranes. In order to load into CD1, the lipid must be removed from a highly hydrophobic membrane and be transferred into the membrane distal antigen-binding domain of the CD1 HC. This process can be facilitated by sphingolipid activator proteins (SAPs or saposins). Saposins may operate either by partially disrupting lipid bilayers making membrane-bound lipids more accessible for loading into CD1. Alternatively, saposins may directly bind and transfer lipids into the CD1-binding pockets.

Winau *et al.*, 2004; Zhou *et al.*, 2004a). Individual saposin isoforms are more adept at loading particular CD1 isoforms than others; Saposin C specifically was required for CD1b presentation of GMM, LAM, and mycolic acid while Saposin B was most efficient in promoting transfer of α-GalCer to human CD1d (Winau *et al.*, 2004; Yuan *et al.*, 2007). Isoelectric focusing experiments suggest that in the case of mCD1d loading, saposins can directly transfer lipids from membrane bilayer structures such as liposomes into the groove of CD1d (Zhou *et al.*, 2004a). The same study also identified another endosomal lipid modifying protein, GM2 activator, as capable of transferring α-GalCer onto CD1d molecules. Thus, for CD1 molecules localized in lysosomes, successful antigen loading likely depends on the combination of optimal pH and assistance of saposins.

3. GROUP I CD1: CD1a, b, AND c MOLECULES AND T CELLS

The human CD1 genes are organized into Group 1 (CD1a, b, c), Group 2 (CD1d), and Group 3 (CD1e) based on sequence similarity and organization in the locus. Group 1 genes appear to have been deleted during evolution in muroid rodents (Dascher and Brenner, 2003) but have been found in all other mammalian species studied (Dascher, 2007). Studies on the nature of CD1a, b, and c in humans have focused on expression on professional APC, the presentation of lipid antigens from *M. tuberculosis*, and the activation of CTL and Th1-like T cells. CD1a, b, and c-restricted T cells have extensive diversity in their TCRs and may provide adaptive immunity against microbes, indicating a fundamental difference in their nature compared to CD1d-restricted iNKT cells.

3.1. Expression of CD1 on DC and other professional APC

CD1 proteins were first defined based on their expression on immature thymocytes, leading to their designation as CD1 (Bernard and Boumsell, 1984; McMichael *et al.*, 1979; Reinherz *et al.*, 1980). In addition, CD1a, b, and c are primarily expressed on professional APC (DCs and B cells) and in this feature resemble MHC Class II more than the broadly expressed MHC Class I. Although human blood monocytes lack expression of CD1a, b, and c, all three of the Group I CD1 isoforms are upregulated during the process of myeloid DC differentiation. CD1a, b, and c expression on myeloid DC often serve as DC markers (Brigl and Brenner, 2004) but the pattern of expression may vary among DC subsets. Studies on human DC employ *in vitro* differentiation systems to complement analysis *in vivo*. Depending on the culture conditions, blood monocyte-derived DC can express CD1a, b, and c or alternatively, lack expression of CD1a but still

express CD1b and c. Human blood monocytes ($CD14^+$) cultured with GM-CSF and IL-4 (Kasinrerk *et al.*, 1993; Porcelli *et al.*, 1992) develop into CD1a, b, and c expressing DC. Furthermore, when cultured in the presence of GM-CSF and IL-15 or TGF-β1, upregulation of Langerin and E-cadherin along with CD1a occurs, typical of the Langerhans DC phenotype (Geissmann *et al.*, 1998; Mohamadzadeh *et al.*, 2001). In addition to differentiation from blood monocytes, $CD34^+$ hematopoietic precursors from adult or fetal (cord) blood, when stimulated with cytokines (GM-CSF, stem cell factor (SCF), and TNF-α), also differentiate into to several DC subsets (Caux *et al.*, 1996; Gatti *et al.*, 2000). Some $CD34^+$ precursors differentiate into DC that acquire CD1a or alternatively may differentiate into a subset that lacks Langerin and expresses CD1a, b, and c (Banchereau *et al.*, 2000; Caux *et al.*, 1992, 1996). Although CD1a can be expressed on several DC subsets, it has been used widely as a key marker expressed of Langerhans cells and of Langerhans cell histiocytosis (Crawford *et al.*, 1989; Fithian *et al.*, 1981; Sholl *et al.*, 2007). Human Langerhans cells appear to express high levels of CD1a, moderate levels of CD1c but typically lack CD1b and CD1d in situ (Caux *et al.*, 1992; Gerlini *et al.*, 2001; Nestle *et al.*, 1993; Ochoa *et al.*, 2008). Dermal DCs express CD1a, b, and c (Ochoa *et al.*, 2008).

In mice, the separation of myeloid DC into subsets with defined functions has been highly informative. CD8α is one of the most important phenotypic markers for the subset of murine splenic DC that are capable of producing high levels of IL-12p70, enabling Th1 T cell polarization *in vitro* (Hochrein *et al.*, 2001; Maldonado-Lopez *et al.*, 1999; Ohteki *et al.*, 1999). However, while human DC can also produce significant levels of IL-12, CD8α is not expressed on human DC. This raises the question of whether IL-12 production is a function of a particular human DC subset, and if so, whether another marker identifies such a population. Strikingly, CD1a expression has been found to serve as a maker for human myeloid DC derived either from blood monocytes or $CD34^+$ precursors that are major IL-12p70-producing Th1 T cell-polarizing DC (Gogolak *et al.*, 2007) (Cernadas *et al.*, 2009). Correspondingly, human DC lacking CD1a have been noted to lack IL-12 production and instead to produce IL-10 and polarize Th0/Th2 T cells (Chang *et al.*, 2000; Sacchi *et al.*, 2007) (Cernadas *et al.*, 2009). The differentiation of both $CD1a^+$ and $CD1a^-$ DC from blood monocytes or $CD34^+$ precursors is typical for *in vitro* culture systems using GM-CSF. These cells have the typical appearance of immature DC that express CD1a, b, and c as well as CD40 and DC-SIGN (CD209) (Porcelli *et al.*, 1992; Sallusto and Lanzavecchia, 1994), although it should be noted that DC-SIGN, once considered a DC marker (Engering *et al.*, 2002; Geijtenbeek *et al.*, 2000), now has been suggested to be expressed mainly on macrophages *in vivo* (Ochoa *et al.*, 2008). When stimulated with CD40L or LPS, these DC undergo typical changes of DC maturation and

upregulate MHC Class II and CD83 at the cell surface (Hava *et al.*, 2008; Sallusto *et al.*, 1995). Studies using these *in vitro* DC differentiation systems have also revealed that lipids and peroxisome proliferator-activated receptor γ (PPARγ) activation influence the expression of CD1 molecules during the DC differentiation process. While serum lipids that stimulate PPARγ activation reduced the expression of CD1a, b, and c, they increased the expression of CD1d (Gogolak *et al.*, 2007; Leslie *et al.*, 2008; Szatmari *et al.*, 2004). Other serum factors may also influence CD1 expression as heparin has been noted to enhance CD1a expression on DC, even in the presence of other serum factors (Xia and Kao, 2002). Thus, CD1 upregulation occurs during myeloid DC differentiation and then may be regulated by factors such as PPARγ stimulators. The expression patterns of CD1a, b, c, and d not only reflect the professional antigen-presenting capacity of the DC, but in the case of CD1a, also correlates with their IL-12-producing capability. CD1a, b, and c molecules can also be upregulated following exposure of monocytes to microbial TLR agonists as it also induces their differentiation to DC (Roura-Mir *et al.*, 2005). Few studies have examined plasmacytoid DC (pDC), but they were noted to lack expression of CD1a, b, c, or d (Liu, 2005).

CD1c is unique as the only Group I CD1 isoform that is expressed on particular subsets of B cells such as in the mantle zones of lymph nodes, in the marginal zones of spleen (Smith *et al.*, 1988), and on circulating B cells (Plebani *et al.*, 1993; Small *et al.*, 1987).

3.2. TCRs of CD1a, b, and c-restricted T cells

Group I CD1a, b, and c-specific T cells recognize antigen by their TCR αβ (Porcelli *et al.*, 1989, 1992). Transfection of the αβTCRs showed that the TCR confers specificity for both lipid antigen and CD1 isoform (Grant *et al.*, 1999, 2002), analogous to peptide antigen-specific and MHC-restricted recognition. Importantly, sequence analysis of a panel of αβ TCRs from microbial antigen-specific individual CD1 a, b, and c-restricted T cells reveals they have extensive germline (V and J) and junctional diversity reflecting template-independent N nucleotides that together encode clonally unique, diverse sequences (Grant *et al.*, 1999). Furthermore, mutational analyses of the TCRβ CDR3 loop predicted to interact with bound lipid antigen abrogated recognition as did mutation of the TCRβ CDR1 loop predicted to interact with the CD1 α-helices. While no TCRαβ-CD1a, b, c cocrystal atomic structure has yet been solved, both modeling (Grant *et al.*, 2002) and CD1b mutagenesis (Melian *et al.*, 2000) suggest a TCR diagonal orientation on the CD1 antigen complex, similar to the orientation of TCR recognition of MHC peptide complexes. If these predictive models are correct, it would suggest a different interaction compared to that of the determined Vα14 Jα18 iNKT cell TCR interaction with CD1d which

displays an unusual parallel orientation focused at the edge of the CD1d α-GalCer antigen complex (Borg *et al.*, 2007).

A number of studies found that γδ T cells recognize CD1. Several human T cell clones that expressed Vδ1-containing TCRs were self-reactive and recognized CD1c in the absence of foreign lipid antigens (Faure *et al.*, 1990; Spada *et al.*, 2000). Interestingly, since γδ T cells are known to be a large percentage of intestinal intraepithelial lymphocytes, an analysis of their specificity revealed that two thirds of the intraepithelial γδ T cells were CD1 reactive (Russano *et al.*, 2007). A majority of the γδ T cells expressed a Vδ1 gene rearrangement, but in addition to CD1c, these clones recognized both self and exogenously added lipids on CD1a, c, and d transfectant APC.

3.3. Effector functions of CD1a, b, and c-restricted T cells

CD1a, b, and c-restricted T cells appear to have a broad range of functions that include most of the effector capabilities of MHC Class I and II-restricted T cells. Similarly, they may express CD4, CD8 or can be CD4/8 double negative (DN), and thus make up a part of the T cell subsets previously assumed to be MHC restricted. Nearly all of the T cell responses to date have been defined for T cells that recognize mycobacterial antigens including *M. tuberculosis*, *M. leprae*, *M. bovis* bacillus Calmette-Guerin (BCG) and other species. The range of lipid-containing antigens identified is extensive and includes examples from virtually every class of lipid contained in the mycobacterial cell wall, including various mycolates, sulfated trehaloses (sulfolipids), mycoketides, phospholipids, lipopeptides, and diacylglycerols such as LAM and various PIMs (Fig. 1.1). The first microbial lipid antigen-specific Group I CD1-restricted T cells described lacked CD4 and CD8 (DN) (Porcelli *et al.*, 1992). Subsequently, in addition to other DN T cells, both CD8 (Rosat *et al.*, 1999) and CD4 (Sieling *et al.*, 2000) expressing T cells were found. Although the relative frequency of CD1 versus MHC Class I or II reactive T cells in the CD4 and CD8 T cell pools are not known, Kawashima *et al.* (2003) found that a majority of the $CD8^+$ T cells in the circulation that recognize live BCG infected DC were CD1 restricted.

Nearly all of the mycobacterial lipid antigen-specific CD1a, b, and c-restricted T cells secrete Th1-like cytokines including IFN-γ. These include *M. leprae* reactive $CD4^+$ T cells (Sieling *et al.*, 2000), *M. tuberculosis* and BCG reactive $CD8^+$ T cells (Kawashima *et al.*, 2003; Rosat *et al.*, 1999), and *M. tuberculosis* reactive DN T cells (Sieling *et al.*, 1995). Since IFN-γ is a major cytokine important for activating macrophages that are the main cells infected with mycobacteria, these CD1-restricted, mycobacteria-specific T cells may participate in control of infection via their effects on

macrophages. In addition, both the cytokines (IFN-γ and TNF-α) produced by CD1 reactive T cells as well as their upregulation of CD40L can stimulate DC maturation and instruction towards a Th1-polarizing phenotype (Leslie *et al.*, 2002; Vincent *et al.*, 2002).

CD1a, b, and c-restricted recognition of infected CD1 expressing APC has been particularly striking. In several cases, the mycobacterial antigen that is recognized is only synthesized or upregulated after infection. For example, following infection of DC, mycobacteria upregulate the biosynthesis of siderophores, like mycobactin, in order to scavenge iron which is essential for intracellular survival of the bacterium. DDM, a likely breakdown product of mycobactin generated following mycobactin synthesis, is a lipopeptide that is presented by CD1a, allowing the immune system to detect the infected DC via CD1a-restricted $CD8^+$ T cells (Moody *et al.*, 2004). In another example, mycobaceria can produce GMM despite not synthesizing glucose, by using the host as the source for glucose, in order to couple mycobacterial mycolates to glucose. The specific antigen recognized by human CD1-restricted T cells, GMM, thus is formed by the interaction of host and pathogen biosynthetic pathways (Moody *et al.*, 2000a). APC infected with live mycobacteria were recognized by GMM-specific CD1-restricted T cells while APC pulsed with killed organisms were not (Kawashima *et al.*, 2003). Interestingly, in several other examples, recognition of the mycobacteria-infected DC occurs earlier and more efficiently for CD1-specific T cells compared to MHC-restricted T cells because of differential immune evasion strategies (Hava *et al.*, 2008).

Recognition of infected APC also provides an opportunity for CD1-restricted T cells to lyse the infected cells and contribute to control of bacterial growth. Both $CD8^+$ and DN CD1-restricted αβ and γδ T cells are efficient CTL (Beckman *et al.*, 1994, 1996; Porcelli *et al.*, 1989; Rosat *et al.*, 1999; Spada *et al.*, 2000; Stenger *et al.*, 1997). These CTL utilize perforin as well as FasL to mediate cytolysis (Spada *et al.*, 2000; Stenger *et al.*, 1997). Importantly, besides lysis of antigen pulsed or infected APC, cytolytic $CD8^+$ CD1-restricted T cells also exhibit bactericidal effects mediated by granulysin that reduced mycobacterial growth (Stenger *et al.*, 1997, 1998a).

Analyses of live *M. tuberculosis*-infected DC have further revealed distinct roles for CD1 versus MHC-restricted T cells. Following infection, CD1 molecules were found to colocalize with *M. tuberculosis* organisms in phagolysosomes (Hava *et al.*, 2008; Schaible *et al.*, 2000). *M. tuberculosis* lipid antigens were presented rapidly in the first 24 h following infection of DC and then continued to be presented efficiently for at least 72 h thereafter. The *M. tuberculosis* phagolysosome in DC formed rapidly but did not maintain contact with the early endocytic system (van der Wel *et al.*, 2003) and instead rapidly acquired key lysosomal proteins (CD63, LAMP, and saposin). These microbial phagolysosomes are the likely

antigen-loading compartment (Hava *et al.*, 2008). Interestingly, DC maturation occurred rapidly in infected DC and this did not alter CD1-based antigen presentation. In contrast, rapid DC maturation occurred prior to the secretion of immunodominant *M. tuberculosis* protein antigens such as Ag85 and CFP10 resulting in evasion of MHC Class II peptide presentation during the first few days following infection. Together, these results underscore the fact that CD1-mediated lipid antigen presentation may be less dependent on DC maturation compared to MHC-based antigen presentation (Cao *et al.*, 2002; Hava *et al.*, 2008). In antigen pulsed cells, mature myeloid DC-presented protein antigens more efficiently than immature DC, while for CD1-presented lipids, DC maturation had little or no effect on presentation efficiency (Cao *et al.*, 2002). Such differences between CD1 and MHC Class II presentation highlight how these systems of antigen presentation may be distinct and complement one another. Besides differences in the chemical nature of the antigens presented, the CD1 molecules traffic differently and survey many endocytic compartments and utilize different accessory molecules for antigen loading compared to MHC Class I or II. Thus, they provide a distinct opportunity for antigen uptake, sampling, presentation and cell-mediated immunosurveillance.

3.4. Immune evasion of Group I CD1-based antigen presentation

Not surprisingly, microbes that have evolved as successful pathogens that exhibit mechanisms of immune evasion of CD1a, b, and c antigen presentation. Several pathogens (*M. tuberculosis*, *M. bovis* BCG, *L. donovani*, and *Leishmania major*) can block the upregulation of Group I CD1 molecules that normally occurs during the process of monocyte differentiation into DCs in response to cytokines such as GM-CSF and IL-4 (Amprey *et al.*, 2004a,b; Donovan *et al.*, 2007; Giuliani *et al.*, 2001; Mariotti *et al.*, 2002). In most instances, live infection is necessary to prevent CD1a, b, and c expression. In addition, Pertussis toxin blocks the upregulation of CD1a during monocyte to DC differentiation, but does not influence CD1b or c induction (Martino *et al.*, 2006). Besides blocking induction of CD1 expression, some pathogens also appear to selectively downregulate CD1 molecules that are already expressed on DC. For example, HIV-nef, known for its ability to downregulate MHC Class I and II, is also capable of downregulating CD1a (but not CD1b or c) (Shinya *et al.*, 2004). HSV-1 displays a complex pattern *in vitro* of upregulating cell surface CD1 molecules at low multiplicity of infection (MOI) and downregulating expression at high MOI (Raftery *et al.*, 2006). In one report (Stenger *et al.*, 1998a,b), live infection with *M. tuberculosis* downregulated surface CD1 expression in DC. However, CD1 a, b, and c expression was not reduced by live infection of myeloid DC in several other reports (Hava *et al.*, 2008; Henderson *et al.*, 1997) and expression was actually upregulated strongly in another

report (Roura-Mir *et al.*, 2005). CD1 levels were partially downregulated on some but not all donors following infection with Leishmania (Amprey *et al.*, 2004a,b; Donovan *et al.*, 2007) but was not downmodulated significantly by BCG infection (Giuliani *et al.*, 2001). Thus, blocking CD1 upregulation during the process of monocyte differentiation into DC may occur in some instances, and downregulating CD1a, b, and c molecules already expressed on DC appears to be more difficult and the effects may be partial and donor dependent. These differences reflect the battle between host efforts to detect microorganisms and immune evasion efforts of pathogens.

3.5. CD1a, b, and c responses *in vivo* and vaccine potential

The *in vitro* analyses above all support the likely role of CD1a, b, and c-restricted T cells in microbial defense based on their effector functions including Th1-like cytokine secretion, cytolysis and bactericidal activities. However, in humans it has not been possible to directly demonstrate their protective role *in vivo*. Importantly, correlative studies reveal that CD1a, b, and c-restricted T cells are detected and expanded in humans, and both CD1-bearing APC and CD1 reactive T cells are found in tissue lesions *in vivo*. Ulrichs *et al.* (2003) analyzed PPD-positive individuals known to have had contact with tuberculosis patients and found significant IFN-γ ELISPOT responses to lipid antigens, while such responses were absent from PPD-negative subjects. CD1-restricted T cells recognizing *M. tuberculosis* extracts or purified GMM were significantly increased in asymptomatic *M. tuberculosis* donors compared to uninfected controls. Interestingly, CD1-restricted T cells were not found in patients with active tuberculosis infection, but they became detectable within a few weeks after starting antibiotic treatment. Most of the lipid reactive T cells were found in the $CD4^+$ T cell pool. In another study, Gilleron *et al.* (2004) found increased IFN-γ production by T cells stimulated with *M. tuberculosis* sulfoglycolipids from PPD^+ donors and from patients with active pulmonary tuberculosis compared to controls. Studies in leprosy have confirmed the expression of CD1a, b, and c molecules on DC in tuberculoid leprosy lesions in skin, but not in lepromatous lesions (Sieling *et al.*, 1999). Correspondingly, a significantly higher frequency of CD1-restricted IFN-γ-producing T cells could be detected by ELISPOT in the blood of tuberculoid leprosy patients in response to *M. leprae* extracts, compared to lepromatous leprosy patients (Sieling *et al.*, 2005). Together, these studies support the proposal that CD1a, b, and c-restricted T cells accumulate and expand *in vivo* during and after infection with mycobacteria in humans. No CD1-based vaccine trials for mycobacterial infections have been reported in humans; however, studies in Guinea pigs using *M. tuberculosis* lipid extracts as vaccines suggest that improvement in

infection and pathology may be achieved (Dascher *et al.*, 2003) and relate to CD1-restricted mycobacterial lipid antigen responses (Hiromatsu *et al.*, 2002).

4. GROUP II CD1: CD1d-RESTRICTED T CELLS: ACTIVATION, FUNCTION, AND ROLE IN ANTIMICROBIAL IMMUNITY

T cells restricted to CD1d, which are present both in mice and humans, fall into two categories that have been defined on the basis of TCR diversity. Cells of the first subset, commonly referred to as iNKT cells, express a semi-invariant TCR consisting of Vα14 Jα18 paired with Vβ2, 7 or 8.2 in mice and Vα24 Jα18 with Vβ11 in humans. As will be discussed below, iNKT cells exhibit a distinctive memory/effector phenotype, recognize both self and foreign lipid antigens, and have been found to play an important role in the immune response to a range of microbes. CD1d-restricted T cells of the second subset express a much more diverse TCR repertoire and will be referred to as diverse natural killer T (dNKT). The biology of dNKT cells and whether it resembles that of iNKT cells remains largely unknown. In this section, we focus primarily on iNKT cells and their functions, though we have included a discussion of dNKT cells where information is available.

4.1. Activation of iNKT cells

4.1.1. Constitutive activation of iNKT cells in the absence of infection

4.1.1.1. The effector/memory phenotype of resting iNKT cells iNKT cells circulate in a distinctive state of partial activation. Phenotypically, they resemble effector memory T cells, with high surface levels of activation markers such as CD44 and CD69 (Bendelac *et al.*, 1992) (see also Matsuda *et al.*, 2000) and low expression of CD62L. iNKT cells display similar homeostatic maintenance requirements as antigen-experienced T cells, namely, a dependency on cytokines such as IL-15 and IL-7 but no requirement for CD1d, their selecting antigen presentation molecule, for survival in the periphery (Matsuda *et al.*, 2002). Furthermore, neither effector memory T cells nor iNKT cells require classical costimulatory signals to secrete cytokines following TCR engagement (Uldrich *et al.*, 2005). In fact, data suggest that preformed cytokine-encoding mRNA transcripts are present constitutively in the cytoplasm of iNKT cells, gearing them for rapid effector responses (Stetson *et al.*, 2003). However, in contrast to MHC-restricted memory cells, the iNKT cell active state does not require prior recognition of foreign antigens. Indeed, iNKT cells from mice raised in a germ-free environment as well as neonatal iNKT cells isolated from

human cord blood display a similar activated phenotype (D'Andrea *et al.*, 2000; Park *et al.*, 2000; van Der Vliet *et al.*, 2000).

4.1.1.2. Self-reactivity of iNKT cells Autoreactivity has emerged as a defining feature of iNKT cells. Early on, it was demonstrated that iNKT cell hybridomas secrete cytokines upon recognition of CD1d molecules at the surface of thymocytes in the absence of exogenously added antigens (Bendelac *et al.*, 1995). In mice, endosomal trafficking of CD1d is required for development and self-reactivity of iNKT cells, as are lysosomal lipid transfer proteins (Cernadas *et al.*, 2003; Chiu *et al.*, 2002; Elewaut *et al.*, 2003; Zhou *et al.*, 2004a). Thus, it has been assumed that murine iNKT cells are reactive to endogenous lipids acquired by CD1d in the lysosome, not to empty CD1d molecules. In humans, however, the origin of iNKT cell autoreactivity is less dependent on lysosomal trafficking of CD1d for activation in the absence of foreign antigens (Chen *et al.*, 2007).

The identification of self-lipids presented by CD1d has been informative although the range of physiologically relevant antigens is not yet fully understood. Cellular phospholipids can act as self-antigens for iNKT cells. Biochemical analyses have detected endogenous GPI and phosphatidylinositol (PI) molecules inside mouse CD1d (De Silva *et al.*, 2002; Joyce *et al.*, 1998). Although subsequently murine iNKT cells were shown to recognize synthetic mimics of mammalian GPI (Schofield *et al.*, 1999), the finding that GPI-deficient APCs are fully able to elicit murine iNKT cell autoreactivity has challenged the relevance of these lipids as important self-antigens. Separately, PI, PE (phosphatidyl ethanolamine) and phosphatidylglycerol (PG) purified from mammalian cell lines were found to be the major stimulatory self lipids for one iNKT cell hybridoma and at the same time serve as weakly stimulatory for a number of other iNKT cell hybridomas. (Gumperz *et al.*, 2000; Rauch *et al.*, 2003). Endogenous ceramides have also been proposed as iNKT cell antigens. Specifically, iGb3, a GSL of the isoglobo series, was proposed to be the master self-antigen for iNKT cells, driving both positive selection in the thymus and partial activation at the periphery (Zhou *et al.*, 2004b). This hypothesis was based on the observation that mice deficient in β-hexosaminidase (HexB), an enzyme catalyzing the degradation of iGb4 into iGb3, had severely decreased numbers of iNKT cells, and that iNKT cells lacked autoreactivity to $HexB^{-/-}$ APCs. However, the major pathway for biosynthesis of the entire isoglobo series of glycosphingolipids (GSLs) from lactosylceramide is mediated by the enzyme iGb3 synthase. The recent finding that iGb3 $synthase^{-/-}$ mice have normal levels of functional iNKT cells (Porubsky *et al.*, 2007) makes it unlikely that iGb3 is the main selecting self-lipid in murine iNKT cell development. Further, the iNKT cell defect in $HexB^{-/-}$ animals was shown to be non-specific, as similar defects occur for a range of enzymes not directly influencing this pathway. Finally iGb3 has been extremely difficult or impossible to detect in

the thymus or in DCs of mice and humans (Li *et al.*, 2008; Speak *et al.*, 2007), and the gene encoding the iGb3 synthase in humans is nonfunctionnal (Christiansen *et al.*, 2008). Thus, although iGb3 is an antigenic self-lipid, more recent data indicate it is not a required self-lipid antigen for iNKT cell selection or activation. Rather than a single self lipid, it remains possible that a range of self-lipids with varying agonist activity all participate in iNKT cell biology.

4.1.2. Mechanisms of iNKT cell activation in the context of microbial infection

iNKT cells participate in the immune response to a broad range of bacteria, viruses and fungi (Tupin *et al.*, 2007). How this lymphocyte population displaying a limited TCR repertoire and restricted to a nonpolymorphic antigen-presenting molecule can become activated by a vast array of microorganisms is an important question. Do iNKT cells recognize conserved or common foreign lipids, like innate germline-encoded receptors would microbial patterns, or are CD1d and the invariant TCR uncommonly promiscuous in their ability to bind structurally diverse lipid antigens? While the quest for microbial lipids recognized by iNKT cells has yielded a number of candidates, an intriguing alternative mechanism of activation, independent on foreign lipid recognition has recently emerged. Both mechanisms are described in the following subsections (Fig. 1.6).

4.1.2.1. Cognate recognition of CD1d-restricted foreign lipid antigen by iNKT cells A number of microbial lipids can be presented by human CD1a, b, and c molecules and recognized by T cells. Until recently, however, the only known nonmammalian lipid capable of activating iNKT cells in a CD1d-restricted manner was a GSL derived from a marine sponge, α-GalCer, discovered during a screen for compounds with therapeutic activity against liver cancer (Kawano *et al.*, 1997; Kobayashi *et al.*, 1995). The atypical α-linkage connecting the sugar to the sphigosine base is required for iNKT cell recognition, since β-GalCer is not antigenic. The exceptional potency of this synthetic pharmacological antigen has made it a useful study tool, though it is not a physiologically relevant microbial antigen.

Recently, several microbial lipids were discovered that bind to CD1d and activate iNKT cells. Specifically, certain natural GSLs from *Sphingomonas* proteobacteria (Kinjo *et al.*, 2005, 2008; Mattner *et al.*, 2005) and galactosyl diacylglycerols from *B. burgdorferi*, the bacterium responsible for Lyme disease (Kinjo *et al.*, 2006b) can be presented in CD1d and stimulate iNKT cells. Interestingly, although these compounds belong to separate lipid classes and differ with respect to backbone and fatty acid composition, they possess the same α-anomeric linkage connecting their

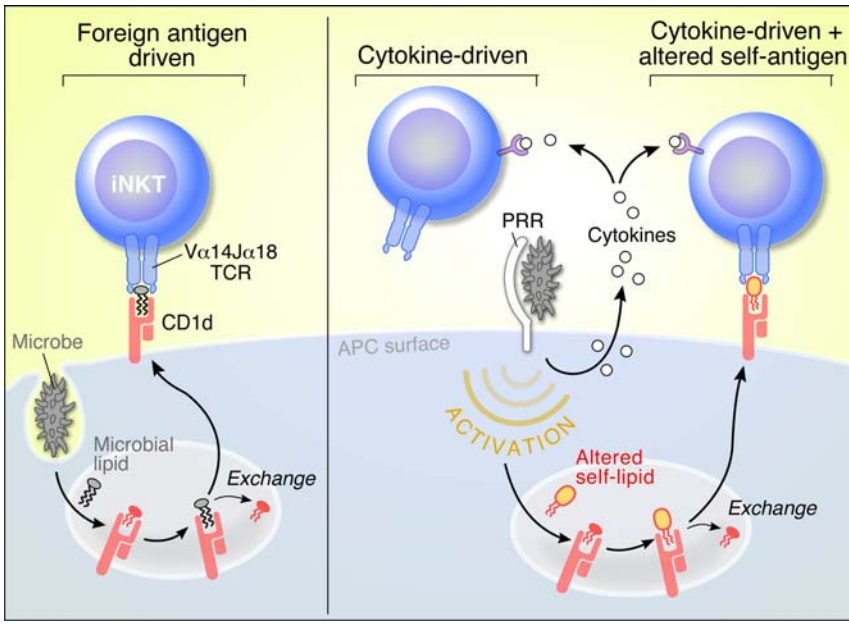

FIGURE 1.6 Mechanisms of iNKT cell activation by microbes. (Left panel) iNKT cells can become activated following cognate, TCR-mediated recognition of foreign microbial lipids presented in CD1d. (Right panel) iNKT cell activation can also occur independently of foreign lipid recognition. iNKT cells can be activated by inflammatory cytokines alone or in together with CD1d-presented altered self-lipids produced by APCs upon ligation of innate pattern recognition receptors (PRRs).

respective sugar headgroups to the lipid backbone as α-GalCer, suggesting this pattern may be an important determinant of iNKT cell recognition.

Other microbial lipids suggested to serve as cognate iNKT cell antigens include some *Leishmania* surface phospholipids (LPG and glycoinositol phospholipids (GIPLs)) (Amprey *et al.*, 2004a,b), and the mycobacterial PIM, in particular PIM4 (Fischer *et al.*, 2004). However, only a small fraction of α-GalCer-reactive cells appear to also recognize LPG and GIPLs. Given the bulky headgroups of these antigens, one might expect that they could not be accommodated between the CD1 α-helices and the TCR and thus would require extensive processing to become antigenic. Furthermore, the antigenicity of PIM4 (Fischer *et al.*, 2004) could not be confirmed using a synthetic version of this lipid (Kinjo *et al.*, 2006b).

The finding that iNKT cells can recognize certain microbial lipids is significant and makes it clear that CD1d, like Group I CD1s, can present foreign lipid antigens. However, it is surprising that despite much effort, so few microbial lipid antigens have been discovered. This observation,

combined with work documenting the response of iNKT cell to viruses that do not encode lipids suggest stimulatory mechanisms independent of microbial lipid recognition must exist.

4.1.2.2. Foreign antigen-independent activation of iNKT cells by microbes
Under homeostatic conditions *in vivo*, self-reactivity is insufficient to prompt iNKT cell effector functions. However, there is evidence that during infection, autoreactivity may be harnessed to allow rapid activation of iNKT cells without the need for foreign lipid recognition. Indeed, weak TCR stimuli provided by endogenous antigens appear to sensitize iNKT cells to inflammatory cytokines elicited by APC responses to innate immune recognition of pathogens. In the context of *Salmonella typhimurium* infection, IL-12 produced by DCs in response to TLR4 stimulation by bacterial LPS elicited iNKT cell activation in a CD1d-dependent but foreign lipid-independent manner (Brigl *et al.*, 2003; Mattner *et al.*, 2005). Activation of iNKT cells by the helminth *Schistosoma* was proposed to occur following a similar indirect pathway (Mallevaey *et al.*, 2006). Recently, DCs recognizing nucleic acids though TLR7 and TLR9 were shown to activate iNKT cells. In these experiments, Type I IFN secretion by TLR9-stimulated DCs combined with CD1d-restricted self-lipid recognition was sufficient for iNKT cell activation (Paget *et al.*, 2007). It was subsequently demonstrated that engagement of nearly any TLR on DCs is sufficient to lead to such iNKT cell activation (Salio *et al.*, 2007).

In addition to creating an inflammatory cytokine milieu, detection of microbes by APCs causes the APC to mature and become more efficient at presenting self-lipids to iNKT cells. Recognition of bacterial products from *Escherichia coli* or *S. typhimurium*, or infection with *M. tuberculosis* or *Listeria monocytogenes* resulted in upregulation of CD1d at the surface of DCs or macrophages, enhancing iNKT cell self-reactivity (Berntman *et al.*, 2005; Raghuraman *et al.*, 2006; Skold *et al.*, 2005). Furthermore, TLR-stimulated APCs modulate their lipid biosynthetic pathways, leading to substitution of weak CD1d-presented self-antigen for more potent endogenous agonists of iNKT cells. Engagement of TLR-4, -7, and -9 was found to alter the expression of several enzymes involved in the biosynthetic pathway of GSLs and APCs exposed to lipid extracts from TLR9-stimulated DCs but not from naïve DCs combined with inflammatory cytokines were capable of activating iNKT cells (Paget *et al.*, 2007; Salio *et al.*, 2007).

Finally, an increasing number of reports suggest that iNKT cells can be activated by cytokines alone, in the absence of any TCR signal. Such bystander activation was observed upon exposure to LPS-induced IL-12 and IL-18 as well as in response to Type I IFNs secreted by human pDCs (Montoya *et al.*, 2006; Nagarajan and Kronenberg, 2007). In the later case, however, only partial iNKT cell activation was achieved. Recently, CD1d-independent iNKT cell activation by TLR9-stimulated or MCMV-infected

DCs was reported to depend on IL-12 but not IL-18 secretion by the activated DCs (Tyznik *et al.*, 2008).

Activation of iNKT cells independent of microbial lipid antigen recognition provides an elegant explanation for iNKT cell activation in response to a vast array of microbes not previously encountered by the host. Their rapid response to nearly all infectious agents places them in the context of innate immunity resulting in the designation of ''innate-like lymphocytes.''

4.2. The CD1d-restricted T cell immune response to infection: Deployment, effector functions and regulation

Rapid deployment of CD1d-restricted T cells provides these cells with the opportunity to shape both the ongoing innate responses and the ensuing adaptive ones. Following infection, iNKT cell are typically elicited earlier than cells of the adaptive immune system but later than innate effectors (Chiba *et al.*, 2008). As will be discussed below, immediate secretion of abundant and diverse cytokines by CD1d-restricted T cells can contribute significantly to selective recruitment and activation of many cells types, as well as to immune response polarization. In addition, most CD1d-restricted T cells have cytotoxic potential, and thus may participate in primary control of intracellular pathogens. Although the mechanisms in place to funnel and regulate the versatile effector functions exerted by these cells are only partially characterized, some data suggest that separate subsets of CD1d-restricted T cells with distinct functions may be elicited under different circumstances. It has also been proposed that the nature and strength of activating stimuli might contribute to shaping CD1d-restricted T cell responses.

4.2.1. Deployment of CD1d-restricted T cells

While the localization and trafficking of dNKT cells has been difficult to assess owing to lack of selective markers, iNKT cells, which can be tracked using α-GalCer-loaded CD1d tetramers, exhibit distinctive tissue distribution and recruitment patterns.

4.2.1.1. Homeostatic distribution of iNKT cells At steady state, murine iNKT cells typically account for ~0.3–1.5% of lymphocytes in primary and secondary lymphoid organs and lymphoid tissues, as determined by α-GalCer-loaded CD1d tetramer staining. The frequency of iNKT cells is the highest in the liver, where they can constitute up to 40% of mononuclear cells (Hammond *et al.*, 2001; Matsuda *et al.*, 2000) (see also Geissmann *et al.*, 2005). iNKT cells are less abundant in humans, and their tissue distribution remains poorly characterized. In peripheral blood, the frequency of iNKT cells varies between donors from undetectable levels to

2.3% of lymphocytes with a mean of 0.2% (Gumperz *et al.*, 2002; Lee *et al.*, 2002). Human iNKT cells are also thought to be enriched in the liver, though their exact frequency, which has been assessed mostly in diseased livers, is probably much lower than in mice (de Lalla *et al.*, 2004). The distinctive overrepresentation of iNKT cells in the liver may point to a special role for iNKT cells in this organ.

4.2.1.2. iNKT cell trafficking during infection A number of studies have reported the rapid accumulation of iNKT cells at sites of injury or infection (Apostolou *et al.*, 1999; Guinet *et al.*, 2002; Hazlett *et al.*, 2007; Ishikawa *et al.*, 2000; Joyee *et al.*, 2007; Kawakami *et al.*, 2001a, 2003). However, the mechanisms and chemotactic signals orchestrating recruitment of these cells *in vivo*, and their correlation to the phenotype of iNKT cells isolated from mice or human peripheral blood have not been addressed.

iNKT cells express a homing receptor repertoire resembling that of effector memory T cells rather than naïve lymphocytes. Specifically, most human iNKT cells lack the adhesion molecule CD62L (L-selectin) and the chemokine receptors CCR7 and CXCR5, which are uniformly expressed by naïve T cells and mediate their entry and trafficking through secondary lymphoid organs. Instead, expression of molecules such as CCR2, CCR5, CCR6, CXCR3, and CXCR4 by most human iNKT cells suggests that they might preferentially circulate through nonlymphoid tissues and home directly to sites of inflammation (Gumperz *et al.*, 2002; Kim *et al.*, 2002; Thomas *et al.*, 2003). Similarly, in mice, while most NK1.1$^+$ TCRβ^+ cells migrate towards the chemokine ligands of CXCR3 and CXCR4, they do not express CD62L, are largely insensitive to CXCR5 ligands and only certain subsets respond to CCR7-binding chemokines (Johnston *et al.*, 2003). Interestingly, a recent study comparing the gene expression profiles of iNKT cells and dNKT cells derived from Vα14 or Vα3.2Vβ9 TCR Tg mice, respectively, found several differences in homing receptor expression, suggesting these cell populations may exhibit distinct trafficking behaviors (Rolf *et al.*, 2008).

In murine models of pulmonary infection with the yeast *Cryptococcus neoformans* or the bacterium *Streptococcus pneumoniae*, recruitment of iNKT cells to the lungs was found to require the chemokine MCP-1 (CCL2), a ligand for both CCR2 and CCR5 (Kawakami *et al.*, 2001a, 2003). Likewise, expression of the chemokine MIP-2 and its receptor CXCR2 were necessary for recruitment of NK1.1$^+$ CD3$^+$ T cells to the spleen of mice in a model of ocular tolerance induction (Faunce *et al.*, 2001). Furthermore, a recent report suggested that MIP-2, in combination with IL-12, also contributed to iNKT cell recruitment to the liver following sulfatide-mediated activation of hepatic dNKT cells and APCs (Halder *et al.*, 2007).

4.2.2. Effector functions of iNKT cells

The variety of mechanisms whereby iNKT cells can contribute clearance of microbes (see Table 1.1) reflects the remarkable versatility of these cells. Through rapid production of an array of cytokines as well as cell-to-cell interactions, iNKT cells can play an important role in selective recruitment, activation and polarization of both the innate (Fig. 1.7) and adaptive (Fig. 1.8) immune system. In addition, many iNKT cells are thought to possess cytotoxic potential, suggesting they may contribute directly to the destruction of intracellular pathogens.

4.2.2.1. Rapid cytokine production and its consequences Following primary TCR stimulation using anti-CD3 antibodies or the model CD1d-restricted lipid antigen α-GalCer, both human and murine iNKT cells rapidly secrete large amounts of cytokines. Among those typically reported are IFN-γ and other Th1-type molecules such as IL-2 and TNF-α, but also, and often simultaneously, Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13

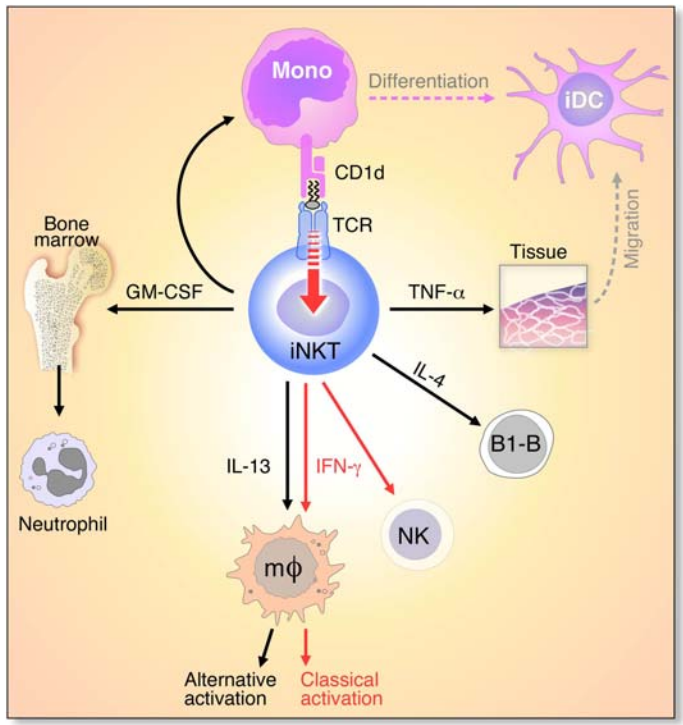

FIGURE 1.7 Activated iNKT cells elicit innate immune responses. Rapid secretion of cytokines by activated iNKT cells leads to mobilization of innate immune effectors such as macrophages (Mϕ), neutrophils, monocytes (Mono), natural killer (NK) cells and B1–B cells.

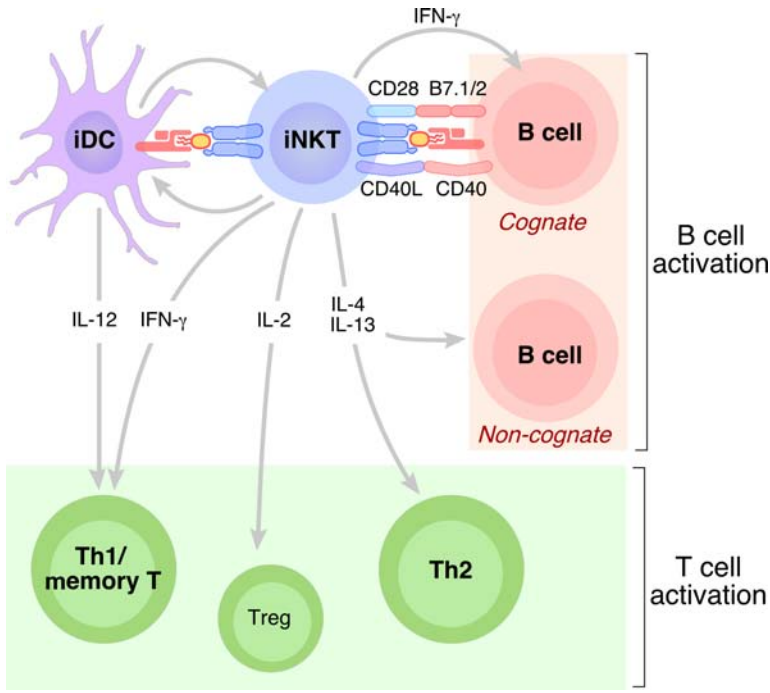

FIGURE 1.8 Activated iNKT cells elicit adaptive immune response. Through cytokine secretion and cell–cell interactions, activated iNKT cells contribute to shaping the adaptive immune response.

(Arase *et al.*, 1993; Gumperz *et al.*, 2002; Matsuda *et al.*, 2000). IL-17, a potent proinflammatory cytokine associated with autoimmune disease, and IL-21, a common γ-chain cytokine with pleiotropic effects have recently been added to the list of factors produced by iNKT cells following activation (Coquet *et al.*, 2007; Lee *et al.*, 2008; Rachitskaya *et al.*, 2008). Moreover, stimulated iNKT cells have been found to secrete the hematopoietic growth factors IL-3 and GM-CSF (Leite-de-Moraes *et al.*, 2002) as well as chemokines such as RANTES (Faunce and Stein-Streilein, 2002). Notably, mRNAs for at least some of these molecules are already present in the cytoplasm of resting iNKT cells, explaining the capacity of these cells to secrete cytokines within hours following stimulation (Stetson *et al.*, 2003). As will be discussed below, the consequences of such an outpour can be numerous, diverse, and affect virtually every arm of the immune system directly or indirectly, ultimately contributing to shape multiple facets antimicrobial responses.

4.2.2.1.1. Recruitment of myeloid lineage cells Secretion of hematopoietic growth factors such as IL-3 and GM-CSF by activated iNKT cells has been found to cause mobilization of myeloid progenitor cells and recruitment

of neutrophils to the periphery. iNKT cells were found to promote myelopoeisis both *in vivo* and *in vitro* via secretion of GM-CSF (Kotsianidis *et al.*, 2006). Furthermore, a single i.v. injection of α-GalCer causes a marked increase in colony-forming unit cells (CFU-S) in the spleen and peripheral blood, as well an efflux of granulocytes from the bone marrow mirrored by an increase of these cells, mostly consisting of neutrophils, in the circulation and, transiently, in the liver (Leite-de-Moraes *et al.*, 2002). Similarly, i.n. administration of α-GalCer leads to accumulation of neutrophils in the lungs (Lee *et al.*, 2008). Consistent with these observations, iNKT cell-mediated neutrophil recruitment appears be a crucial process during the early innate phases of certain antimicrobial immune responses. For instance, impaired neutrophil recruitment to the lungs was proposed to account for the increased susceptibility of iNKT cell-deficient mice to respiratory infection by *S. pneumoniae* and *Pseudomonas aeruginosa* (Kawakami *et al.*, 2003; Nieuwenhuis *et al.*, 2002).

4.2.2.1.2. Differentiation and activation of APCs Through cognate interactions and cytokine secretion, iNKT cells can promote differentiation and activation of APCs. For instance, human NKT cells are able to drive differentiation of autologous peripheral blood monocytes into immature DCs. Secretion of IL-13 and GM-CSF by NKT cells following recognition of self-lipid-loaded CD1d expressed by monocytes was found to be responsible for directing their differentiation *in vitro*. Importantly, the ability to induce the monocyte to DC progression appeared to be unique to NKT cells, since MHC-restricted T cells failed to affect the phenotype of monocytes during coculture (Hegde *et al.*, 2007). Both human and murine CD1d-restricted T cells have also been reported to drive DC maturation following recognition of self or foreign lipids in CD1d. In addition to upregulating surface MHC Class II and costimulatory molecules, activated iNKT cells push DCs to produce IL-12, a cytokine which promotes Th1 polarization (Kitamura *et al.*, 1999; Tomura *et al.*, 1999; Vincent *et al.*, 2002). Moreover, α-GalCer-activated iNKT cells enhance migration of DCs from peripheral sites such as the skin to draining lymph nodes (Gorbachev and Fairchild, 2006). The effects of iNKT cells on DC maturation are potent and have prompted the use of α-GalCer as an adjuvant for enhancement of immune responses to coadministered protein antigens (Fujii *et al.*, 2003; Hermans *et al.*, 2003). Finally, iNKT cells have also been reported to drive macrophage activation (Kim *et al.*, 2008; Nieuwenhuis *et al.*, 2002).

iNKT cell driven activation of APCs may have important consequences on the outcome of antimicrobial immune responses. For example, iNKT cell-induced DC maturation towards a Th1-polarizing APC was recently suggested to be crucial to protection against pulmonary infection with *Chlamydia pneumoniae* in mice (Joyee *et al.*, 2008). In addition,

α-GalCer treatment of mice infected with *P. aeruginosa* was found to markedly enhance the phagocytic activity of alveolar macrophages and contribute significantly to clearance of bacteria from the lungs, presumably in a manner dependent on iNKT cells (Nieuwenhuis *et al.*, 2002). In some cases, activation of APCs by iNKT cells can have immunopathological effects. In a model of chronic obstructive pulmonary disease (COPD) triggered by respiratory viral infection, iNKT cells were found to induce alternative macrophage activation which was central to perpetuation of pathology, as iNKT cell-deficient mice developed significantly reduced chronic disease (Kim *et al.*, 2008).

4.2.2.1.3. NK cell activation Transactivation of NK cells by iNKT cells is rapid and striking. As early as 90 min following i.v. administration of α-GalCer into wild type but not $CD1d^{-/-}$ mice, splenic NK cells upregulate surface expression of the early activation marker CD69 and start secreting large quantities of IFN-γ (Carnaud *et al.*, 1999). By 24 h postinjection, liver NK cells have undergone extensive proliferation and exhibit maximal cytotoxic activity (Eberl and MacDonald, 2000). Secretion of IFN-γ as well as IL-2 and IL-12 by iNKT cells or iNKT-stimulated APCs is thought to contribute to NK cell activation (Carnaud *et al.*, 1999; Eberl and MacDonald, 2000; Metelitsa *et al.*, 2001).

In the context of certain microbial infections, rapid triggering of NK cell functions can depend on iNKT cell activation. For instance, during visceral leishmaniasis, cytotoxicity and IFN-γ production by splenic NK cells is abrogated in iNKT-deficient mice, and might, in part, explain why these mice develop elevated parasitemia (Mattner *et al.*, 2006). iNKT cell-mediated NK activation was also suggested to promote immunity against encephalomyocarditis virus, which is impaired in $CD1d^{-/-}$ mice (Exley *et al.*, 2003).

4.2.2.1.4. B cell help Like $CD4^{+}$ MHC-restricted T cells, activated iNKT cells can provide both non-cognate help and cognate help for B cells presenting lipid antigens in CD1d. Within hours following i.v. injection of α-GalCer, splenic B cells strongly upregulate surface expression of CD69, the costimulatory molecule B7.2 and MHC Class II molecules in wild type but not iNKT cell-deficient mice (Carnaud *et al.*, 1999; Kitamura *et al.*, 2000). Human iNKT cells have also been reported to rapidly activate autologous naïve or memory B cells, and promote their proliferation *in vitro*. Interestingly, the intrinsic autoreactivity of human iNKT cells is sufficient to drive B cell activation, since the addition of exogenous iNKT cell antigens was not required (Galli *et al.*, 2003). In both human and murine systems, iNKT cell-derived Th2 cytokines contributed significantly to such early B cell activation (Galli *et al.*, 2003; Kitamura *et al.*, 2000). Furthermore, iNKT cell activity has been reported to help sustain serological memory following primary immunization with protein antigens, though the mechanism involved is unclear (Galli *et al.*, 2007) (see

also Barral *et al.*, 2008b; Devera *et al.*, 2008; Lang *et al.*, 2006, 2008). Secretion of IL-4 by activated liver iNKT cells has also been shown to rapidly activate peritoneal B1-B cells, a crucial step in the development of contact sensitivity responses (Campos *et al.*, 2003). Importantly, iNKT cells have recently been found to provide cognate help to B cells via recognition of foreign lipids presented by B cells in CD1d. iNKT cell instruction was shown to require both cell-to-cell interactions involving CD40L and the costimulatory molecules B7.1 and B7.2 as well as production of INF-γ. Cognate help by iNKT cells resulted in production of antigen-specific, class-switched antibody responses *in vivo* (Barral *et al.*, 2008b; Leadbetter *et al.*, 2008). Interestingly, some data suggest that iNKT cells may be particularly important for providing help to marginal zone B cells, which express very high levels of CD1d (Belperron *et al.*, 2005).

Notably, iNKT cell-deficient mice are less able to mount specific antibody responses against malarial GPI-anchored antigens or pneumococcal polysaccharides. Pathogen-specific antibody titers during *Borrelia hermisii* or *Trypanosoma cruzi* infection are also reduced in these mice, suggesting iNKT cells help activate B cells in the physiological context of microbial infection (Belperron *et al.*, 2005; Duthie *et al.*, 2005; Hansen *et al.*, 2003b; Kobrynski *et al.*, 2005).

4.2.2.1.5. Activation and expansion of MHC-restricted T cells Through cytokine secretion and cell-to-cell interactions, iNKT cells can contribute to expansion and activation of MHC-restricted T cells. Within hours following i.v. injection of α-GalCer, splenic T cells upregulate the early activation marker CD69 (Carnaud *et al.*, 1999). Specifically, $CD4^+$ and $CD8^+$ memory MHC-restricted T cells ($CD44^{high}$) in the liver, and to a lesser extent, in the spleen, undergo extensive bystander proliferation following such *in vivo* activation of iNKT cells, with IL-12 and IFN-γ production contributing to this effect (Eberl *et al.*, 2000). Similarly, secretion of IL-2 by α-GalCer-stimulated human iNKT cells can promote expansion $CD4^+$ $CD25^+$ regulatory T cells *in vitro* (Jiang *et al.*, 2005). In addition to promoting bystander T cell expansion, iNKT cells, though their maturational effects on DCs, can greatly enhance priming of naïve T cells (Fujii *et al.*, 2003; Hermans *et al.*, 2003; Stober *et al.*, 2003).

4.2.2.1.6. Immune response polarization As discussed earlier, iNKT cells are capable of producing large quantities of both Th1 and Th2 cytokines following activation, and can influence the maturation process of APCs. As a consequence, iNKT cell activation during microbial infection can significantly impact the polarization of subsequent adaptive responses. For instance, the increased susceptibility of iNKT cell-deficient mice to *C. pneumoniae* correlates with a failure to mount protective Th1 responses following pulmonary infection. Instead, splenocytes and lymph node cells from these mice were found to secrete increased amounts of type-II

cytokines such as IL-4 and IL-5 upon *in vitro* restimulation (Joyee *et al.*, 2007). Further investigation has revealed that iNKT cells are responsible for conferring a Th1-polarizing phenotype to DCs following infection (Joyee *et al.*, 2008). iNKT cells are also thought to impact the Th1/Th2 balance during infection with parasites such as *S. mansoni*, *Toxoplasma gondii*, and *L. major* (Faveeuw *et al.*, 2002; Mallevaey *et al.*, 2007; Ronet *et al.*, 2005; Wiethe *et al.*, 2008).

4.2.2.2. Cytotoxicity In addition to secreting cytokines, both human and murine iNKT cells can acquire potent cytotoxic function following activation. Expression of TNF-family receptors such as FasL and TRAIL as well as upregulation of granzyme B and perforin have been reported to contribute to iNKT cell killing of tumor cells, thymocytes, activated T cells and APCs (Arase *et al.*, 1994; Ho *et al.*, 2004; Kaneko *et al.*, 2000; Kawano *et al.*, 1998; Nicol *et al.*, 2000; Nieda *et al.*, 2001; Yang *et al.*, 2000). Furthermore, human iNKT cells have been found to express granulysin, an antimicrobial peptide present in cytotoxic granule, with activity against bacteria, parasites and fungi (Gansert *et al.*, 2003). Although the role of iNKT cell cytotoxicity during infection remains to be assessed *in vivo*, these findings suggest that iNKT cells likely contribute to direct control of intracellular pathogens.

4.2.3. Effector functions of dNKT cells

The effector functions of dNKT cells, in contrast to those of iNKT cells, remain poorly characterized. Although there is often a strong positive correlation between the susceptibility of $CD1d^{-/-}$ mice, which lack all NKT cells, and that of $J\alpha18^{-/-}$ mice, that are deficient only in iNKT cells, there are now several examples of microbes that cause disease in $CD1d^{-/-}$ but not $J\alpha18^{-/-}$ animals (Tupin *et al.*, 2007). This suggests that the immune functions of dNKT cells may differ from those of iNKT cells.

Consistent with this idea, a recent molecular profiling study has revealed a number of similarities and differences in expression of effector function-related genes between these two subsets. Specifically, dNKT cells and iNKT cells shared constitutive expression of IFN-γ transcripts as well as elevated expression of genes involved in cytolytic activity, suggesting rapid cytokine secretion and cytotoxicity may also be part of the dNKT cells' arsenal. In contrast, dNKT and iNKT cells differed in their chemokine and integrin expression profiles, suggesting they may exert their functions at distinct anatomical sites (Rolf *et al.*, 2008). Furthermore, dNKT cells may, by the nature of their TCR diversity, have the potential for recognition of a broader array of antigens and for unique clonal expansion.

4.2.4. Regulation of iNKT cell function

The systems in place to steer and orchestrate the functional responses of these multipotent lymphocytes are beginning to be understood. Findings suggest that phenotypically distinct subsets of iNKT cells exist that may be differentially elicited and exert more specialized functions. Furthermore, different activating signals and interaction with polarized inflammatory environments might also contribute to hone the iNKT cell response.

4.2.4.1. iNKT cell subsets with specialized functions Phenotypically distinct subsets of iNKT cells have been defined on the basis of coreceptor expression. Most iNKT cells are either $CD4^-$ $CD8^-$ (DN) or $CD4^+$, while only a very small fraction of iNKT express only the CD8 coreceptor (Benlagha *et al.*, 2000; Gumperz *et al.*, 2002). Evidence of functional differences between these subsets is most compelling in humans. Specifically, following α-GalCer stimulation, Th1 cytokines are abundantly secreted by all human iNKT cell subsets, while only the $CD4^+$ subset simultaneously produces Th2 cytokines (Gumperz *et al.*, 2002; Lee *et al.*, 2002; Takahashi *et al.*, 2002). Furthermore, although both iNKT cell subsets possess cytolytic capabilities, cytotoxicity is more prominent in DN iNKT cells, which frequently express constitutively high levels of perforin (Gumperz *et al.*, 2002). The presence of CD4 has been proposed to contribute to the functional differences between subsets, since cytokine secretion and proliferation, but not cytotoxicity of $CD4^+$ iNKT cells is markedly reduced in the presence of anti-CD4 blocking antibodies (Chen *et al.*, 2007; Thedrez *et al.*, 2007). In addition to their distinct cytokine profiles and cytotoxic functions, human iNKT cell subsets express slightly different repertoires of chemokine receptors. Specifically, CCR1, CCR6, and CXCR6 are found mostly on DN and $CD8^+$ iNKT cells, while CCR4 is more prominent on $CD4^+$ cells, indicating that their migratory patterns may differ (Gumperz *et al.*, 2002; Kim *et al.*, 2002; Lee *et al.*, 2002). Finally, human iNKT cell subsets also differ in their ability to help B cells, with $CD4^+$ iNKT cells inducing higher levels of immunoglobulin production than DN iNKT cells (Galli *et al.*, 2003).

In mice, iNKT cell subsets with can be distinguished based on anatomical localization and differential expression of CD4 and NK1.1. Unlike in humans, murine $CD4^+$ and $CD4^-$ iNKT cells produce comparable levels of Th2 cytokines (Coquet *et al.*, 2008). Nevertheless, recent findings have revealed otherwise complex heterogeneity in the cytokine responses of murine iNKT cell subsets. Of particular interest, this study identified a $CD4^-$ $NK1.1^-$ population secreting the cytokine IL-17, a potent proinflammatory cytokine (Coquet *et al.*, 2008). Data also suggest that DN iNKT cells from the liver exert more potent antitumor activities than $CD4^+$ cells or iNKT cells from other organs (Crowe *et al.*, 2005).

4.2.4.2. Cytokine polarization-mediated by different stimuli In addition to functional specialization of subsets, there is evidence that the nature of activating stimuli and the context in which these stimuli are delivered may help steer the iNKT cell response. For instance, while the pharmacological agonist α-GalCer induces secretion of both IFN-γ and IL-4, structural variants of this lipid have been found to skew the iNKT cell response. Truncation of either the phytosphingosine or the acyl chain of α-GalCer resulted in an iNKT cell bias towards Th2 cytokine production (Goff *et al.*, 2004; Miyamoto *et al.*, 2001; Oki *et al.*, 2004), as did the introduction of double bonds into α-GalCer's acyl chain (Yu *et al.*, 2005). Compounds bearing other modifications such as substitution of the glycosidic oxygen for a methylene group or insertion of an aromatic residue in either fatty acyl chain-activated iNKT cells to secrete Th1 rather than Th2 cytokines (Franck and Tsuji, 2006; Fujio *et al.*, 2006). Stereochemistry of the phytosphingosine chain also appears to alter iNKT cell polarization (Park *et al.*, 2008). While these differences suggest that physiological lipid antigens may also skew iNKT cell responses, why structural variations affect the cytokines secreted is unclear. One possibility is that differing stability of CD1d–lipid complexes affects the strength or duration of the iNKT cell TCR signal which in turn biases responses towards Th1 or Th2 cytokine production (Oki *et al.*, 2004). Alternatively, iNKT cell polarization has been proposed to also depend on which cell type is acting as the APC (Bezbradica *et al.*, 2005), thus, preferential uptake of different lipids by different cells may also explain specific iNKT cell skewing.

Certain foreign-antigen-independent stimuli such as LPS, IL-2, IL-12, or IL-18 combined with IL-12 appear to activate cytotoxic functions preferentially over cytokine secretion (Gumperz *et al.*, 2002; Leite-De-Moraes *et al.*, 1999; Nagarajan and Kronenberg, 2007). Furthermore, expression of certain costimulatory molecules or pretreatment of DCs with cytokines can affect iNKT cell functions, suggesting the inflammatory environment also helps to shape iNKT cell responses (Chen *et al.*, 2007; Hayakawa *et al.*, 2001; Matsumoto *et al.*, 2004; Minami *et al.*, 2005).

5. ROLE OF CD1d-RESTRICTED NKT CELLS IN SPECIFIC MICROBIAL INFECTIONS

A number of studies have assessed the roles of CD1d-restricted T cells in immunity to microbial infections. These reports often rely on either the use of J$\alpha 18^{-/-}$ mice (a.k.a. J$\alpha 281^{-/-}$), which lack the Jα gene segment necessary to form the invariant TCR and are thus deficient in iNKT cells specifically (Cui *et al.*, 1997); or CD1d$^{-/-}$ mice, which lack both iNKT and dNKT cell populations (Mendiratta *et al.*, 1997; Smiley *et al.*, 1997). In addition, the impact of iNKT cell activation on infection has been

evaluated by studying the effect of prophylactic or therapeutic administration of α-GalCer, a potent iNKT cell agonist. α-GalCer-loaded CD1d tetramers are also routinely used for *ex vivo* FACS-based identification of these cells. In contrast, due to the lack of specific markers or antigens, the role of dNKT cells has remained relatively unexplored. A wealth of evidence implicating CD1d-restricted T cells in the immune responses against bacteria, parasites, viruses and fungi is accumulating (Tables 1.1 and 1.2). It is also becoming clear that pathogens have evolved various strategies to evade recognition by these lymphocytes.

5.1. CD1d-restricted T cells in immunity to bacteria

5.1.1. Streptococcus pneumoniae

Mice lacking iNKT cells are highly susceptible to infection with *S. pneumoniae*, a gram-positive bacteria and significant human pathogen causing a spectrum of diseases including pneumonia and meningitis. Recruitment of neutrophils, which is critical for bacterial clearance, is impaired in J$\alpha 281^{-/-}$ mice following i.t. infection with *S. pneumoniae*. In addition, these mice develop elevated bacterial loads in their lungs and survive significantly less time than wild-type mice (Kawakami *et al.*, 2003). Adoptive transfer of liver mononuclear cells from wild type but not from INF-$\gamma^{-/-}$ mice rescues iNKT-deficient mice, suggesting that iNKT cell secretion of IFN-γ is important for protection in this model (Nakamatsu *et al.*, 2007).

5.1.2. *Borrelia* spp

Borrelia spp. are spirochetes responsible for vector-borne diseases including relapsing fever and human Lyme-borreliosis, a disease transmitted by infected ticks. In North America, *Borrelia burgdoferi* is the predominant cause of Lyme disease, which can result in arthritis as well as cardiac and nervous system disorders. Relapsing fever can be caused by several *Borrelia* species including *B. hermsii* and *B. recurrentis*.

CD1d-deficiency renders resistant mice susceptible to *B. burgdoferi*. CD1d$^{-/-}$ C57BL/6 mice develop persistent arthritis in the knees and tibiotarsal joints 1 week following intradermal inoculation. Strong anti-borrelial IgG2a antibody responses typically found in mice susceptible to developing autoimmune arthritis, but not resistant in mice, are mounted in CD1d$^{-/-}$ mice. Also, bacterial dissemination to the bladder can be detected in CD1d$^{-/-}$ animals but not in wild-type controls (Kumar *et al.*, 2000). In addition, a recent report demonstrated that both human and murine iNKT cells directly recognize CD1d-restricted galactosyl diacylglycerols antigens from *B. burgdorferi* (Kinjo *et al.*, 2006b). Although CD1d-restricted T cells alone are not sufficient to confer protection, since certain strains of mice are susceptible to disease despite expressing CD1d,

TABLE 1.1 Protective roles of NKT cells during infection

		Experimental model			
	Microorganism	Route of infection	Mouse strain	NKT cell functions possibly contributing to protection	References
Bacteria	*Streptococcus pneumoniae*	i.t.	B6	Neutrophil recruitment, IFN-γ secretion	Kawakami *et al.* (2003), Nakamatsu *et al.* (2007)
	Borrelia burgdorferi	i.d.	B6 or B6/129	Prevention of possibly detrimental IgG2a responses	Kinjo *et al.* (2006b)
	Pseudomonas aeruginosa	i.n.	B6 or BALB/c	Neutrophil recruitment, enhancement of alveolar macrophage phagocytosis	Nieuwenhuis *et al.* (2002)
		i.ves.	B6	–	Minagawa *et al.* (2005)
	Sphingomonas yanoikuyae	i.p.	B6	–	Kinjo *et al.* (2005)
	Sphingomonas capsulata	i.v. (low dose)	B6	–	Mattner *et al.* (2005)
	Ehrlichia muris	i.p.	B6	–	Mattner *et al.* (2005)
	Chlamydia pneumoniae	i.n.	B6 or BALB/c	Enhancement of Th1 responses	Joyee *et al.* (2007)
	Listeria monocytogenes	i.v. or oral	B6/129	Regulation of Th1 responses	Arrunategui-Correa and Kim (2004)
Parasites	*Leishmania major*	i.v.	B6	NK cell activation via IFN-γ secretion	Mattner *et al.* (2006)
	Leishmania donovani	i.v.	BALB/c	–	Amprey *et al.* (2004a)

(*continued*)

TABLE 1.1 (*continued*)

	Microorganism	Experimental model: Route of infection	Experimental model: Mouse strain	NKT cell functions possibly contributing to protection	References
	Plasmodium berghei	i.p.	BALB/c	IFN-γ secretion, enhancement of antibody response	Hansen *et al.* (2003a), Gonzalez-Aseguinolaza *et al.* (2000, 2002)
	Trypanosoma cruzi	i.p.	B6	Enhancement of antibody responses	Duthie and Kahn (2006), Duthie *et al.* (2002, 2005); Vitelli-Alevar *et al.* (2006)
	Toxoplasma gondii	i.p.	B6	–	Smiley *et al.* (2005)
Viruses	HSV-1	cut.	B6	–	Grubor-Bauk *et al.* (2003, 2008)
	HSV-2	i.vag.	B6	–	Ashkar and Rosenthal (2003)
	HBV	–	HBV-Tg	Induction of type-I and -II IFNs in the liver	Kakimi *et al.* (2000)
	Influenza A	i.n.	B6	–	Ho *et al.* (2008); De Santo *et al.* (2008)
	RSV	i.n.	B6	–	Johnson *et al.* (2002)
	EBV	–	–	–	
	VZV	–	–	–	
	ECMV-D	i.p.	BALB/c	Induction of IL-12 and NK cell activation	Exley *et al.* (2001, 2003), Ilyinskii *et al.* (2006)
Fungi	*Cryptococcus neoformans*	i.t.	B6	–	Kawakami *et al.* (2001)

Note: Infections listed in this table are either exacerbated in CD1d$^{-/-}$, Jα18$^{-/-}$ mice or iNKT cell-deficient humans (for EBV and VZV) or/and ameliorated by α-GalCer treatment.
Abbreviations: i.n., intranasal; i.t, intratracheal; i.p., intraperitoneal; i.v., intravenous; i.vag, intravaginal; i.ves, intravesicular; cut., cutaneous; s.c., subcutaneous; B6, C57BL/6; –, not known or not applicable.

TABLE 1.2 Detrimental roles of NKT cells during infection

		Experimental model			
	Microorganism	Route of infection	Mouse strain	Detrimental NKT cell functions	References
Bacteria	*Sphingomonas capsulata*	i.v. (high dose)	B6	Explosive cytokine secretion leading to septic shock	Mattner *et al.* (2005)
	Novosphingobium aromaticivorans	i.v.	B6 or NOD	Transactivation of autoreactive T cells and B cells causing primary biliary cirrhosis	Mattner *et al.* (2008)
	Chlamydia muridarum	i.n.	B6 or BALB/c	Enhancement of detrimental Th2 responses	Joyee *et al.* (2007), Bilenki *et al.* (2005)
	Salmonella choleraesuis	i.p.	B6	–	Ishigami *et al.* (1999)
Parasites	*Leishmania donovani*	i.v.	B6	Detrimental immune polarization, defective hepatic recruitment of CTL	Stanley *et al.* (2008)
	Toxoplasma gondii	i.p.	BALB/c	–	Nakano *et al.* (2001, 2002)
Viruses	HBV	–	HBV-Tg/RAG$^{-/-}$	Recognition of stress-induced ligands on hepatocytes via the NKG2D receptor	Baron *et al.* (2002), Vilarinho (2007)
	Coxsackievirus B3	i.p.	BALB/c	–	Huber *et al.* (2003)

Note: Infections listed in this table are either ameliorated in CD1d$^{-/-}$, Jα18$^{-/-}$ mice or iNKT cell-deficient humans (for EBV and VZV) or/and exacerbated by α-GalCer treatment.
Abbreviations: i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; B6, C57BL/6; –, not known or not applicable.

collectively, these data indicate that CD1d-restricted T cells participate in the immune response to *B. burgdoferi* and promote resistance to the disease. Control of the related bacterium *B. hermsii* is similarly impaired in CD1d$^{-/-}$ C57BL/6 mice, which mount weakened specific antibody responses and develop a higher pathogen burden in the blood than wild-type animals (Belperron *et al.*, 2005).

5.1.3. Pseudomonas aeruginosa

P. aeruginosa is a gram-negative bacterium abundant in soil and water. It is a pathogen for healthy individuals as well as for immunocompromised hosts. *P. aeruginosa* is responsible for a variety of diseases including dermatitis, keratitis, urinary tract or gastrointestinal infection. Fatal systemic bacteremia in immunocompromised patients and frequent pneumonia in patients with cystic fibrosis or other obstructive lung disorders can also be consequences of *P. aeruginosa* infection. In healthy individuals, *P. aeruginosa* infections are limited mainly through neutrophil-mediated defense mechanisms. The role of CD1d-restricted T cells in *P. aeruginosa* immunity has been assessed in murine models of bacterial pneumonia, urinary tract infection (UTI) and keratitis.

- *Pneumonia*: In the case of *P. aeruginosa*-induced pulmonary disease, an early study found that CD1d$^{-/-}$ animals of both BALB/c and C57/BL6 genetic backgrounds were significantly less able to clear bacteria from their lungs than wild-type controls. This phenotype was explained by impaired neutrophil recruitment due to decreased levels of the neutrophil chemoattractant MIP-2 in the BALF of CD1d$^{-/-}$ mice. Furthermore, activation of iNKT cells by pretreatment with α-GalCer lead to rapid clearance of pulmonary bacteria through enhancement of alveolar macrophage phagocytosis (Nieuwenhuis *et al.*, 2002). However, a subsequent study performed using both CD1d$^{-/-}$ and Jα281$^{-/-}$ C57/BL6 mice failed to confirm the earlier data, finding no role for CD1d-restricted T cells in host resistance to *P. aeruginosa* (Kinjo *et al.*, 2006a). Differences in bacterial strain (D4 vs. PAO-1) or route of infection (i.n. vs. i.t.) may contribute to these contrasting findings.
- *UTI*: iNKT activation enhances immunity to *P. aeruginosa* UTI. Wild-type, C57/BL6 mice infected intravesically with *P. aeruginosa* were found to rapidly clear bacteria from their kidneys following i.p. administration of α-GalCer (Minagawa *et al.*, 2005). The role of CD1d-restricted T cells in natural immunity to this type of infection remains to be investigated in CD1d$^{-/-}$ or Jα281$^{-/-}$ mice.
- *Keratitis*: Recent data suggest that NKT cells contribute to the early inflammatory response to ocular infection with *P. aeruginosa*. NKT cells accumulated rapidly in the corneal stroma and the conjunctiva of

susceptible B6 mice following infection, and levels of IFN-γ, an important cytokine for disease resolution, were reduced in Jα281$^{-/-}$ mice. Whether NKT cell-deficiency increases the severity of keratitis was not addressed, though corneal perforation developed more rapidly in animals depleted of both NK and NKT cells (Hazlett *et al.*, 2007).

5.1.4. *Sphingomonas* spp

Sphingomonas are gram-negative α-proteobacteria ubiquitously present in the environment. *Sphingomonas* species are generally not considered human pathogens, although rare cases of nosocomial infections and septic shock have been reported (Hsueh *et al.*, 1998; Kilic *et al.*, 2007). In addition, cross-reactive immune responses against certain members of the Sphingomonadacae family have been correlated with development of primary biliary cirrhosis (PBC), a fatal autoimmune liver disorder (Olafsson *et al.*, 2004; Selmi *et al.*, 2003).

iNKT cells have been shown to exert both protective and detrimental effects in the immune response to *Sphingomonas*. Compared to wild-type mice, both CD1d$^{-/-}$ and Jα281$^{-/-}$ animals develop higher bacterial burdens in their lungs and liver following inoculation with low doses of *Sphingomonas*, suggesting iNKT cell activation contribute to bacterial clearance (Kinjo *et al.*, 2005; Mattner *et al.*, 2005). However, injection of high doses of *Sphingomonas* i.v. causes septic shock in wild type but not NKT cell-deficient mice, highlighting the importance of NKT cells in promoting pathological cytokine storms (Mattner *et al.*, 2005). Furthermore, recent findings indicate that development of murine autoimmune PBC resulting from chronic infection with the Sphingomonadacae *Novosphingobium aromaticivorans* required NKT cells. Unlike wild-type mice, CD1d$^{-/-}$ mice did not develop the antimitochondrial autoantibodies typical of PBC, and were also protected against liver inflammation, bile duct damage and granuloma formation. Conversely, Vα14 TCR Tg mice, which have increased numbers of iNKT cells, display more severe liver disease than wild-type mice following infection. Thus, in the PBC model, iNKT cell activity was hypothesized to be critical for transactivation of autoreactive T and B cells, and the ensuing breach in self-tolerance (Mattner *et al.*, 2008).

The importance of iNKT cells in models of *Sphingomonas* disease is thought to result from cognate recognition of bacterial lipid antigens by these cells. Indeed, converging findings from several groups indicate that iNKT cells recognize GSL antigens expressed by various *Sphingomonas* species. Importantly, these lipids closely resemble the synthetic iNKT cell antigen α-GalCer, with whom they share the α-anomeric linkage attaching the glycan headgroup the sphingosine base (Kinjo *et al.*, 2005; Mattner *et al.*, 2005; Sriram *et al.*, 2005).

5.1.5. *Ehrlichia* spp

iNKT cells participate in the immune response against *Ehrlichiae*, a family of small, gram-negative, obligate intracellular bacteria that have recently emerged as human pathogens. *In vitro*, iNKT cell were activated by heat-killed *Ehrlichia muris* in a CD1d-dependent, TLR signaling-independent manner, suggesting the cognate recognition of a microbial lipid. *In vivo*, a significant fraction of iNKT cells upregulated CD69 and produced IFN-γ following i.v. injection of *E. muris*. Furthermore, in two different models of Erlichiosis, CD1d$^{-/-}$ mice developed increased bacterial loads in the spleen and liver indicating that *in vivo* activation of CD1d-restricted T cells contributes to host protection again murine ehrlichiosis (Mattner *et al.*, 2005; Stevenson *et al.*, 2008). However, one study found that despite controlling bacterial infection, CD1d-restricted T cells contributed to *Ehrlichia*-induced toxic shock (Stevenson *et al.*, 2008).

5.1.6. *Chlamydia* spp

Several findings indicate that CD1d-restricted T cells can be elicited during infection with *Chlamydia* spp., important human pathogens responsible for a range of diseases including genital, ocular, rheumatological, and pulmonary conditions. However, whether activation of these cells is protective or harmful varies between different infection models. iNKT cells rapidly accumulate in the lungs of mice following i.n. administration of *C. pneumoniae*. In this model, both CD1d$^{-/-}$ and Jα18$^{-/-}$ mice fail to control bacterial loads in their lungs and loose weight significantly faster than wild-type controls. In contrast, these same animals show enhanced resistance to i.n. infection with *Chlamydia muridarum*, an alternative model of chlamydial pneumonia. Consistently, activation of iNKT cells by pretreatment with α-GalCer is protective in the first model and detrimental in the second (Bilenki *et al.*, 2005; Joyee *et al.*, 2007). To explain these results, the authors hypothesized that the different organisms elicit distinct subsets of iNKT cells, which, in one case, promote protective Th1-biased responses, and in the other, enhance detrimental Th2 polarization (Yang, 2007).

5.1.7. Listeria monocytogenes

L. monocytogenes are rod-shaped gram-positive bacteria that cause rare but potentially severe disease in pregnant women and immunocompromised individuals. *Listeria* is ubiquitous in the environment and is generally acquired following ingestion of contaminated food products. *L. monocytogenes* infection is one of the few to cause exacerbated disease in CD1d$^{-/-}$ mice but not in Jα18$^{-/-}$ mice, suggesting that dNKT cells, which, unlike iNKT cells, are absent from CD1d$^{-/-}$ but not Jα18$^{-/-}$ animals, play a protective role. Indeed, despite an early report that

anti-CD1d treatment of mice ameliorated survival following infection with *L. monocytogenes* (Szalay *et al.*, 1999a), a later, more conclusive study found that mice deficient for CD1d, unlike wt animals, failed to rapidly control bacterial burdens in the spleen and liver, developed severe intestinal inflammation and tissue damage and suffered weight loss following either i.v. or oral administration of *L. monocytogenes*. This phenotype was attributed to deregulation of Th1 responses, which were exacerbated in the colon and small intestine but absent from the liver and spleen of $CD1d^{-/-}$ mice. This suggests that CD1d-restricted T cells could function both to dampen intestinal response while promoting lymphoid organ inflammation (Arrunategui-Correa and Kim, 2004). In contrast, $J\alpha18^{-/-}$ mice, which lack only iNKT cells but possess dNKT cells, were not more susceptible to listeriosis than wild-type animals, based on survival and CFU in host organs (Emoto *et al.*, 2006; Ranson *et al.*, 2005). Although direct comparison of these studies is complicated by the use of different bacterial stains, infectious doses and parameters of susceptibility, collectively, the data suggest that dNKT cells, not iNKT cells are important for resistance to listeriosis.

5.1.8. *Salmonella* spp

CD1d-restricted T cells become activated during infection with various species of *Salmonella*, a group of facultative intracellular gram-positive bacteria causing a range of diseases in humans. However, these cells are not required for protection and in some cases can even be detrimental during the immune response to these pathogens. In the context of infection with *S. typhimurium*, a murine model for human typhoid fever, activation of splenic iNKT cells has been noted based on the upregulation of surface CD69 expression and the production of IFN-γ (Berntman *et al.*, 2005; Brigl *et al.*, 2003; Mattner *et al.*, 2005). Nevertheless, neither $CD1d^{-/-}$ nor $J\alpha18^{-/-}$ mice display increased susceptibility to disease (Berntman *et al.*, 2005; Mattner *et al.*, 2005). Furthermore, the absence of iNKT cell in $J\alpha281^{-/-}$ mice prevented hepatic injury following infection with *S. choleraesuis*, implicating these cells in *Salmonella*-induced liver damage.

5.1.9. Mycobacterium tuberculosis

It is estimated that one third of the world's population is infected with *M. tuberculosis* with 9–10 million new cases and approximately 2 million deaths per year. Nearly half of the new cases are in human immunodeficiency virus (HIV)-infected individuals and more than 5% are multidrug resistant tuberculosis (MDR TB), defined as tuberculosis resistant to the best first line drugs, isoniazid and rifampin-based drugs (2002–2007, 2008; Organization, 2008). Now, cases also resistant to multiple second-line drugs, referred to as extensively drug resistant (XDR TB), are also emerging. *M. tuberculosis* causes pulmonary disease as well as

disseminated infection. Control involves innate and adaptive immunity including both MHC Class I and II protein reactive T cells, inflammatory cytokines, intracellular localization of growth and killing of the organisms in macrophages and the formation of granulomatous lesions. Although most individuals control the initial infection, they continue to harbor viable microorganisms that may reactivate later in life and cause latent tuberculosis. This may occur spontaneously, with increasing age or debilitation or as coinfection with HIV. Although Robert Koch identified the bacillus that causes tuberculosis in 1882 and BCG was introduced as a vaccine 39 years later, the need for more effective vaccines and drugs for tuberculosis remains one of the most important challenges in public health today. The recent discovery of CD1-mediated T cells recognition of lipid antigens from the mycobacterial cell wall offers a series of new insights into cell-mediated immunity against this organism. In humans, many examples illustrate that lipid antigens from the cell wall of mycobacteria can be presented by CD1a, b, and c and activate Th1 cytokine-producing T cells and CTL and directly recognize *M. tuberculosis*-infected APC and are bactericidal to the organisms. These studies and the finding of expanded CD1-restricted *M. tuberculosis*-specific T cells following infection or immunization in humans support the likely role of Group I CD1a, b, and c molecules in tuberculosis (see Section 3.3). Studies on CD1d-restricted T cells in humans revealed that activated iNKT cells express granulysin and can kill M. tubercule bacilli *in vitro* (Gansert *et al.*, 2003). CD1d expressing DCs and other mononuclear cells were found in the granulomas of infected patients, also supporting a potential role for iNKT cells in tuberculosis infection in man.

Mice lack the genes encoding CD1a, b, and c but have been studied extensively in regard to CD1d-restricted NKT cells. Injection of *M. tuberculosis* lipids, especially PIMs, in mice rapidly induce granulomas in which the predominant lymphocytes are iNKT cells (Apostolou *et al.*, 1999; Gilleron *et al.*, 2001). One report suggested that iNKT cells may directly recognize certain PIMs (Fischer *et al.*, 2004); however, the specific antigenic recognition of PIMs by the iNKT TCR could not be confirmed using synthetic antigens (Kinjo *et al.*, 2006b). Evidence supporting the role of iNKT cells in murine tuberculosis infection *in vivo* includes studies showing that treatment of mice with anti-CD1d mAb resulted in exacerbation of infection with increased CFU at early time points following infection (Szalay *et al.*, 1999b). However, CD1d-deficient mice were not more susceptible to infection with *M. tuberculosis* than control mice (Behar *et al.*, 1999a; Sousa *et al.*, 2000). In contrast, activation of iNKT cells with α-GalCer protected susceptible mice from tuberculosis and treatment with α-GalCer reduced the bacterial burden in the lungs, diminished tissue injury and prolonged survival (Chackerian *et al.*, 2002). Moreover, iNKT cells were found to directly recognize *M. tuberculosis*-infected APC

in vitro and to reduce CFU, supporting a role for iNKT cells in microbial killing. Importantly, the adoptive transfer of iNKT cells from uninfected mice significantly reduced the bacterial burden in the lungs of mice infected by the aerosol route (Sada-Ovalle *et al.*, 2008).

Several reports have also examined infection with *M. bovis* BCG in mice. Interestingly, iNKT cells were rapidly activated reaching a peak at d7–8 postinfection and produced copious IFN-γ (Chiba *et al.*, 2008; Dieli *et al.*, 2003). However, 2 weeks following activation, the iNKT cells entered an unresponsive or anergic phase and no longer produced IFN-γ (Chiba *et al.*, 2008). Mice deficient in iNKT cells produced significantly higher numbers of granulomas and revealed signs of caseation (Dieli *et al.*, 2003) but did not have higher levels of organisms. These studies underscore the complex nature of the iNKT cell response in mycobacterial infections. They indicate the activation and involvement of iNKT cells in mycobacterial infection but suggest that, at least in the mouse model, their absence is not essential to control of the infection (Skold and Behar, 2003).

5.2. CD1d-restricted T cells in immunity to parasites

5.2.1. Schistosoma mansoni

The helminth *S. mansoni* is an important human pathogen responsible for schistosomiasis, a chronic disease that is endemic to many parts of the developing world. Aquatic *S. mansoni* cercariae infect humans through the skin and gain access to the bloodstream. They undergo sexual replication in mesenteric vessels and produce large quantities of eggs against which immunopathological responses are mounted. *S. mansoni* worms can remain in the body for years, causing damage to organs and impairing cognitive development in children.

CD1d-restricted T cells participate in the immune response against *S. mansoni*. Three weeks following percutaneous injection of parasites, iNKT cells become activated and accumulate in the liver and spleen of wild-type mice. *S. mansoni* egg-pulsed DC elicit both Th1 and Th2 production by iNKT cells *in vitro* and *in vivo* in a CD1d-dependent fashion. However, extraction and fractionation of lipids from *S. mansoni* eggs failed to identify a lipid antigen recognized by iNKT cell, and HexB-dependent endogenous glycolipid processing appeared to be required for activation, suggesting that activation of iNKT cells by *S. mansoni* may occur independently of a foreign lipid antigen (Mallevaey *et al.*, 2006). Furthermore, parasite eggs are not present at the initial time of iNKT cell activation, and schistosomules and adult worms, which are, do not stimulate iNKT cells *in vitro*. Thus, although *S. mansoni* eggs may drive late iNKT cell responses, the mechanisms initiating iNKT cell activation in this model remain unclear.

Worm and egg burdens of infected CD1d$^{-/-}$ and Jα18$^{-/-}$ mice are comparable to those of wild-type animals, indicating that neither iNKT nor dNKT cells play a crucial role in limiting disease (Mallevaey *et al.*, 2007). Yet, polarization of late antiparasitic immune responses appeared to be dysregulated in NKT cell-deficient mice. Interestingly, while iNKT cells promoted Th1 responses, dNKT cells enhanced a Th2 bias. Specifically, wild-type splenocytes from mice infected for 7 weeks proliferated and produced IFN-γ, IL-4, IL-5, and IL-10 following antigen-specific restimulation *in vitro*. In the same assay, splenocytes from Jα18$^{-/-}$ mice, which lack only iNKT cells, secreted IL-4, -5 and -10 but failed to produce IFN-γ, while CD1d$^{-/-}$ cells, which lack all CD1d-restricted T cells made IFN-γ but no IL-4, -5 or -10. Consistently, antiparasite antibody IgG1: IgG2a isotype ratios and IgE titers were elevated in Jα18$^{-/-}$ mice but reduced in CD1d$^{-/-}$ mice compared to wild-type controls (Faveeuw *et al.*, 2002; Mallevaey *et al.*, 2007). Thus, iNKT cells and dNKT cells appear to exert opposite regulatory functions in this model.

5.2.2. *Leishmania* spp

CD1d-restricted T cells contribute to protection against cutaneous leishmaniasis caused by the protozoan parasite *L. major*. iNKT cells accumulate in popliteal lymph nodes of BALB/c, C57/BL6 and DBA/2 mice 7 days following subcutaneous footpad injection of *L. major* promastigotes (Ishikawa *et al.*, 2000). Although recovery from cutaneous disease occurs with similar kinetics in CD1d$^{-/-}$, Jα18$^{-/-}$ and wild-type mice, Jα18$^{-/-}$ animals develop elevated parasite burdens in the footpad and spleen during the acute phase of infection. Furthermore, the absence of iNKT cells renders mice more susceptible to systemic infection by *L. major* following i.v. injection. Defective NK cell activation in the absence of iNKT cell-derived INF-γ was proposed to account for elevated parasite burdens in the spleen of these mice (Mattner *et al.*, 2006).

The role of CD1d-restricted T cells in protection against visceral leishmaniasis varies between mouse strains. In BALB/c mice, *L. donovani* infection is worsened in the absence of CD1d-restricted T cells, as evidenced by increased parasite burdens in the spleen and liver, as well as defective granulomatous responses in CD1d$^{-/-}$ mice (Amprey *et al.*, 2004a,b). However, although hepatic iNKT cells upregulate CD69 and secrete IFN-γ in response to *L. donovani* infection in C57/BL6 mice, a recent report found no exacerbation of disease in either CD1d$^{-/-}$ or Jα18$^{-/-}$ mice of C57/BL6 genetic background. In fact, treatment of infected mice with α-GalCer significantly impaired parasite clearance from the liver. The reasons for disease exacerbation were not clear, although the authors suggested that changes in immune polarization or defective recruitment of CTLs to the liver might be responsible (Stanley *et al.*, 2008).

5.2.3. *Plasmodium* spp

The activity of CD1d-restricted T cells contributes to immune protection against the protozoan parasite *Plasmodium*, which causes malaria in humans. Prophylactic activation of iNKT using α-GalCer protects both BALB/c and C57/BL6 mice against infection of the liver by *P. yoelii* or *P. berghei* sporozoites injected intravenously. Secretion of INF-γ by iNKT cells is required for resistance to hepatocyte infection, since neither $J\alpha18^{-/-}$ nor INF-$\gamma^{-/-}$ mice are protected by α-GalCer treatment (Gonzalez-Aseguinolaza *et al.*, 2000). Similarly, α-GalCer was shown to enhance the development of protective antigen-specific $CD8^+$ T cell responses when coadministered with experimental malaria vaccines (Gonzalez-Aseguinolaza *et al.*, 2002).

Although artificial activation of iNKT cells promotes antimalarial immunity, the impact of CD1d-restricted T cells on the natural course of infection is less clear and varies between mice of differing genetic backgrounds. While the liver iNKT cell population becomes activated and expands following i.v. injection of *P. yoelii* sporozoites, $CD1d^{-/-}$ C57/BL6 mice develop similar parasite loads in the liver and blood as wild-type controls (Soulard *et al.*, 2007). In a model of cerebral malaria induced by infection with the *P. berghei* ANKA strain, NKT cell-deficient C57/BL6 showed no increased susceptibility, while BALB/c $CD1d^{-/-}$ mice succumbed more rapidly to disease than wild-type animals. Inter-strain genetic variability within the natural killer complex (NKC) accounted for the difference in susceptibility between the strains and correlated with the ability of iNKT cells to regulate the Th1/Th2 polarization of T cell responses (Hansen *et al.*, 2003a). In addition to cytokine production, recognition of *Plasmodium* GPI anchors by CD1d-restricted T cells has been suggested to contribute to B cell help for production of antibodies against various GPI-linked protein antigens, though these findings are controversial (Hansen *et al.*, 2003b) (see also Molano *et al.*, 2000; Romero *et al.*, 2001).

5.2.4. *Trypanosoma* spp

Activation of iNKT cells promotes immunity against infection with *T. cruzi*, the protozoan parasite responsible for Chagas disease. NKT cells become activated in the liver and spleen of mice following i.p. inoculation with parasites (Antunez and Cardoni, 2004; Duthie and Kahn, 2002). At sublethal doses, both C57/BL6 $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice develop elevated levels of parasitemia during the acute phase of disease, though they recover with similar kinetics as wild-type mice (Duthie *et al.*, 2002). At high infective doses, the phenotype of NKT cell-deficient mice is more striking. $J\alpha18^{-/-}$ mice develop exacerbated inflammatory responses, decreased specific antibody titers and die rapidly

following infection. Interestingly, in contrast to Jα18$^{-/-}$ mice, CD1d$^{-/-}$ animals are not more susceptible to disease than wild-type animals. These data suggest that dNKT and iNKT cells may respond differently to *T. cruzi* infection (Duthie *et al.*, 2005). The protective role of iNKT cells against *T. cruzi* infection is further supported by the finding that α-GalCer treatment confers protection to wild-type and NK cell-deficient mice, which are otherwise highly susceptible to disease (Duthie and Kahn, 2002, 2006). Furthermore, clinical data have correlated elevated levels of NKT cells with limited disease progression (Vitelli-Avelar *et al.*, 2006).

In contrast to their importance in *T. cruzi* immunity, CD1d-restricted T cells appear to play a minor role in the context of infection with *T. brucei* or *T. congolense*, related organisms causing sleeping sickness in humans (Shi *et al.*, 2006). Interestingly however, a recent report suggested that regulatory T cells may downmodulate otherwise protective functions CD1d-restricted T cells. In this study, wild type but not CD1d$^{-/-}$ mice depleted of CD25^{+} cells were resistant to *T. congolense* infection (Wei and Tabel, 2008).

5.2.5. Toxoplasma gondii

The immune response to *T. gondii*, the protozoan parasite responsible for toxoplasmosis, is complex and must strike the right cytokine balance to resolve infection without causing lethal immunopathology. NKT cells are elicited during *T. gondii* infection; however, whether activation of these cells is protective or harmful varies between mice of differing genetic backgrounds and different parasite strains. Initial reports found that antibody depletion of both NK and NKT cells, but not NK cells alone, from BALB/c mice ameliorated survival chances following i.p. infection with the mildly virulent *T. gondii* Beverly strain, suggesting that NKT cell activity was detrimental (Nakano *et al.*, 2001, 2002). In contrast, CD1d-restricted T cells were found to be required for survival following oral administration of low doses of ME49 strain *T. gondii* cysts to C57/BL6 but not BALB/c mice, with CD1d$^{-/-}$, and to a lesser extent Jα18$^{-/-}$ C57/BL6 mice, displaying decreased survival chances. Interestingly, while intestinal parasite burdens were only moderately elevated in CD1d$^{-/-}$ animals, these mice showed exacerbated ilieal immunopathology, with increased Th1 cytokine levels and more activated CD4^{+} T cells (Smiley *et al.*, 2005). However, in a model of *T. gondii* inflammatory bowel disease triggered by oral administration of high doses of 76K strain cysts, iNKT cells appeared to play a detrimental role. Unlike Smiley *et al.*, this group found that Jα18$^{-/-}$ C57/BL6 mice survived better than wild-type mice following infection (Ronet *et al.*, 2005).

5.3. CD1d-restricted T cells in immunity to viruses

5.3.1. Herpesviruses (HSV-1, HSV-2, MCMV, VZV, and EBV)

Several studies indicate that CDd-restricted T cells may be important in immunity against certain herpesviruses, a large family of DNA viruses including HSV-1, HSV-2, CMV/MCMV, VZV, and EBV.

Following skin infection with HSV-1 (SC16 strain), both CD1d$^{-/-}$ and Jα18$^{-/-}$ C57/BL6 mice develop exacerbated disease. Although these mice are not more susceptible to the less virulent KOS HSV-1 strain (Cornish *et al.*, 2006), SC16 HSV-1 infection causes them to develop larger and more abundant zosteriform lesions, increased viral load, and nearly fourfold-elevated expression of latency-associated transcripts (LATs) in sensory neurons of the dorsal root ganglia, indicating more extensive viral spread across the spinal cord. Survival following infection with SC16 HSV-1 was affected in both CD1d$^{-/-}$ and Jα18$^{-/-}$ mice, though the phenotype of Jα18$^{-/-}$ mice was milder. These data suggest that both dNKT and iNKT cells play a role in control of SC16 HSV-1 infection (Grubor-Bauk *et al.*, 2003, 2008). SC16 HSV-1 interference with CD1d recycling pathways in human APCs, resulting in decreased CD1d surface levels and evasion of iNKT cell recognition further reflects the immune pressure exerted on HSV-1 by the CD1d recognition system (Raftery *et al.*, 2006; Yuan *et al.*, 2006). CD1d-restricted T cells also appear to promote control of HSV-2, since vaginal infection with HSV-2 is exacerbated in CD1d$^{-/-}$ mice (Ashkar and Rosenthal, 2003). Furthermore, α-GalCer treatment of MCMV-infected mice reduced viral replication in visceral organs, although Jα18$^{-/-}$ mice were not markedly more susceptible to infection than wild-type animals (van Dommelen *et al.*, 2003). Finally, clinical observations have correlated increased patient susceptibility to VZV and EBV with deficiencies in CD1d-restricted T cells (Levy *et al.*, 2003; Pasquier *et al.*, 2005; Rigaud *et al.*, 2006).

5.3.2. Hepatitis viruses (HBV and HCV)

Because CD1d-restricted T cells are particularly abundant in the liver (20–30% of intrahepatic lymphocytes), their role in the immune response against hepatitis viruses is especially intriguing. Although the lack of practical and robust murine models for hepatitis virus infection has hindered progress in this field, some data indicate that NKT cells become activated and can contribute to both antiviral immunity and liver immunopathology. In an initial report, i.v. injection of α-GalCer into HBV-transgenic mice was found to rapidly inhibit viral replication *in vivo*. Induction of both Type I and Type II IFNs in the liver by activated iNKT cells was critical for this antiviral effect (Kakimi *et al.*, 2000). In contrast, another study reported that adoptive transfer of dNKT cells specifically, but not iNKT cells, was sufficient for development of acute

hepatitis in HBV-transgenic $Rag^{-/-}$ mice (Baron *et al.*, 2002). Interestingly, recognition of stress-induced ligands by NKG2D expressed at the surface of dNKT cells was required for activation of these cells and development of disease (Vilarinho *et al.*, 2007). Increased frequency and Th2 cytokine production by iNKT cell has also been suggested to promote cirrhosis in humans chronically infected with HBV or HCV (de Lalla *et al.*, 2004). Upregulated CD1d expression at the hepatocyte–biliary border and on infiltrating mononuclear cells of HCV-infected patients may account for CD1d-restricted T cell activation (Durante-Mangoni *et al.*, 2004). Furthermore, recent findings suggest that Ito cells, a specialized hepatic DC subset, express high levels of CD1d and might be important for activating iNKT cells in the liver during infection (Winau *et al.*, 2007).

5.3.3. Human immunodeficiency virus

Several reports indicate that iNKT cells are prime targets for HIV infection and are selectively depleted during the first year following HIV infection in humans (Motsinger *et al.*, 2002; van Der Vliet *et al.*, 2000, 2002). Interestingly, treatment with IL-2 or by highly active antiretroviral therapy (HAART) was found to rapidly restore iNKT cell populations in infected patients (Moll *et al.*, 2006; van der Vliet *et al.*, 2006). Whether these cells have antiviral effects is not known. Nevertheless, the HIV proteins Nef and gp120 were found to downregulate surface expression of CD1d, suggesting that CD1d-restricted T cells may in fact exert immune pressure on the virus (Chen *et al.*, 2006; Cho *et al.*, 2005; Hage *et al.*, 2005).

5.3.4. Influenza A virus

Activation of iNKT cells promotes immunity against influenza A infection. iNKT-deficient C57BL/6 mice are highly susceptible to the Puerto Rico/8/34 (PR8) influenza A virus. Following intra-nasal injection of high doses of PR8, both CD1d and $Ja18^{-/-}$ mice are severely impaired in the development of PR8-specific T and B cell responses, fail to control viral replication, and die within 8 days. Adoptive transfer of iNKT cells into $Ja18^{-/-}$ but not $CD1d^{-/-}$ mice restores the ability of the animals to control the infection indicating that CD1d-dependent iNKT cell activation is important for anti-viral immunity in this model (De Santo *et al.*, 2008). In addition, intraperitoneal injection of α-GalCer into C57/BL6 mice intranasally infected with human influenza enhances early innate immune responses, diminishing weight loss and pulmonary viral titers (Ho *et al.*, 2008). The role of CD1d-restricted T cells has also been studied in the context of development of heterosubtypic immunity between different influenza A subtypes. In this study, $CD1d^{-/-}$ C57/BL6 mice were as efficient as wild-type mice in mounting cross-protective antiviral immunity, suggesting that CD1d-restricted T cells are not required for memory immune responses in this context. Furthermore, survival upon challenge

infection was comparable in the presence or absence of CD1d (Benton *et al.*, 2001). Together, these data indicate that activation of CD1d-restricted T cells, though beneficial, is not required for immunity to influenza A virus.

5.3.5. Respiratory syncythial virus (RSV)

CD1d-restriced T cells participate in the immune response against RSV, a single-stranded RNA virus causing seasonal epidemics of respiratory infection. α-GalCer treatment of BALB/c mice infected with RSV diminished illness-linked weight loss. However, iNKT cell activation also delayed viral clearance and RSV-infected $CD1d^{-/-}$ BALB/c mice lost less weight than wild-type controls, revealing contrasting protective and harmful effects in this model. In contrast, in C57/BL6 mice, CD1d-deficiency leads to mildly exacerbated illness and a slight delay in viral clearance, suggesting that NKT cells promote antiviral resistance in these mice (Johnson *et al.*, 2002).

5.3.6. Diabetogenic encephalomyocarditis virus (EMCV-D)

CD1d-restricted T cells play an important role in protection against the EMCV-D, a rodent virus causing paralysis, myocarditis and diabetes. Both $CD1d^{-/-}$ BALB/c and $CD1d^{-/-}$ C57/BL6 mice developed exacerbated disease following i.p. infection. Treatment with α-GalCer prevented disease. Rapid induction of IL-12 and NK cell activation, which is critical for protection, depended on CD1d-dependent immune responses (Exley *et al.*, 2001, 2003; Ilyinskii *et al.*, 2006).

5.3.7. Coxsackievirus B3 (CVB3)

CD1d-restricted T cells might play an important role in the pathogenicity of CVB3. In BALB/c mice, myocarditis induced by CVB3 is obliterated in $CD1d^{-/-}$ but not in $J\alpha18^{-/-}$ mice. These data suggest that $V\alpha14^{-}$ CD1d-restricted T cells contribute to CVB3 disease (Huber *et al.*, 2003).

5.3.8. Lymphocytic choriomeningitis virus (LCMV)

CD1d-restricted T cells are not able to control LCMV, a rodent virus that occasionally causes disease in immunocompromised subjects. Although the absence of CD1d affects the cytokine balance during the immune response to LCMV (Roberts *et al.*, 2004), the kinetics of viral clearance and the mounting of cellular immunity against LCMV are comparable in wild-type and $CD1d^{-/-}$ mice (Spence *et al.*, 2001). Interestingly, APCs from LCMV-infected mice are able to activate iNKT cells *in vitro* in a CD1d-independent manner. Activation, however, is rapidly followed by iNKT cell death. *In vivo*, this is reflected by a rapid and persistent loss of iNKT cells following infection (Lin *et al.*, 2005). Thus, failure to control

infection in this case may include a significant element of viral immune evasion in animals that possess NKT cells.

5.4. CD1d-restricted T cells in immunity to fungi

Only a few studies have investigated the role of iNKT cells in antifungal immunity. To date, all have focused on the response against *C. neoformans*. The contribution of iNKT cells to immunity against other significant fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* remains to be assessed.

5.4.1. Cryptococcus neoformans

C. neoformans is a ubiquitous yeast-like fungus and an important opportunistic pathogen. Inhaled *C. neoformans* cells can cause granulomatous lesions in the lung. Hematogenous dissemination to the central nervous system may result in severe and sometimes fatal meningoencephalitis. $CD4^+$ T cell-mediated immunity is thought to be critical in limiting infection, with AIDS and immunocompromized patients being particularly prone to develop cryptococcosis infection.

iNKT cells participate in the murine immune response to *C. neoformans*. iNKT cells accumulate within a week in the lungs of intratracheally infected mice, in a partially MCP-1-dependent manner. In $J\alpha281^{-/-}$ mice, which are iNKT cell-deficient, clearance of the fungi from the lungs is measurably delayed and DTH responses are obliterated (Kawakami *et al.*, 2001a). Furthermore, α-GalCer treatment of mice systemically infected with *C. neoformans* decreases the fungal burden in the lung and spleen and enhances pathogen-specific Th1 responses (Kawakami *et al.*, 2001b,c).

5.5. Evasion of CD1d-restricted T cell recognition

As significant roles for CD1d-mediated microbial detection and host defense are defined, also surfacing rapidly are the mechanisms evolved by pathogens to evade recognition by this system. Most, though not all, evasion strategies described to date involve interference with presentation of endogenous or exogenous lipids by CD1d molecules. For microbial evasion of CD1a, b, and c, see Group I CD1 section above.

5.5.1. Enhanced CD1d internalization

A common tactic adopted by pathogens to reduce surface levels of CD1d is to promote its rapid internalization. Kaposi sarcoma-associated herpesvirus (KSHV) encodes a set of proteins known as ''modulators of immune recognition'' (MIRs). MIR-1 and MIR-2 are expressed during the lytic replication phase of KSHV, and function as membrane-bound ubiquitin ligases that target lysines in the cytoplasmic tails of transmembrane

proteins. Ubiquitinated molecules are rapidly internalized and sent to lysosomal compartments for degradation. MHC Class I molecules, the costimulatory molecule B7.2 and the leucocyte adhesion molecule ICAM-I are all targets of the MIR proteins (Coscoy and Ganem, 2000, 2001; Coscoy *et al.*, 2001). Similarly, MIR-1 and MIR-2 were found to be responsible for downregulating CD1d at the surface of KSHV-infected cells. Transient transfection of MIR-2 into uninfected cells leads to ubiquitination and internalization of surface CD1d, as well as to reduced recognition by CD1d-restricted T cell clones (Sanchez *et al.*, 2005).

Similarly, HIV-1-encoded proteins such as gp120 and Nef, which is known to reduce surface levels of both CD4 molecule and certain MHC Class I alleles, have been found to bind the cytoplasmic tail of CD1d and accelerate its endocytosis, resulting in decreased CD1d surface expression and impaired recognition by CD1d-restricted T cells (Chen *et al.*, 2006; Cho *et al.*, 2005) (see also Hage *et al.*, 2005). Although the mechanism of action of these proteins is not precisely understood, Nef-mediated CD1d downregulation may also involve retention of newly synthesized CD1d in the *trans*-Golgi network and it is thought to require recognition of multiple tyrosine-based motifs in the cytoplasmic tail of target molecules (Chen *et al.*, 2006; Cho *et al.*, 2005).

5.5.2. Block in delivery of CD1d to the cell surface

A number of viruses are able to sequester CD1d molecules intracellularly, impairing the delivery of these molecules to the plasma membrane. HSV-1 was found to both block traffic of newly synthesized CD1d molecules to the surface and retain naturally recycling CD1d molecules at the limiting membrane of lysosomes (Yuan *et al.*, 2006). The viral factors HSV-1 employs to disrupt CD1-trafficking are not known.

5.5.3. Destabilization of CD1d

The intracellular bacteria *Chlamydia trachomatis* has also been found to interfere with CD1d expression at the surface of infected cells. *C. trachomatis* encodes a protein known as chlamydial proteasome-like activity factor (CPAF) that is secreted into the host cells' cytoplasm. CPAF degrades RFX5, the transcription factor responsible for enhancing transcription of MHC Class I heavy chains and β2m. While *C. trachomatis* infection is not thought to affect CD1d gene expression, the lack of β2m is believed to cause immature CD1d molecules to accumulate in the ER. CPAF can also physically associate with the cytoplasmic tail of CD1d heavy chain and trigger its proteolytic degradation. As a result, the expression of CD1d at the surface of *C. trachomatis*-infected cells is drastically reduced (Kawana *et al.*, 2007).

5.5.4. Other mechanisms

Varicella virus (VV) and vesicular stomatitis virus (VSV) have been reported to interfere with CD1d antigen presentation. Infection of cells with either VV or VSV leads to impaired recognition of endogenous lipids by CD1d-restricted T cells without causing a detectable decrease in surface levels of CD1d. Viral modulation of specific cellular MAPK signaling pathways and expression of the VV kinase B1R and one of its targets, the H5R transcription factor, have been suggested to contribute to deregulating intracellular trafficking routes of CD1d molecules (Renukaradhya *et al.*, 2005; Webb *et al.*, 2006).

6. CONCLUSIONS

The breadth of functions and reactivities of CD1-restricted T cells is large and rapidly expanding. The earlier concept that T cell reactivity was limited to the peptides that bind MHC molecules has now been revised to include recognition of lipid antigens that can bind CD1a, b, c, and d molecules. These four isoforms differ significantly in the nature of the T cells they activate and complement one another in their intracellular trafficking and lipid-binding capacity. The evolution of the CD1 antigen-binding groove has specialized for binding hydrocarbon chains by London forces rather than binding peptides by hydrogen bonding and electrostatic interactions. The size and number of CD1 hydrophobic channels have been found to bind relatively short single chain lipids to enormously long chains of more than 60 C chains. CD1a and d have size constrained closed channels that can each accommodate lipids of up to approximately 22 C whereas the three hydrophobic channels of CD1b are interconnected by a fourth hydrophobic tunnel resulting in to potential to bind very long hydrocarbon chain that traverse from one interconnected channel to the next. For MHC Class I and II, their ability to bind peptides depends markedly on the ability of peptides to be delivered to their antigen-loading compartments in the ER or the lysosomes, respectively. By comparison, the CD1 isoforms survey the major subcompartments of the endocytic system with CD1a focusing on the ERC and CD1b and CD1d mainly localizing to late endosomes and lysosomes. This allows one CD1 isoform or another to lie in wait and be available for binding lipids in virtually any endocytic compartement to which they are internalized and corresponding traffic to intersect with and bind CD1 which can then deliver it to the plasma membrane for recognition by T cells. The differences in trafficking and loading of CD1 molecules compared to MHC Class I and II provide a distinct opportunity to elicit T cell responses and mediate host defense. The challenge for microbes to evade another antigen presentation pathway at the same time as MHC Class I and II provides a further hurdle necessary for pathogens to succeed.

The pathway of antigen presentation by CD1 molecules in still unfolding, but it is already clear that many components of lipid metabolism are adopted for immunological use by this system. The mechanisms of proteolysis used for turning over proteins such as the proteasome and lysosomal proteases are co-opted and used extensively for the generation of peptides for MHC Class I and II, respectively. By comparison, lipid uptake systems (lipoprotein particle receptors) are co-opted by cells to take up exogenous lipids as are saposins to facilitate lipid transfer and loading into CD1.

Most of the specificity for lipid binding has emphasized the characteristics of the acyl chain, but interactions between the polar head of the amphipathic antigens with the opening of the hydrophobic channels and the α helices at the membrane distal end are also important. The specificity for the fine structure and the stereochemistry of the polar headgroups for recognition by the TCR underscores the antigen-specific basis of T cell recognition. In contrast to the range of distinctively microbial and unique structures of lipids from the cell wall of *Mycobacteria* that bind to CD1a, b, and c, only a few microbial antigens have been isolated that are presented by CD1d to activate NKT cells. Several of these microbial NKT antigens utilize an α-anomeric carbon link to the acyl chain that is reminiscent of the pharmacological model antigen α-GalCer. However, iNKT cells can be activated not only by specific microbial lipid antigens, but also by inflammatory cytokines (IL-12, IL-18, Type I IFNs) in the context of infection and TLR activation of the APC. This latter strategy may allow the activation of iNKT cells in a vast array of infections, even without foreign antigen recognition by the iNKT TCR. The CD1a, b, and c-restricted T cells appear to be functionally like MHC-restricted adaptive Th1 T cells and CTL but with specificity for recognition of lipids antigens rather than proteins. In contrast, iNKT cells have been considered to be innate-like lymphocytes based on the limited diversity of their TCR and the rapid nature of their response. They participate in immunity against a wide range of pathogens including bacteria, fungi, parasites, and viruses. They not only act early to control infection but through their multiple effect on other leukocytes including DCs, B cells, and other T cells, they can also strongly influence later adaptive responses. In some cases iNKT cells are indispensible in the control of infection, while in other instances they may make only a limited contribution to host defense or instead mediate immunopathology that damages tissue and harms the host. The roles of iNKT cells in tumor immunity and in both preventing and mediating inflammation and autoimmunity are not covered in this review. But together, these examples make it clear that as T cells recognizing a distinct class of antigens, CD1-restricted T cells already are implicated in virtually every helpful and harmful immunologic process previously defined as T cell mediated. The potential for manipulating CD1a, b, c, or d-restricted T cell responses as therapeutic or preventative vaccines for infection and

cancer or to prevent or ameliorate autoimmunity are among the opportunities that the future holds. The structure and the nature of lipids are essential to microbial and mammalian life and now are also inherent as targets for cell-mediated immune responses.

ACKNOWLEDGMENTS

We thank Duarte Barral, Manfred Brigl, Elizabeth Leadbetter, and Raju Tatituri for critical reading of the manuscript, as well as Rosa Grenha and Luis Leon for help with creating the figures for this chapter.

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CHAPTER 2

How the Immune System Achieves Self–Nonself Discrimination During Adaptive Immunity

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Contents

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Advances in Immunology, Volume 102
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)01202-4

Abstract

We propose an "Avidity Model of Self–Nonself Discrimination" in which self–nonself discrimination is achieved by both central thymic selection and peripheral immune regulation. The conceptual framework that links these two events is the understanding that both in the thymus and in the periphery the survival or the fate of T cells is determined by the avidity of the interactions between T cell receptors (TCRs) on T cells, specific to any antigens and MHC/antigen peptides presented by antigen-presenting cells (APCs). We envision that the immune system achieves self–nonself discrimination, during adaptive immunity, not by recognizing the structural differences between self versus foreign antigens, but rather by perceiving the avidity of T cell activation. Intrathymic deletion of high avidity T cell clones responding to the majority of self-antigens generates a truncated peripheral self-reactive repertoire composed of mainly intermediate and low but devoid of high avidity T cells compared with the foreign-reactive repertoire. The existence of intermediate avidity self-reactive T cells in the periphery represents a potential danger of pathogenic autoimmunity inherited in each individual because potentially pathogenic self-reactive T cells are included in the pool of intermediate avidity T cells and can often be functionally activated to elicit autoimmune diseases. The distinct composition of peripheral T cell repertoires to self versus to foreign antigens provides a unique opportunity for the immune system to discriminate self from nonself, in the periphery, by selectively downregulating intermediate avidity T cells to both self and foreign antigens. Selective downregulation of the intermediate avidity T cell populations containing the potentially pathogenic self-reactive T cells enables the immune system to specifically control autoimmune diseases without damaging the effective anti-infection immunity, which is, largely, mediated by high avidity T cells specific to the infectious pathogens. In this regard, it has been recently shown that Qa-1-restricted $CD8^{+}$ T cells selectively downregulate

intermediate avidity T cells, to both self and foreign antigens, and as a consequence, specifically dampen autoimmunity yet optimize the immune response to foreign antigens. Selective downregulation of intermediate avidity T cells is accomplished via specific recognition, by the Qa-1-restricted $CD8^+$ T cells, of particular Qa-1/self-peptide complexes, such as Qa-1/Hsp60sp, which function as a common surrogate target structure and preferentially expressed on the activated intermediate avidity T cells. This regulatory pathway thus represents one example of the peripheral mechanisms that the immune system evolved to complete self–nonself discrimination that is achieved, imperfectly, by thymic negative selection, in order to maintain self-tolerance. The conceptual framework of the "Avidity Model" differs from, but contains intellectual wisdom of certain conceptual elements of, the "Tunable Activation Thresholds Hypothesis," the "Danger Model," and the "Ergotypic Regulation Phenomenon." It provides a unified and simple paradigm to explain various seemingly unrelated biomedical problems inherent in immunological disorders that cannot be uniformly interpreted by any currently existing paradigms. The potential impact of the conceptual framework of the "Avidity Model" on our understanding of the development and control of commonly seen autoimmune diseases is also discussed.

1. INTRODUCTION

By discriminating self from nonself and controlling the magnitude and class of immune responses, the immune system mounts effective immunity to virtually any foreign antigens but avoids harmful immune responses to self (Cohn, 2004; Jiang and Chess, 2006). Immunologically relevant clinical problems often occur due to failure of either process.

How the immune system achieves self–nonself discrimination remains a central conundrum in *Immunology*. Understanding the mechanism of peripheral self-tolerance is the key to the solution of a major biomedical problem of how to selectively shut off the unwanted immune response to self-antigens but retain the normal on going immune responses to foreign pathogens intact.

The immune system accomplishes self–nonself discrimination, in part, by employing cell-surface recognition molecules which, when activated by nonself ligands leads to the elaboration of inflammatory molecules and/or the death of adjacent cells. For example, the cells of the innate immune system (NK cells, macrophages, dendritic cells (DCs), and granulocytes) employ sets of germ-line encoded receptors that specifically recognize conserved molecular patterns found predominately in microorganisms and other nonself pathogens and particles. Thus, recognizing

complement regulatory proteins and the toll-like or pathogen-associated molecular pattern recognition molecules (PAMPs), which define precise differences between foreign pathogens and self cells, by the innate receptors is one approach that the immune system achieves self–nonself discrimination (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000, 2002). In contrast, the adaptive immune system operates predominantly on the somatically generated antigen receptors, which are clonally distributed on T and B cells. These antigen receptors are generated by random processes and the biological design of the adaptive immune system is based on clonal selection of lymphocytes expressing receptors with particular antigenic specificities. Although the antigen receptors on T or B cells enable these cells to distinguish an almost infinite number of distinct self and foreign antigens, they cannot determine to which antigens the T and B cells should and to which antigens these cells should not respond. As a consequence, the adaptive immune system cannot achieve self–nonself discrimination by just simply using antigen receptors on effector T or B cells to distinguish self from nonself by recognizing the structural differences between self and foreign antigens. In fact, the antigen receptors are randomly generated (Davis, 2004; Litman *et al.*, 1999) without any ''knowledge'' of what is self and what is foreign. If there is any bias during the ontogeny of the adaptive immune system, it is towards self-recognition because all T cells are self-referential in the sense that they are positively selected for survival on self-peptide/s bound to major histocompatibility complex (MHC) molecules during thymic positive selection (Bevan, 1977; von Boehmer and Kisielow, 1990; Waldmann, 1978; Zinkernagel, 1978) before thymic negative selection, in which thymocytes expressing T cell receptor (TCR) of high avidity to the majority of self-antigens are deleted (Hengartner *et al.*, 1988; Kappler *et al.*, 1987; Pircher *et al.*, 1991). It is generally accepted that thymic negative selection eliminates the ''imminent danger'' of pathogenic autoimmunity in the periphery and is the major mechanism of self-tolerance.

Intrathymic deletion of high avidity self-reactive T cells generates a truncated peripheral self-reactive repertoire composed of mainly intermediate and low but devoid of high avidity T cell clones compared with the foreign-reactive repertoire, which possesses T cells with a full array of avidity. Thus, from the onset, the compositions of T cell repertoires to self versus to foreign antigens differ from each other. We propose that the distinct composition of peripheral T cell repertoires to self versus to foreign antigens provides a unique opportunity for the immune system to discriminate self from nonself, in the periphery, by selectively downregulating intermediate avidity T cells to both self and foreign antigens. Selective downregulation of intermediate avidity T cell populations containing the potentially pathogenic self-reactive T cells (Anderton *et al.*, 2001; Han *et al.*, 2005; Jiang *et al.*, 2003; Zehn and Bevan, 2006)

enables the immune system to employ a unified and simple mechanism to specifically control autoimmune diseases without damaging the on going anti-infection immunity, which is, largely, mediated by high avidity T cells specific to the foreign pathogens (Chen *et al.*, 2007; Jiang *et al.*, 2005; Wu *et al.*, 2009).

It is our thesis that self–nonself discrimination is achieved by central thymic selection followed by peripheral immune regulation because the consequence of thymic negative selection determines how the adaptive immunity is regulated in the periphery to achieve self-tolerance. Selective downregulation of intermediate avidity T cells specific to both self and foreign antigens is a unified mechanism that the immune system evolved to complete self–nonself discrimination in the periphery that is initiated by thymic negative selection.

2. IS PERIPHERAL IMMUNE REGULATION NECESSARY FOR SELF–NONSELF DISCRIMINATION?

2.1. A few facts

The pioneering work of Burnet and Medawar demonstrated that introducing a foreign antigen into animals during the neonatal period induces immunological tolerance to that foreign antigen and the animal will not make immune responses to reject the same antigen during adulthood (Billingham *et al.*, 1953; Burnet and Fenner, 1949). The definition of self versus nonself is therefore arbitrary to the immune system because foreign antigens presented during fetal life are thereafter considered self.

Since then understanding the mechanisms of self-tolerance has been a major focus of biomedical research in immunology. It is generally accepted that thymic negative selection, in which thymocytes expressing TCR of high avidity for MHC/self-peptide complexes are deleted (Hengartner *et al.*, 1988; Kappler *et al.*, 1987; Pircher *et al.*, 1991) eliminates ''imminent danger'' of pathogenic autoimmunity in the periphery and is the major mechanism of self-tolerance.

However, in order to provide a peripheral T cell repertoire with sufficiently large size and maximum flexibility to respond effectively to foreign antigens, in addition to the ''innocent'' self-reactive T cells with truly low avidity, thymic negative selection also allows a large fraction of self-reactive T cells with higher or intermediate avidity to be released into the periphery under normal circumstances (Bouneaud *et al.*, 2000; Jiang *et al.*, 2003; Sandberg *et al.*, 2000). The existence of the ''intermediate avidity'' self-reactive T cells in the periphery represents a potential danger of pathogenic autoimmunity inherited in each individual because these T cells can often be functionally activated when they encounter

self-peptides presented at a sufficient level in the periphery and some may differentiate into potentially pathogenic effector cells to initiate an autoimmune disease (Anderton *et al.*, 2001; Han *et al.*, 2005; Jiang *et al.*, 2003; Zehn and Bevan, 2006). We thus propose that self–nonself discrimination must continue in the periphery after thymic negative selection and a major function of peripheral regulatory mechanisms is to selectively downregulate immune responses to self-antigens without damaging the ongoing responses to foreign pathogens in order to maintain self-tolerance (Chen *et al.*, 2007; Jiang *et al.*, 2005; Wu *et al.*, 2009).

In this regard, it has been proposed that recurrent interactions would modify the sensitivity of T cells to ambient signals and, therefore, prevent ''resting'' lymphocytes from becoming activated and differentiating into effector cells (''Tunable Activation Thresholds Hypothesis'') (Grossman and Paul, 2001; Grossman *et al.*, 2004). The intermediate avidity thymic escapees that have the potential to react to self could thus ''ignore'' the endogenous self-ligands that are repetitively and constantly presented to them in the periphery and become tolerant to self. However, although the intermediate avidity self-reactive thymic escapees would thus be tolerant to self they could be functionally activated by a rapidly increased level of self-ligands presented (Grossman and Paul, 2001; Grossman *et al.*, 2004), such as ''danger signals'' (Matzinger, 1994, 2002) generated from an inflammatory process during an active infection or injury. To ensure self-tolerance, additional peripheral regulatory mechanisms are required to control the clonal expansion of self-reactive T cells with TCRs of avidity that are not sufficiently high to be eliminated intrathymically, but high enough to initiate pathogenic autoimmunity in the periphery.

The peripheral regulatory mechanisms involve processes intrinsic to the antigen activation and differentiation of T cells which include antigen activation-induced cell death (Lenardo *et al.*, 1999) and antigen-induced expression of costimulatory molecules including CD40L, CD28, and CTLA-4, which dictate whether immunity or anergy ensues (Chambers *et al.*, 2001; Durie *et al.*, 1994; Klaus *et al.*, 1994; Koulova *et al.*, 1991; Lenschow *et al.*, 1996). In addition, the functional activation and differentiation of the $CD4^+$ T cells into the Th1 and Th2 subsets (Coffman and Mosmann, 1991; Mosmann and Coffman, 1989; Mosmann *et al.*, 1986) or Tr1 and Tr3 subsets (Groux *et al.*, 1997; Levings and Roncarolo, 2000; Roncarolo and Levings, 2000) phenotypically identified, in part, by the elaboration of distinct cytokines are also considered an important aspect of the intrinsic mechanisms of regulation. Intrinsic mechanisms are usually induced by specific antigen but affect the immune responses either specifically or nonspecifically. In general, the intrinsic mechanisms have evolved to control the magnitude and class of immune responses to ensure an optimal immune response to foreign antigens by avoiding the collateral damage due to excessive reactions or an improper response due

to an inadequate class of an immune response. In the case of affecting immune responses by elaborating negative cytokines, such as IL-10 and TGF-β, the intrinsic mechanisms, by dampening all immune responses in general, could also nonspecifically ameliorate pathogenic autoimmunity (Jiang and Chess, 2004, 2006). In this regard, how to localize an antigen-specific, cytokine-mediated immune regulation to specifically target the relevant tissue or organ is a major challenge in attempt to employ mechanism of controlling the magnitude and class of immune responses to treat and prevent autoimmune disease. For example, it would, at least, require precise identification of specific self-antigen/s involved in each given autoimmune disease, which are largely undetermined at the present time, and the *in vivo* control of the migration and trafficking of relevant pathogenic as well as regulatory cells during an autoimmune process.

Superimposed on the intrinsic mechanisms of homeostatic regulation are the extrinsic regulatory mechanisms mediated by distinct T subsets of regulatory $CD4^+$ and $CD8^+$ T cells, which may suppress the outgrowth of potentially pathogenic self-reactive T cells in the periphery. The extrinsic regulatory mechanisms either function to discriminate self from nonself, therefore, specifically control pathogenic autoimmunity without damaging the on going immune responses to foreign pathogens, or alternatively, function to control the magnitude and class of immune responses which could also nonspecifically ameliorate pathogenic autoimmunity (Jiang and Chess, 2004, 2006). We emphasize that both self–nonself discrimination and the control of magnitude and class of immune response, executed by either intrinsic or extrinsic regulatory mechanisms, must function in concert to ensure an optimal function of the immune system.

The central issue we intend to address in this essay will be how the peripheral adaptive immunity is regulated at a biological system level, which enables the immune system to discriminate self from nonself in order to maintain self-tolerance without damaging its capacity to react to the invasions of foreign pathogens.

2.2. An "Avidity Model of Peripheral T Cell Regulation"

During the past few years, an ''Avidity Model of Peripheral T Cell Regulation'' has been proposed and tested, which may provide a unified conceptual framework to understand how immune responses, to both self and foreign antigens, are regulated in the periphery to achieve self–nonself discrimination (Chen *et al.*, 2007; Jiang and Chess, 2000; Jiang *et al.*, 2005; Wu *et al.*, 2009). The concept of the ''Avidity Model'' first emerged from the observations that $CD8^+$ T cells were involved in amelioration of experimental allergic encephalomyelitis (EAE) by selectively downregulating certain but not all self-peptide 1-9Nac MBP (myelin basic protein) reactive T cells enriched with encephalitogenic clones of higher growth

potential to MBP (Jiang *et al.*, 1992, 2003). This set of regulatory $CD8^+$ T cells were initially discovered to be involved in the resistant to EAE that were induced during the first episode of the disease (Jiang *et al.*, 1992; Koh *et al.*, 1992). It was later shown that the interaction between the $CD8^+$ T cells and the target T cells is restricted by the MHC Class 1b molecule, Qa-1 (Jiang *et al.*, 1995, 1998, 2001). More severe symptoms of EAE develop, in a much less controllable fashion, during the relapse in CD8 knock out mice (Koh *et al.*, 1992) or the re-induction of EAE in Qa-1 knock out mice (Hu *et al.*, 2004), indicating that Qa-1-restricted $CD8^+$ T cells play an important role in maintaining peripheral self-tolerance. The experiments that directly led to the formulation of the ''Avidity Model'' were the further observations that the Qa-1-restricted $CD8^+$ T cells were found to inhibit the immune response to a conventional antigen Hen egg lysozyme (HEL) when it functions as a self-antigen in HEL transgenic (TG) mice, but enhance the immune response to the same antigen when it functions as a foreign antigen in wild-type (WT) mice (Jiang *et al.*, 2005). Thus, the Qa-1-restricted $CD8^+$ T cells are involved in both the maintenance of peripheral tolerance to self-antigen and the optimization of T cell responses to foreign antigens.

Using a panel of HEL-specific $CD4^+$ T cell clones with different avidity, it was shown that the susceptibility of the target T cells to the downregulation by the $CD8^+$ T cells is determined by the avidity of the interactions that activates the T cell clones. In a wide range of the antigen doses used to activate the T cell clones, Qa-1-restricted $CD8^+$ T cells selectively downregulate the HEL-specific clones of intermediate but not high or low avidity, regardless whether these clones are derived from WT or from HEL TG mice (Jiang *et al.*, 2005). It was concluded from these studies that the strategy used by the Qa-1-restricted $CD8^+$ T cells to accomplish their tasks *in vivo* is to selectively downregulate activated T cells of intermediate avidity specific to both self and foreign antigens. Thus, the susceptibility of activated T cells to the downregulation by the Qa-1-restricted $CD8^+$ T cells is determined by the avidity of T cell activation, regardless of whether the T cells are activated by self or by foreign antigens (Jiang *et al.*, 2005).

The idea that perceiving the avidity of T cell activation can be translated into peripheral T cell regulation is the essence of the ''Avidity Model.'' The cellular mechanism that defines how perceiving the avidity of T cell activation is translated into peripheral T cell regulation and the molecular structures recognized by regulatory T cells that enable them to discriminate self from nonself in the periphery are the key issues of the regulatory T cell biology. Because the recognition of T cell targets is blocked by mAbs to Qa-1, CD8, and TCR (Jiang *et al.*, 1995, 1998, 2001, 2005), the actual target structure that is recognized by the TCR of $CD8^+$ T cells is likely to be certain self-peptides, presented by Qa-1, preferentially

expressed on target T cells as a function of intermediate avidity T cell activation (Jiang and Chess, 2000, 2006; Jiang *et al.*, 2005). In this regard, it is known that the predominant peptide bound to the MHC Class 1b molecule Qa-1, is Qdm, a hydrophobic peptide derived from leader sequence of MHC Class 1a molecules (Aldrich *et al.*, 1992; Lowen *et al.*, 1993). This peptide binds with high affinity and accounts for the majority of the peptides associated with Qa-1. Qa-1/Qdm interacts with CD94/NKG2A on NK cells and inhibits NK activity (Cotterill *et al.*, 1997; Kurepa *et al.*, 1998; Lu *et al.*, 2007). We have classified Qdm or Qdm-like peptides that could interact with CD94/NKG2A receptor and inhibit NK activity, when bound to Qa-1, as ''type A'' peptides (Chen *et al.*, 2007). Qa-1 can also bind other self-peptides, however, including those derived from heat shock proteins (Imani and Soloski, 1991) and preproinsulin leader sequences (Chun *et al.*, 1998). In this regard, human studies have shown that a signal peptide derived from the leader sequence of a stress protein Hsp60 (Hsp60sp) is capable of competing with B7sp peptide, the human counterpart of Qdm, for occupancy of HLA-E, the human counter part of Qa-1 (Michaelsson *et al.*, 2002). Interestingly, the resultant HLA-E/Hsp60sp complex does not interact with CD94/NKG2A and therefore is not capable of inhibiting the NK activity. Qa-1 binding peptides that do not interact with CD94/NKG2A, when coupled with Qa-1, were then classified as ''type B'' peptides (Chen *et al.*, 2007). It has been recently shown in mice that Hsp60sp, one of the type B peptides, presented by the MHC class 1b molecule Qa-1, is a surrogate target structure, preferentially expressed at a higher level on the intermediate avidity T cells and specifically recognized by the Qa-1-restricted $CD8^+$ T cells (Chen *et al.*, 2007). These studies provide a molecular and cellular mechanism suggesting that selective downregulation of intermediate avidity T cells by the Qa-1-restricted $CD8^+$ T cells can be accomplished via the specific recognition of Qa-1/Hsp60sp, expressed at a higher level on intermediate avidity T cells, by the $CD8^+$ T cells. The studies showed that intermediate avidity T cells have a significantly higher ratio of Qa-1/Hsp60sp versus Qa-1/Qdm, reflected by the differential expression of Hsp60 protein and the Qdm containing MHC Class 1a protein. This leads to the predominant expression of the surrogate structure Qa-1/Hsp60sp, a specific target for the regulatory $CD8^+$ T cells, on the surface of intermediate avidity T cells. Thus, preferential expression of particular surrogate target structures, which can be specifically recognized by the regulatory T cells, on the activated T cells of intermediate avidity, enables the immune system to distinguish high avidity T cells from intermediate avidity T cells in the periphery to achieve self–nonself discrimination by perceiving the avidity of T cell activation. The biological significance of this concept was further demonstrated by the ability of Hsp60sp-loaded relevant DCs to induce a Qa-1-restricted $CD8^+$ T cell-mediated protection from autoimmune encephalopathy in the EAE

model (Chen *et al.*, 2007). It is currently unclear if the Hsp60sp is the only peptide that serves as the target structure for the Qa-1-restricted $CD8^+$ T cells or if it is representative of a class of self-peptides that are capable of competing with Qdm or Qdm-like peptide/s for the occupancy to Qa-1, and serve as the target structures for the Qa-1-restricted $CD8^+$ T cells.

The regulatory pathway of selective downregulation of intermediate avidity T cells by the Qa-1-restricted $CD8^+$ T cells is envisioned to be a series of sequential cellular events as illustrated in Fig. 2.1. It is initiated by the activation of naïve T cells during the primary immune response in which the TCRs on T cells interact with MHC/antigen-peptide complexes presented by conventional antigen-presenting cells (APCs). One of the consequences of the T cell activation is the differential expression of a specific surrogate ''target structure,'' such as Qa-1/Hsp60sp, on the surface of target T cells (Chen *et al.*, 2007). Importantly, the expression of the surrogate ''target structure,'' which is recognized by the TCR on regulatory T cells, is a biological consequence of the intermediate avidity T cell activation (Chen *et al.*, 2007; Jiang *et al.*, 2005). During the induction phase, since T cells are not professional APCs, the professional APCs, such as DCs may be recruited and function to provide costimulatory molecules to prime the regulatory $CD8^+$ T cells. Alternatively, the regulatory $CD8^+$ T cells could also be directly primed by the relevant Qa-1 expressing DCs which are capable of cross presenting the common surrogate target structure, such as Qa-1/Hsp60sp, by engulfing and processing Hsp60 protein released by damaged cells involved in the inflammatory process during the primary immune response initiated by infections or injuries. The surrogate ''target structure'' presented by the inducer cells, including intermediate avidity T cells or relevant DCs, triggers the regulatory $CD8^+$ T cells to differentiate into effector cells, which in turn downregulate any activated T cells presenting the same target structures during the secondary immune response, which could be triggered by different antigens. The link between the induction and effector phases of Qa-1-restricted $CD8^+$ T cell-mediated pathway is the common surrogate target structures, such as Qa-1/Hsp60sp, expressed on both inducing and target cells regardless of which antigens these cells are triggered by. As a consequence, intermediate avidity T cells activated by any self and foreign antigens are targeted for the downregulation whereas high avidity T cells activated by any foreign antigens would escape the downregulation. This is one approach that the immune system achieves self–nonself discrimination in the periphery. On the other hand, it is conceivable that DCs expressing Qa-1/Hsp60sp on their surface may also be targeted by the regulatory $CD8^+$ T cells for downregulation during the secondary immune response. These DCs may either be killed or anergized, in the latter case, anergized DCs may be involved in the intrinsic mechanisms that could subsequently participate in control of the magnitude and class of immune responses.

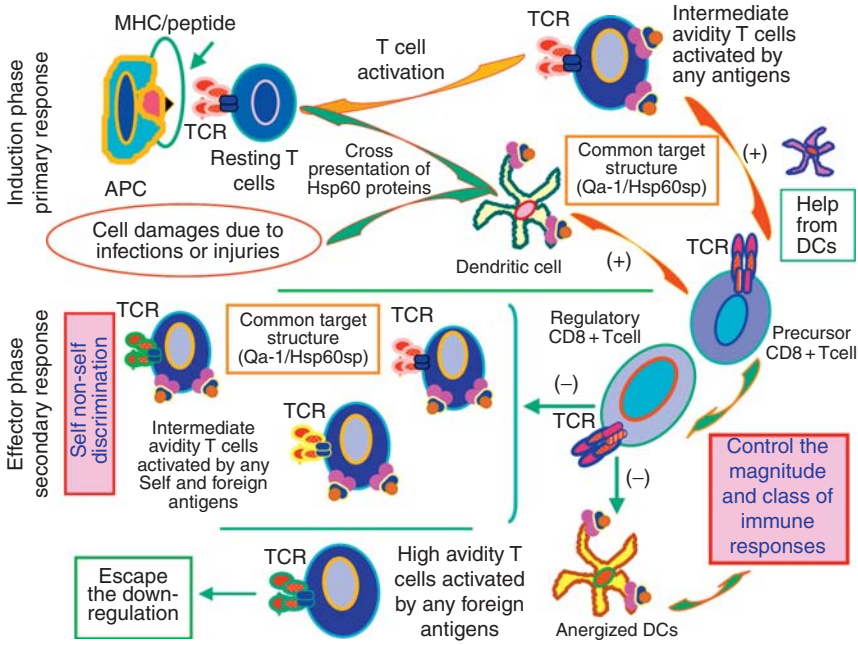

FIGURE 2.1 The cellular events of Qa-1-restricted $CD8^+$ T cell mediate pathway. It is initiated by the activation of naïve T cells during the primary immune response in which the TCRs on T cells interact with MHC/antigen-peptide complexes presented by conventional APCs. One of the consequences of the T cell activation is the differential expression of a surrogate target structure, such as Qa-1/Hsp60, on the surface of target T cells. Importantly, the expression of the surrogate target structure, specifically recognized by the TCR on regulatory T cells, is determined by the intermediate avidity interactions of T cell activation. During the induction phase, since T cells are not professional APCs, the professional APCs, such as dendritic cells (DCs) may be recruited and function to provide costimulatory molecules to prime the regulatory $CD8^+$ T cells. Alternatively, the regulatory $CD8^+$ T cells could also be directly primed by the relevant Qa-1 expressing DCs which are capable of cross presenting the common surrogate target structure, such as Qa-1/Hsp60sp, by engulfing and processing Hsp60 protein released by damaged cells during the primary immune response initiated by infections or injuries. The surrogate target structure presented by the inducer cells, including intermediate avidity T cells or relevant DCs, triggers the regulatory $CD8^+$ T cells to differentiate into effector cells, which in turn downregulate any activated T cells presenting the common surrogate target structures during the secondary immune response, which could be triggered by different antigens.

Taken together, despite the fact that, at a biological system level as well at molecular and cellular levels, the major characteristic features of the specificity and the biological function of the Qa-1/HLA-E-restricted $CD8^+$ T cells have been identified, there remain other biological features

of this pathway to be investigated. For example, it is unclear whether the Qa-1-restricted $CD8^+$ T cells are a lineage-specific subset of $CD8^+$ T cells or they instead represent a subset of conventional $CD8^+$ T cells expressing a unique set of αβ TCRs specific for particular self-peptide/s associated with Qa-1/HLA-E. In addition, the effector mechanism of the Qa-1/HLA-E-restricted $CD8^+$ T cells remained to be identified.

2.3. The implications of the conceptual framework of the "Avidity Model" on our understanding of peripheral T cell regulation

The conceptual framework of the ''Avidity Model'' offers a few unique insights to understand the peripheral T cell regulation and self–nonself discrimination.

1. The ''Avidity Model'' predicts that the specificity of the regulation is not at the level of antigens that activate the target T cells. The specificity is at the level of recognizing or sensing a common consequence of intermediate avidity T cell activation regardless of which antigens the target T cells are triggered by (Chen *et al.*, 2007; Wu *et al.*, 2009). It thus differs from the ''Idiotypic Model'' (Jerne, 1974), which functions to distinguish immune response to each individual antigen, via recognition of countless idiotypic peptide/s derived from the V regions of either TCRs or immunoglobulins generated by activated T or B cells (Batchelor *et al.*, 1989; Dorf and Benacerraf, 1984; Gammon and Sercarz, 1990). The ''Avidity Model'' functions to discriminate two types of immune responses, antiself versus antiforeign, by a unified and simple mechanism of selectively downregulating intermediate avidity T cells to both self and foreign antigens. This is achieved by recognition of common surrogate target structures expressed on T cells as a function of intermediate avidity of their own TCR-ligand interactions (Chen *et al.*, 2007; Jiang and Chess, 2000; Jiang *et al.*, 2005). Thus, the immune system discriminates self from nonself, during adaptive immunity, not by recognizing the structural differences between self versus foreign antigens, but rather by perceiving the avidity of T cell activation. Conceptually, the ''Avidity Model'' contains some elements of ''ergotypic regulation'' in that both types of regulation recognize the consequence of T cell activation. But it also differs from ''ergotypic regulation'' because the ''ergotypic regulation'' does not consider the avidity of T cell activation (Cohen *et al.*, 2004; Lohse *et al.*, 1989). The ''Avidity Model'' thus represents an alternative approach that enables the immune system to control peripheral immunity with sufficient specificity but does not demand a huge repertoire for the regulatory T cells. For example, in the Qa-1-restricted $CD8^+$ T cell pathway, the

actual target structures that are recognized by the $CD8^+$ T cells are certain self-peptide/s, such as Hsp60sp, presented by Qa-1, differentially expressed on target T cells as a function of intermediate avidity T cell activation. Since the diversity of self-peptides binding to Qa-1 that are responsible for rendering T cells susceptible to the downregulation by $CD8^+$ T cells is limited, the model enables the immune system to regulate immune responses to almost infinite diverse self and foreign antigens in a sufficiently specific and effective but also a straightforward and simple way. Furthermore, because the specificity of the regulation is not at the level of the antigens that activate the target T cells, control of autoimmune diseases could thus be achieved independent of the knowledge of the particular self-antigens involved, in any given autoimmune disease, that are largely undetermined at the present time (Wu *et al.*, 2009).

2. The model also predicts that the immune system employs a unified mechanism of suppression to regulate peripheral immune responses to both self and foreign antigens, which appears to have opposing effects: maintaining self-tolerance while optimizing, or at least not damaging, the immune response to foreign antigens. Because the compositions of the naïve peripheral TCR repertoires to self and foreign antigens are different due to thymic selection, the biological consequences of selective downregulation of the intermediate avidity T cells to self and foreign antigens are also different (Chen *et al.*, 2007; Jiang and Chess, 2000; Jiang *et al.*, 2005) (see Fig. 2.2). This forms the conceptual framework for a new paradigm to explain, at a biological system level, how the immune system achieves self–nonself discrimination, during adaptive immunity, without the necessity to distinguish self from nonself in the periphery at the level of T cell regulation.
3. The conceptual framework of the ''Avidity Model'' may also well be suited for other peripheral regulatory pathways. In this regard, particularly interesting and relevant may be the NK cells. The central molecule that links the NK pathway to the T cell regulation is Qa-1. It is well known that Qdm, the predominant Qa-1 binding peptide derived from leader sequence of conventional MHC Class 1a molecules (Aldrich *et al.*, 1994), binds with high affinity and accounts for the majority of the peptides associated with Qa-1, which interacts with CD94/NKG2A on NK cells and inhibits NK activity (Cotterill *et al.*, 1997; Kurepa *et al.*, 1998; Lu *et al.*, 2007). Since activated T cells usually express high level of Qa-1/Qdm on their surface, it becomes an essential mechanism by which the nonspecific and unnecessary killing of activated T cells by NK cells can be avoided. However, it was recently shown that peptides, including Hsp60sp, can compete with Qdm for binding to Qa-1 (Chen *et al.*, 2007) or HLA-E (Michaelsson *et al.*, 2002), and the resultant Qa-1/Hsp60sp complex is the specific target structure recognized by

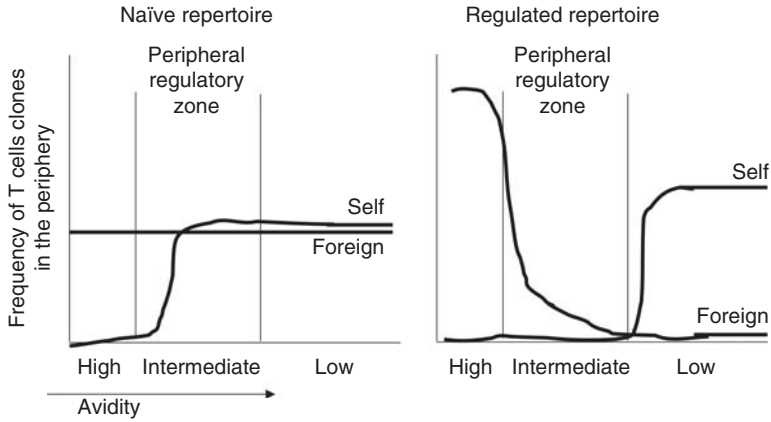

FIGURE 2.2 The selective downregulation of intermediate avidity T cells by the $CD8^+$ T cells shapes the peripheral T cell repertoire to both self and foreign antigens during the evolution of an immune response. Because the compositions of the naïve peripheral TCR repertoires to self and foreign antigens are different due to thymic negative selection, the biological consequences of selective downregulation of the intermediate avidity T cells to self and foreign antigens are also different (Chen *et al.*, 2007; Jiang *et al.*, 2005). Intrathymic deletion of high avidity self-reactive T cell clones generates a truncated peripheral self-reactive repertoire mainly composed of intermediate and low but devoid of high avidity clones compared with the foreign-reactive repertoire (Hengartner *et al.*, 1988; Kappler *et al.*, 1987; Pircher *et al.*, 1991). Thus, selective downregulation of the intermediate avidity T cell populations containing potentially pathogenic self-reactive T cells (Anderton *et al.*, 2001; Han *et al.*, 2005; Jiang *et al.*, 2003; Zehn and Bevan, 2006) provides a unified and simple mechanism to specifically control autoimmune diseases without damaging the effective anti-infection immunity, which is, largely, mediated by high avidity T cells specific to the infectious pathogens.

the Qa-1-restricted $CD8^+$ T cells. It has also been shown that the ratio of Qa-1/Hsp60sp versus Qa-1/Qdm is determined by the avidity of T cell activation (Chen *et al.*, 2007). Thus, when a significantly higher Qa-1/Hsp60sp versus Qa-1/Qdm ratio preferentially occurs in intermediate avidity T cells which reflects a decreased level of Qa-1/Qdm on the surface of intermediate avidity T cells, it leads to the increase of their susceptibility to, not only Qa-1-restricted $CD8^+$ T cell killing, but also to NK killing (Cotterill *et al.*, 1997; Kurepa *et al.*, 1998). This may represent another possible mechanism guided by the conceptual framework of the ''Avidity Model'' to discriminate self from nonself in the periphery, which connects innate immunity (NK effector cells) to adaptive immunity (target T cells of intermediate avidity) (Chen *et al.*, 2007; Jiang and Chess, 2000; Jiang *et al.*, 2001).

Qa-1/HLA-E-restricted $CD8^+$ T cell-mediated pathway is the only regulatory mechanism that is currently identified under the conceptual

framework of the ''Avidity Model'', which functions to discriminate self from nonself in the periphery by selectively downregulating the intermediate avidity T cells. As indicated above, it is conceivable that more mechanisms of this kind may exist and remain to be discovered and identified.

3. SELF–NONSELF DISCRIMINATION ACHIEVED DURING THYMIC NEGATIVE SELECTION DETERMINES HOW THE ADAPTIVE IMMUNITY IS REGULATED IN THE PERIPHERY TO MAINTAIN SELF-TOLERANCE

3.1. The immune system discriminates self from nonself during adaptive immunity, not by recognizing the structural differences between "self" versus "foreign" but by perceiving the avidity of T cell activation

Unlike the mechanisms employed by the immune system to discriminate self from nonself during innate immunity (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2000, 2002), the immune system appears to utilize a completely different approach to achieve self–nonself discrimination during adaptive immunity in both the thymus and the periphery. The basic question is: ''Does the immune system discriminate self from nonself, during adaptive immunity, by distinguishing what is self from what is foreign?'' We envision that the immune system does not ''know'' the differences between the distinct structures of a self-antigen versus a foreign antigen during thymic negative selection because, as mentioned earlier in this essay, foreign antigens presented during fetal life are thereafter considered self (Billingham *et al.*, 1953; Burnet and Fenner, 1949). Furthermore, the immune system does not ''know'' the differences between T cells reacting to a self-antigen versus to a foreign antigen at the level of peripheral T cell regulation either. For example, Qa-1-restricted $CD8^+$ T cells regulate the peripheral immune responses to achieve self–nonself discrimination by selectively downregulating activated T cells of intermediate avidity to both self and foreign antigens, regardless of which antigen the target T cells are triggered by (Chen *et al.*, 2007; Jiang *et al.*, 2005; Wu *et al.*, 2009). We, thus, propose that one major mechanism that the immune system uses to achieve self–nonself discrimination is to perceive, at different stages of development, differentially, the avidity of T cell activation to any antigens, both in the thymus and in the periphery. In the thymus, the immune system negatively selects for intermediate and low avidity T cells by eliminating high avidity T cells (Hengartner *et al.*, 1988; Kappler *et al.*, 1987; Pircher *et al.*, 1991), whereas in the periphery, it selects for high and low avidity T cells by eliminating

intermediate avidity T cells (Chen *et al.*, 2007; Jiang and Chess, 2000; Jiang *et al.*, 2005).

The preservation of low avidity self-reactive T cells, by allowing these cells to survive both the thymic and peripheral selection, is biologically significant. It is not only because low avidity to self MHC is an essential requirement for MHC restriction in T cell response achieved by thymic positive selection (Bevan, 1977; Bevan and Fink, 1978; von Boehmer and Kisielow, 1990; Waldmann, 1978; Zinkernagel, 1978), but also based on the fact that these low avidity self-reactive T cells would have high or intermediate avidity to foreign antigens (Goldrath and Bevan, 1999; Kawai and Ohashi, 1995; Sandberg *et al.*, 2000) due to the sufficient plasticity of their TCRs (Garcia *et al.*, 1998). These low avidity self-reactive T cells are thus preserved as a naive pool for the foreign repertoire in the periphery (Jiang *et al.*, 2005).

3.2. A theory for why and how intermediate avidity self-reactive T cells escape thymic negative selection

The activation state of thymocytes in the thymus is crucial for thymic negative selection, which is determined by two major parameters: the avidity of the TCRs and the level of self-peptides presented (Anderton and Wraith, 2002; Grossman and Paul, 2001; Grossman *et al.*, 2004). The avidity of a thymocyte is determined by the affinity of its TCRs, dictated by the structures generated by VDJ rearrangement, and the density of the TCRs expressed. The level of self-peptides presented in the thymus is determined by the number and the affinity of the MHC/self-peptide complex expressed on the surface of APCs (Peterson *et al.*, 1999), which could be influenced by the concentrations of extracellular antigens in the circulation, the expression of intracellular antigens in the thymic APCs as well as the capacities of the APCs to process and present self-antigens. In general, high-level presentation of antigens tends to activate thymocytes with a wide spectrum of avidity covering all high and the majority of intermediate range. In contrast, low-level presentation of antigens tends to only activate thymocytes of high avidity. The intermediate-level presentation of antigens might activate thymocytes with high and certain extent of intermediate avidity (Chidgey and Boyd, 1997; Cook *et al.*, 1997; Grossman and Paul, 2001; Rees *et al.*, 1999; Wang *et al.*, 1998).

Each individual thymocyte must undergo negative selection in the thymus to enable the immune system to respond to virtually any foreign antigens but avoid harmful responses to self in the periphery. The education process of the thymocytes could, in principle, be accomplished either by recognizing one or a set of ''common'' peptides, which could represent all peripheral self-antigens in the thymus (Ignatowicz *et al.*, 1996; Nossal, 1994), or by ''seeing'' the actual peripheral, tissue-restricted or

age-dependent, self-peptides expressed in the thymus, or by both. Discovery of the autoimmune regulator (Aire) gene (Mittaz *et al.*, 1999; Nagamine *et al.*, 1997) provided the first direct evidence for the latter hypothesis. Thus, Aire, functions as a transcription factor, which promotes the ectopic expression of peripheral tissue-restricted antigens, from a particular set of organs, in medullary epithelial cells in the thymus (Anderson *et al.*, 2002; Liston *et al.*, 2004) and also enhances the antigen-presentation capacity of these cells (Anderson *et al.*, 2005). It is unclear if ''Aire'' is an isolated unique event or it represents a class of genes controlling the expression and presentation of all presentable peripheral self-antigens in the thymus. Nevertheless, the discovery of the Aire gene makes it possible to predict that thymic APCs, under the control of Aire or other not yet identified Aire-like genes, express and present a full array of peripheral tissue-restricted or age-dependent self-antigens that the thymocytes would, otherwise, have no access to, during thymic negative selection.

Independent of how peripheral self-antigens are ''seen'' by the thymocytes, thymic negative selection has evolved to delete all self-reactive T cells that could potentially be functionally activated in the periphery in order to eliminate any ''imminent danger'' of pathogenic autoimmunity. This could be achieved either by a higher level presentation of self-antigens in the thymus than in the periphery (Yagi and Janeway, 1990) or by a lower or less ''stringent'' threshold of activation state of thymocytes for apoptosis in the thymus than for active function of these cells in the periphery (Pircher *et al.*, 1991). Thus, in general, self-reactive T cells that could be functionally activated by endogenous self-antigens in the periphery would be guaranteed to be activated in the thymus and undergo apoptosis to be deleted. Self-reactive T cells that could not be sufficiently activated in the thymus due to insufficient presentation of self-antigens and escape thymic negative selection would not be, for the same reason, functionally activated in the periphery by the biological exiting endogenous self-antigens either. In addition, ''Tunable Activation Thresholds Mechanism'' enables the thymic escapees to ''ignore'' the endogenous self-ligands that are repetitively and constantly presented to them in the periphery (Grossman and Paul, 2001; Grossman *et al.*, 2004). We, thus, envision that under biologically normal circumstances there would be no pathogenic autoimmunity in the periphery unless the system is perturbed by, for example, ''danger signals'' (Matzinger, 1994, 2002) generated from an inflammation during an active infection or injury (see Section 4.1.2).

On the other hand, thymic negative selection has also evolved to provide a peripheral T cell repertoire with sufficiently large size and maximum flexibility in order to respond effectively to foreign antigens. A threshold for the activation of thymocytes, therefore, must be established during thymic negative selection to determine the degree of

activation, which would allow apoptotic cell death to occur. The immune system uses self-antigen as a standard to establish this threshold. We propose that in general, thymocytes of high avidity specific to self-antigens presented at any level must be deleted and thymocytes of low avidity specific to self-antigens presented at any level, which could be activated at a baseline or very low level, must be preserved. The flexible area would be the self-reactive thymocytes of intermediate avidity, in which the flexibility of the threshold of the activation state of the thymocytes is determined by the level of a given self-peptide presented under normal biological circumstances. Thus, thymocytes of intermediate avidity that have the potential to be specifically activated by self-antigens presented at low levels would be deleted whereas thymocytes of intermediate avidity that have the potential to be specifically activated by self-antigens presented at high levels would be preserved. In Fig. 2.3, a line, which is drawn between the intersection of the high end of the low TCR avidity at the lowest biological level of antigen presented and the intersection of the low end of the high TCR avidity at the highest level of antigen presented, represents a hypothetical threshold of the activation state of the thymocytes. We propose that thymocytes with an activation state beyond the threshold undergo apoptosis and thymocytes with an

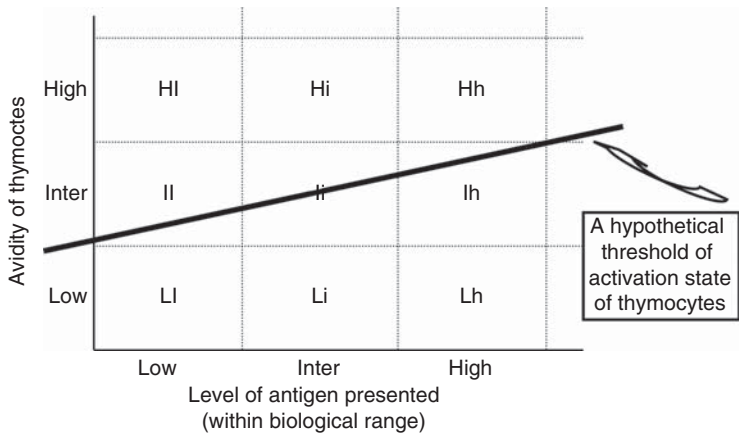

FIGURE 2.3 A hypothetical threshold of the activation state of thymocytes to undergo apoptosis during thymic negative selection. The *Y*-axis represents TCR avidity, which is determined by the affinity dictated by the structures of the TCRs and the density of TCRs expressed on each thymocyte. The *X*-axis represents the level of self-antigens presented in the thymus, which is determined by the number and the affinity of the MHC/self-peptide complex expressed on the surface of APCs, as well as the presentational capacity of the APCs. Thymocytes of activation state beyond the threshold undergo apoptosis and thymocytes of activation state below the threshold are spared from apoptosis and released into the periphery.

activation state below the threshold are spared from apoptosis. In this regard, as discussed above, the threshold of the activation state of thymocytes for apoptosis in the thymus could be lower or less ''stringent'' than the threshold of the activation state of mature T cells for their function in the periphery (Pircher *et al.*, 1991) so that the thymic escapees would not be functionally activated in the periphery under normal biological circumstances. We envision that thymocytes, which escape thymic negative selection, are all the thymocytes of low avidity and at least half of the thymocytes of intermediate avidity, which are specific to the self-antigens presented at different levels.

At this point, a third parameter that must be considered is the effect of costimulatory molecules (Buhlmann *et al.*, 2003; Foy *et al.*, 1995) as well as their respective receptors involved in T cell activation. There is currently little information available to assess the functional relationship of these molecules in the thymus versus in the periphery. In this regard, we envision that, in principle, molecular interactions involved in activation, such as CD28/B7 and CD40/CD40L, are likely higher or at least comparable in the thymus than in the periphery whereas molecular interactions involved in inactivation, such as CTLA-4/B7, might be lower or the same in the thymus than in the periphery. The rationale for defining these molecules as a separate parameter that influences the activation state of thymocytes is based on the consideration that manipulating the functions of these molecules is more feasible than manipulating TCR avidity and the level of antigen presented during an immune response in the periphery. The former is known to be relatively ''fixed'' by TCR VDJ rearrangement (Hackett *et al.*, 1992; Jolly *et al.*, 1996; Slifka and Whitton, 2001; Wagner and Neuberger, 1996) and the latter is usually the primary cause of an immunological disorder (see Section 4.1.2). This third parameter may thus provide a potential window to practically enhance or block the peripheral immune responses of certain kind for therapeutic purpose, but the price to pay would be the nonspecific effects of such therapy on normal on going immune responses. On the other hand, this third parameter may also be the most determining factor that could affect the avidity of T cell activation in the periphery to enable the intermediate avidity T cells to escape the peripheral regulatory mechanisms, leading to a pathogenic autoimmunity. In principle, this type of the pathogenic autoimmunity could be triggered by certain particular infections or some immunological therapies, which can potently affect the costimulatory pathways, even when the peripheral regulatory mechanisms are biologically sound (see Section 3.3).

Taken together, the view that self–nonself discrimination is a continuous process involving both thymic negative selection and peripheral immune regulation requires an understanding of how these two general events are connected. The ''Avidity Model'' provides a unified conceptual

framework to understand the biological inevitability that the consequence of thymic negative selection determines how the adaptive immunity is regulated in the periphery to accomplish self–nonself discrimination. Deletion of high avidity self-reactive T cells followed by the release of intermediate and low avidity self-reactive T cells during thymic negative selection enables the immune system to accomplish two major biological goals: elimination of ''imminent danger'' of pathogenic autoimmune and generation of a optimal peripheral foreign-reactive T cell repertoire with maximum size and flexibility. An important concept ought to be established: the physical link between thymic negative selection and peripheral immune regulation is the fact that release of intermediate avidity self-reactive T cells into the periphery, which contain potentially pathogenic self-reactive T cells (Anderton *et al.*, 2001; Han *et al.*, 2005; Jiang *et al.*, 2003; Zehn and Bevan, 2006), is a biological consequence of thymic negative selection (Bouneaud *et al.*, 2000; Jiang *et al.*, 2003; Sandberg *et al.*, 2000) and must be specifically dealt with by peripheral regulatory mechanisms.

3.3. Can the intermediate avidity self-reactive T cells that are released from thymic negative selection and activated in the periphery gain elevated avidity to escape the peripheral downregulation?

Selective downregulation of intermediate avidity T cell populations containing the potentially pathogenic self-reactive T cells enables the immune system to employ a unified mechanism to specifically control autoimmune diseases without inhibiting the overall on going anti-infection immunity (Chen *et al.*, 2007; Jiang and Chess, 2000, 2006; Jiang *et al.*, 2005). This type of mechanisms must deal with the question of whether it is powerful enough to control the pathogenic autoimmunity that is mediated by the intermediate avidity T cells which might gain an elevated avidity following peripheral activation to enable them to escape the peripheral downregulation. In this regard, the Qa-1-restricted $CD8^+$ T cell-mediated pathway has been shown to be inherently built with sufficient flexibility to particularly deal with the possibility that intermediate avidity T cells may gain certain elevated avidity when activated in the periphery.

First, Qa-1-restricted $CD8^+$ T cells have been shown to downregulate the intermediate avidity T cells activated by a wide range of antigen doses (Jiang *et al.*, 2005). Biochemical experiments further showed that there were no significant differences among the ratio between Qa-1/Hsp60sp and the Qa-1/Qdm expressed when the same HEL-specific clones were activated with varying doses of antigen HEL ranging from 1 to 50 μM under standard T cell culture conditions (Chen *et al.*, 2007). This observation indicates that one of the biological consequences of T cell activation,

the differential expression of Qa-1/Hsp60sp versus Qa-1/Qdm, is predominantly dependent on the avidity of TCRs on T cells, within a wide range of antigen doses used to activate T cells in an environment providing relatively constant costimulation. It thus appears that variation of this consequence of peripheral T cell activation determined by the avidity of TCRs on activated T cells is quite limited, *in vivo*, within a biological range of antigen presented because somatic hypermutation is rare in TCR postthymic selection in the periphery (Hackett *et al.*, 1992; Jolly *et al.*, 1996; Slifka and Whitton, 2001; Wagner and Neuberger, 1996). The limited variation of this biological consequence provides a necessary functional stability for the pathway to selectively downregulate intermediate avidity self-reactive T cells *in vivo*, which could be activated under variable conditions and become potentially pathogenic. However, to what extent the extremely high and low doses of antigens, the drastic change of intensity and duration of costimulation as well as the possible ''altered peptide-ligand'' interactions could influence this particular biological outcome of activation of T cells with fixed TCR avidity (affinity and density of TCRs expressed on each T cell) is unknown and needs to be further investigated.

Second, an overlapping zone of avidity between the low boundary of the activation state of thymocytes for apoptosis in the thymus and the high boundary of the activation state of T cells for peripheral downregulation exists in the periphery and functions to safe guard self-tolerance. In analyzing the HEL-reactive repertoire in HEL TG and WT mice it has been observed that HEL reactive clones bearing a common canonical TCR Vβ motif GTGQ are susceptible to the downregulated by the $CD8^+$ T cells in the periphery (Jiang *et al.*, 2005). Interestingly, although GTGQ motif frequently appears in the HEL-reactive repertoire in WT mice where HEL functions as a foreign antigen (Cabaniols *et al.*, 1994; Cibotti *et al.*, 1994; Jiang *et al.*, 2005), this motif has never been found in HEL-reactive repertoire in HEL TG mice where HEL functions as a self-antigen (unpublished observation). This observation suggests that clones bearing GTGQ motif are self-reactive and are deleted during thymic negative selection. Since the avidity of an actual T cell clone bearing GTGQ motif measured by ED_{50} is 3 μM (Jiang *et al.*, 2005), the low boundary of avidity for activation state of HEL reactive thymocytes to be intrathymically deleted is likely at least 3 μM or lower. On the other hand, it has been demonstrated that the high boundary of avidity for peripheral downregulation of HEL reactive clones by the Qa-1-restricted $CD8^+$ T cells is 1 μM (Jiang *et al.*, 2005). These observations, therefore, indicate the possibility that the low boundary of avidity for thymocytes to undergo apoptosis during thymic negative selection could be much lower than the high boundary of avidity of T cell activation state for peripheral T cell regulation. This creates an overlapping zone in the periphery between the low boundary of the activation

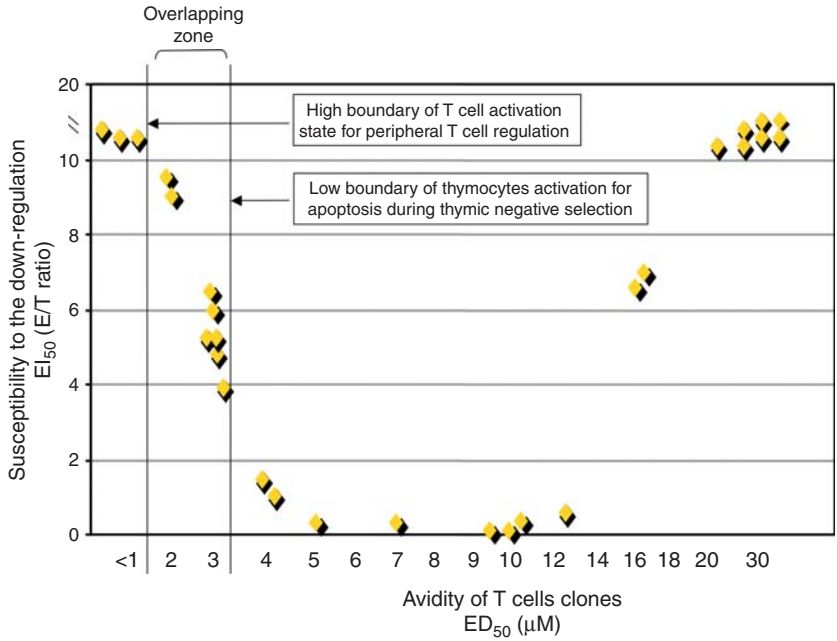

FIGURE 2.4 An overlapping zone of avidity in the periphery between the low boundary of the activation state of thymocytes for apoptosis in thymus and the high boundary of the T cell activation state for peripheral downregulation exist in the periphery. Since the low boundary for thymocytes to undergo apoptosis during thymic negative selection is lower than the high boundary of T cell activation state for peripheral T cell regulation. This creates an overlapping zone in the periphery between the low boundary of the activation state of thymocytes for apoptosis in the thymus, which allows intermediate avidity T cells to escape into the periphery, and the high boundary of the T cell activation state for downregulation. The overlapping zone provides a safe guard for maintaining the peripheral self-tolerance. Each dot represents one actual HEL clone with its susceptibility to the downregulation by the Qa-1-restricted $CD8^+$ T cells (Chen *et al.*, 2007; Jiang *et al.*, 2005).

state of thymocytes for apoptosis in thymus, which allows intermediate avidity T cells to escape into the periphery, and the high boundary of the T cell activation state for downregulation (Fig. 2.4). The existence of such an overlapping zone is important to understand the functional relationship between thymic selection and peripheral regulation. For example, if the intermediate avidity self-reactive T cells, which escaped thymic negative selection, were functionally activated in the periphery, even though these clones may gain certain elevated avidity, they would hardly pass the overlapping zone to escape peripheral downregulation. The overlapping zone thus provides a second level safe guard, superimposed on the window of the regulation with sufficient flexibility of the conditions

for intermediate avidity T cell activation described above, to maintain the peripheral self-tolerance. In general, the T cell clones fallen into the overlapping regulatory zone would be mainly foreign reactive and much fewer are self-reactive clones with avidity that are originally close to the low boundary during thymic negative selection but have gained an elevated avidity when accidentally activated in the periphery.

We envision that if the self-reactive T cells are not only pushed outside the regulatory window but also exceed the high boundary of overlapping zone, autoimmune diseases would likely occur, even when the regulatory mechanism itself functions normally. Such circumstances may likely occur due to extremely potent costimulation during a particular infection that enables these clones to escape the peripheral downregulation. It may also happen during immunological therapies to manipulate the costimulatory pathways, such as anti-CTLA-4 treatment in tumor vaccine therapy and provides a possible interpretation of the inverse relationship between the control of tumor and the increased tendency of organ-specific pathogenic autoimmunity (Hernandez *et al.*, 2001; Hurwitz *et al.*, 2000; van Elsas *et al.*, 1999) (see Section 4.1.2).

It is important to emphasize that, conceptually, the intermediate avidity T cells described in the ''Avidity Model'' (Jiang and Chess, 2000, 2006; Jiang *et al.*, 2005) represent a rather large pool of thymic escapees that have the avidity lower than those deleted in the thymus but cover a wide spectrum of avidity. In general, the spectrum of the avidity could extend from a high end close to the low boundary of the threshold of thymic negative selection to a low end, which might be near the high boundary of the threshold of thymic positive selection. We envision that the process and presentation of the physiologically significant self-peptides during thymic negative selection could be biologically programmed so that the threshold of thymic negative selection would vary for different self-peptides. In this regard, it has been shown in several studies that certain self-reactive T cells with ''low avidity,'' presumably at the low end of the intermediate avidity that we refer to, can be activated in the periphery to initiate autoimmune diseases (Anderton *et al.*, 2001; Jiang *et al.*, 2003; Zehn and Bevan, 2006), such as 1-9NacMBP-specific encephalitogenic clones in B10PL mice. On the other hand, in some autoimmune diseases, pathogenic self-reactive T cells appear to have much higher avidity to self-antigens, probably at the high end of the spectrum of the intermediate avidity, such as certain pathogenic diabetic clones in NOD mice (Han *et al.*, 2005). The observations that Qa-1-restricted $CD8^+$ T cells protect animals from both EAE and T1D (Hu *et al.*, 2004; Wu *et al.*, 2009) are consistent with the notion that this regulatory pathway selectively downregulate intermediate avidity self-reactive T cells, which are covered by a rather wide spectrum of avidity in the periphery.

4. THE IMPACT OF THE CONCEPTUAL FRAMEWORK OF THE "AVIDITY MODEL" ON THE DEVELOPMENT AND CONTROL OF PATHOGENIC AUTOIMMUNITY

4.1. Self–nonself discrimination and autoimmune disease

Based on the conceptual framework of the ''Avidity Model,'' defects of self–nonself discrimination at either central or peripheral levels could cause pathogenic autoimmunity in the periphery with distinct characteristic features.

4.1.1. Autoimmune disease develop as the consequence of failure of central thymic selection

Defects in genes primarily involved in thymic negative selection or some genes controlling general homeostasis in the thymus can lead to the release of high avidity self-reactive T cells, specific to a wide array of self-antigens expressed in multiple organs. These high avidity self-reactive T cells are capable of being functionally activated by endogenous self-antigens presented at any levels, which are biologically available in the periphery. Different degrees of rather immediate and global autoimmunity develop as a direct consequence of the activation of high avidity self-reactive T cells in the periphery, such as symptoms seen in Autoimmune polyglandular syndrome type 1 (APECED) (Nagamine *et al.*, 1997) or in Aire deficient mice (Chen *et al.*, 2005; Mittaz *et al.*, 1999). In general, pathogenic autoimmunity due to failure of thymic negative selection, which are usually rare and lethal genetic defects, develops spontaneously and early in life and reveals a rather drastic, global and diffused autoimmunity affecting multiple organs.

One consequence of failure of thymic negative selection, due to the release of high avidity self-reactive T cells, is the formation of a self-reactive T cell repertoire containing the high avidity portion that is indistinguishable from the foreign-reactive repertoire. It is unlikely that peripheral regulatory mechanisms would evolve to specifically control this type of rare genetic defect, which requires mechanisms that permit discrimination between high avidity T cells specific to self versus to foreign antigens and no working hypothesis has ever been proposed to deal with this problem. Nevertheless, the intrinsic mechanisms that control the magnitude and class of immune response, such as Th1 versus Th2 or Tr1 and Tr3 cells, may play major roles in nonspecific amelioration of overwhelming global pathogenic autoimmunity caused by the genetic defect of thymic negative selection (Jiang and Chess, 2006). In this regard, the studies of $CD25^{+}Foxp3^{+}$ Tregs have been focused on understanding the problems of defect of $Foxp3^{+}$ Tregs in the thymus and shown a potent negative regulation by the $Foxp3^{+}$ Tregs on the

''global autoimmunity'' as well as the excessive immune responses to foreign pathogens (Sakaguchi, 2004, 2005).

A cautionary note should be taken here. Mice possessing defective genes, that negatively control the activation state of T cells, almost universally show a lethal, ''global autoimmunity,'' such as in CTLA-4 KO mice (Chambers *et al.*, 1997, 2001), TGF-β KO mice (Christ *et al.*, 1994), and Foxp3 KO scurfin mice (Schubert *et al.*, 2001). Overwhelming inflammatory process associated with elevated T cell responses across the board to both self and foreign antigens by uncontrolled functions of T effector cells, directly caused by the defect of above genes in T cells, may play a major role in the ''global autoimmunity'' seen in these animals. However, whether the systemic defect of the relevant genes could also affect thymic negative selection leading to the ''global autoimmunity'' in these animals, as discussed above, is unclear, although CTLA-4 has been thought to be primarily involved in control of the T cell activation in the periphery (Chambers *et al.*, 1997, 2001). In this regard, $Foxp3^+$ Tregs have shown to be a potent mechanism that suppresses the ''global autoimmunity'' in scurfin mice (Fontenot *et al.*, 2003; Khattri *et al.*, 2003; Kretschmer *et al.*, 2005). However, whether the ''global autoimmunity'' in scurfin mice is caused by, particularly, the defect of $Foxp3^+$ Tregs (Kim *et al.*, 2007), or by a systemic defect of *Foxp3* gene in general, as in the cases of the relevant defective genes in CTLA-4 KO mice (Chambers *et al.*, 1997, 2001) or TGF-β KO mice (Christ *et al.*, 1994), remains a debatable issue (see Section 4.2).

4.1.2. "Danger signals" and peripheral regulation play central roles in the development and control of organ-specific autoimmune diseases

On the other hand, as a consequence of thymic negative selection, peripheral self-reactive T cell repertoire is mainly composed of intermediate and low avidity T cells. As discussed above, self-reactive T cells of low avidity are ''innocent'' and constitute the major portion of the naïve peripheral T cell repertoire reactive to foreign antigens. The fact that self-reactive T cells of intermediate avidity are part of the naturally formed peripheral T cell repertoire (Bouneaud *et al.*, 2000; Jiang *et al.*, 2003; Sandberg *et al.*, 2000) is biologically significant because it represents a potential danger of pathogenic autoimmunity inherited in each individual. However, based on the conceptual framework of the ''Avidity Model,'' postthymic negative selection, there would be no pathogenic autoimmunity in the periphery under biologically normal circumstances unless the system is perturbed by the environmental insults (see Section 3.2), which could then be ultimately controlled by peripheral regulatory mechanisms. We, thus, propose that different from pathogenic autoimmunity caused by the

defect of central thymic negative selection, the commonly seen organ-specific autoimmune diseases usually occur after the thymic negative selection is completed and are caused by defects of peripheral regulation and triggered by the environmental insults.

We envision that, self-reactive T cells of intermediate avidity, which cannot be adequately activated in the thymus to be deleted, due to insufficient presentation of self-ligands, would not be functionally activated in the periphery either, unless they encounter a rapidly increased level of self-ligands presented by the professional APCs (Grossman and Paul, 2001; Grossman *et al.*, 2004). A rapid increase of the level of self-ligands presented could occur in the periphery either during certain infections, due to molecular mimicry between infectious agents and self-antigens (Benoist and Mathis, 2001; Zhao *et al.*, 1998) or when large amount of self-antigens is released from the injured cells at the inflammation sites, as a consequence of unrelated infections or injuries (Matzinger, 1994, 2002). In addition, increased expression of costimulatory or MHC molecules on professional APCs as a consequence of the release of certain cytokines, such as INF-γ, from the inflammation sites may also play a crucial role in the accidental activation of intermediate avidity self-reactive T cells. Thus, in the absence of an adequate peripheral regulation, ''danger signals,'' generated from the ''dangerous self,'' during infections or injuries (Matzinger, 1994, 2002) play a central role in initiating a pathogenic autoimmunity. We further propose that because infections or injuries are usually confined within the organ affected, an active autoimmunity evoked by such insults, from a biologically normal peripheral T cell repertoire, is likely organ specific, which differs from the more diffused pathogenic autoimmunity caused by the defect of thymic negative selection. However, in certain cases, if the infection or injury occurs in multiple organs, autoimmunity in more than one organ could be seen in such patients.

Taken together, in order for an organ-specific autoimmune disease to occur, two hits are necessary: dysfunction of peripheral T cell regulation and a rapid, high-level presentation of particular self-antigen to the intermediate avidity self-reactive T cells. The former provides a condition for the development of organ-specific autoimmune diseases and the later determines which organ-specific autoimmune disease would occur. This explains why Qa-1 KO mice do not spontaneously develop unprovoked autoimmune disease in the first incidence (Hu *et al.*, 2004). However, in comparison with WT mice, although Qa-1 KO mice do require an experimental induction, like in the WT mice, to develop the first episode of EAE, they lost the capacity to resist the re-induction of EAE due to the defect of Qa-1-restricted $CD8^+$ T cells (Hu *et al.*, 2004). The phenotype of the Qa-1 KO mice precisely reveals how peripheral self-tolerance is maintained in a biological context of the most commonly

seen cases in real life, compared with the control of rarely seen, drastic and lethal global pathogenic autoimmunity that is primarily caused by genetic defect of thymic negative selection.

Consistent with this notion are the observations that in addition to an apparent clinical antitumor effect, treatment of mice with B16 melanoma GM-CSF vaccine and anti-CTLA-4 exclusively resulted in vitiligo, an autoimmune response restricted to melanocytes. Similarly, mice receiving the prostate cancer-GM-CSF vaccine and anti-CTLA-4 develop autoimmune prostatitis but no signs of global autoimmunity while the tumor is controlled (Hernandez *et al.*, 2001; Hurwitz *et al.*, 2000; van Elsas *et al.*, 1999). In this regard, a central question in tumor immunology is whether recognition of tumor antigens by the immune system leads to activation (i.e., immune surveillance) or tolerance, which depends on the nature of the tumor antigens (Pardoll, 2003). There is an important connection between tumor-antigen and self-antigen that determines the relationship between autoimmunity and antitumor immunity (Jiang and Chess, 2008). If the tumor antigens are, behaving like foreign antigens, capable of eliciting effective antitumor immunity, the tumor will be eliminated as a consequence of immune surveillance against tumor. However, if the tumor antigens behave like self-antigens, the tumor can either fail to activate or only activate T cells with intermediate avidity. This could occur because high avidity T cells to the particular self-antigens are deleted by thymic negative selection. The biological significance of eliciting intermediate avidity antitumor immunity during tumorigenesis cannot be ignored because the consequence of such immunity is to induce tolerance to the tumor based on the conceptual framework of the ''Avidity Model.'' The intermediate avidity antitumor T cells would be inhibited by the normal peripheral regulatory mechanisms, which evolved to control the potentially pathogenic autoimmunity. In this regard, blockade of the regulatory pathways, or elevating the avidity of antitumor T cells to enable them to escape the regulatory pathways to rescue the antitumor T cells, may be potentially necessary antitumor therapies, in combination with other approaches although the tendency of autoimmune disease may increase in these patients (Jiang and Chess, 2008). In this regard, anti-CTLA-4 in combination with tumor vaccine may render the antitumor T cells, as well as the relevant self-reactive T cells, to escape the peripheral downregulation by potently elevating the avidity of these cells, which are initially activated by a rapidly increased level of the relevant ''self-antigen'' derived from the vaccine tumor cells. This is evidence supporting the hypothesis that biologically available self-antigens presented in the periphery are not sufficient to functionally activate any self-reactive T cells released from natural thymic negative selection, which functional activation requires a rapid increase of self-ligand presented. Otherwise, blockade of CTLA-4 pathway to break the

peripheral regulation, in this case, would likely initiate a rather global but not organ-specific pathogenic autoimmunity, while the particular tumor is under the control.

One note is taken here, the primary task of the Qa-1-restricted $CD8^+$ T cells is to control the pathogenic autoimmunity, yet, it strengthens or at least, does not damage the normal on going anti-infection immunity mediated, largely, by high avidity T cells which naturally escape the downregulation by the Qa-1-restricted $CD8^+$ T cells (Jiang and Chess, 2008). In this regard, the function of Qa-1-restricted $CD8^+$ T cells favors but is not a deciding factor for the T cell affinity maturation to foreign antigens (Chen *et al.*, 2007; Jiang *et al.*, 2005; Wu *et al.*, 2009). Since the proportion of high avidity versus intermediate avidity T cells during an immune response to foreign antigen varies, this regulatory pathway may have various effects on the overall immune responses to foreign antigens, depending on the particular antigens and experimental systems used. Thus, in principle, the major effect of the regulation of Qa-1-restricted $CD8^+$ T cells *in vivo* is a decreased immune response to self-antigen associated with an increased or unchanged immune response to foreign antigen. Similarly, lacking the regulation of Qa-1-restricted $CD8^+$ T cells *in vivo* could be an increased immune response to self-antigen in combination with a decreased or unchanged immune response to foreign antigen. This could be reflected by an increased self-reactivity associated with a basically unchanged antiforeign reactivity in Qa-1 KO mice compared with WT mice (Hu *et al.*, 2004). An important point needs to be mentioned, the measurement of overall avidity of an immune response should be both the absolute magnitude of T cell proliferation and the ED_{50} of an antigen dose response curve (Jiang *et al.*, 2005; Wu *et al.*, 2009).

In summary, the ''Avidity Model'' provides a unified conceptual framework of the cellular and molecular basis to understand the development and control of pathogenic autoimmunity, particularly the commonly seen, organ-specific autoimmune diseases. We propose that the immune system evolved to be engaged in such a way that biologically available self-antigens would not be presented at a sufficient level in the periphery to functionally activate any self-reactive T cells, which escape thymic negative selection. Peripheral self-tolerance is therefore, indeed, established by thymic negative selection (Hengartner *et al.*, 1988; Kappler *et al.*, 1987; Pircher *et al.*, 1991) and reinforced by the ''Tunable Activation Thresholds Mechanism'' (Grossman and Paul, 2001; Grossman *et al.*, 2004). However, the peripheral regulatory mechanisms have also evolved to maintain peripheral self-tolerance by selectively downregulating intermediate avidity self-reactive T cells, which are released from the thymus as a biological consequence of thymic negative selection and could often be functionally activated during environmental insults, such

as infections or injuries. In this regard, the conceptual framework of the ''Danger Model'' (Matzinger, 1994, 2002) provides a solution to answer the central question of how the escaped intermediate avidity self-reactive T cells could be functionally activated in the periphery to elicit autoimmune diseases. Since the potentially pathogenic self-reactive T cells are included in the pool of intermediate avidity self-reactive T cells released from the natural thymic negative selection (Anderton *et al.*, 2001; Han *et al.*, 2005; Jiang *et al.*, 2003; Zehn and Bevan, 2006), the potential for pathogenic autoimmunity during an infection or injury is great. Selective downregulation of intermediate avidity T cells is one mechanism that the immune system evolves to specifically deal with this biologically inherited problem without paying the price to damage normal anti-infection and antitumor immunity (Chen *et al.*, 2007; Jiang *et al.*, 2005; Wu *et al.*, 2009).

4.2. $CD25^{+}Foxp3^{+}$ Tregs and autoimmune disease

It is impossible to discuss autoimmune disease without talking about $CD25^{+}Foxp3^{+}$ Tregs. As mentioned earlier, the central issue we intend to address in this essay is how the peripheral adaptive immunity is regulated at a biological system level, which enables the immune system to discriminate self from nonself, in the periphery, in order to maintain self-tolerance without damaging its capacity to react to the invasions of foreign pathogens. In this regard, there has been no unified conceptual framework to characterize the precise relationship between thymic negative selection and peripheral immune regulation, which is the basis for understanding self–nonself discrimination versus the control of the magnitude and class of immune responses. Absence of a unified conceptual framework has led to confusion, at both theoretical and experimental levels, in the field of immune regulation. The typical example is the prevailing theory of the role of ''specialized Tregs'' in control of the function of the immune system, which has dominated and profoundly influenced the general thinking in the field of immune regulation for the past two decades. In this regard, from 2006 to 2008, there have been more than 2,000 research articles regarding the $CD25^{+}Foxp3^{+}$ Tregs published in the major *Immunology* journals, representing a mainstream thinking and the major trend of research in the field of immune regulation. ''Specialized Tregs'' have been characterized by the expression of, initially, the CD25 molecule (Sakaguchi *et al.*, 1985, 1995; Shevach, 2001) and recently, the transcriptional factor Foxp3 (Fontenot *et al.*, 2003; Khattri *et al.*, 2003; Kretschmer *et al.*, 2005), known to regulate T cell activation and expression of certain cytokines (Brunkow *et al.*, 2001; Schubert *et al.*, 2001). The Tregs were postulated to be a naturally occurring, lineage-specific T cell subset with a rather huge and diverse TCR repertoire similar to

conventional $CD4^+$ T cells (Sakaguchi, 2004; von Boehmer *et al.*, 2003). Selection of these cells by certain self-antigens expressed on stromal cells in the thymus has been proposed as the mechanism for the development of the Tregs (Apostolou *et al.*, 2002; Kronenberg and Rudensky, 2005; Sakaguchi, 2004; von Boehmer *et al.*, 2003). However, the precise nature of the self-peptides involved has not been elucidated. Nevertheless, the ''$CD25^+Foxp3^+$ Tregs'' have been considered to be a ''specialized'' key mechanism mediating self-tolerance by suppression (Fontenot and Rudensky, 2005; Jaeckel *et al.*, 2006; Sakaguchi, 2004, 2005). In this regard, although the ''specialized Tregs'' can be activated and induced by both self and foreign antigens, the specificity of the suppression is unclear and the target cells as well as the molecular interactions between target cells and the Tregs are unknown (Cohn, 2004; Jiang and Chess, 2006). In fact, the *in vitro* assay that has been universally employed to monitor the effector phase of suppression mediated by the ''specialized Tregs'' does not seem to involve TCR nor to be MHC restricted (Shevach, 2002; Thornton and Shevach, 2000), although it has been shown that the *in vivo* suppression could be antigen specific (Apostolou and von Boehmer, 2004; Kretschmer *et al.*, 2005; Kronenberg and Rudensky, 2005). At the effector phase, the suppression is, at least in part, cell-contact dependent, but the surface molecules involved in the cell contact are unidentified (Shevach, 2002). In addition, IL-10 and TGF-β, which participate in the intrinsic mechanisms of suppression, have also been implicated in suppression by the ''specialized Tregs.''

More biologically significant are the observations that, at a system level, while suppressing the autoimmunity, these cells are also found to suppress the overall immune responses to foreign antigens, including antibacteria, antivirus, antifungi, and antiparasite responses in infectious disease (Hori *et al.*, 2002; Sakaguchi, 2003, 2004), as well as anti-allo response in organ transplantation (Sakaguchi, 2004; Waldmann and Cobbold, 2001; Wood and Sakaguchi, 2003; Zhai and Kupiec-Weglinski, 1999). Thus, the suppression appears to be global, covering the entire spectrum of overall immune responses to both self and foreign antigens. These observations strongly suggest that the ''specialized Tregs'' are unlikely to control autoimmunity by means of self–nonself discrimination. Instead, overwhelming evidence suggests that the ''specialized Tregs'' may nonspecifically ameliorate pathogenic autoimmunity by controlling the magnitude and class of immune responses, much like the regulation mediated by conventional Th1 versus Th2 or Tr1 and Tr3 cells (Cohn, 2004; Jiang and Chess, 2006).

Why have $CD25^+Foxp3^+$ Tregs been considered as a ''specialized'' and key mechanism of self-tolerance? There are two central observations that this conclusion is based on. First, 2–4 days' thymectomized mice developed spontaneous ''global pathogenic autoimmunity,'' which can

be corrected by adoptively transfer of naïve $CD4^+$ T cell populations containing but not being depleted of $CD25^+$ T cells (Asano *et al.*, 1996; Sakaguchi *et al.*, 1985, 1995). Second, in Foxp3-deficient surfin mice the spontaneously developed ''global pathogenic autoimmunity'' can be corrected by adoptive transfer of the ''specialized $CD25^+Foxp3^+$ Tregs'' (Fontenot *et al.*, 2003; Khattri *et al.*, 2003; Kretschmer *et al.*, 2005). In this regard, a typical example that precisely reveals the function of the $Foxp3^+$ Tregs is their ability to control inflammatory bowel disease, an inflammation in the gut caused by an excessive immune response against a bacteria infection (Sakaguchi, 2005). So far, there has been no experimental evidence showing that $Foxp3^+$ Tregs are capable of selectively suppressing overall immune responses to self but not to foreign antigens. Thus, $Foxp3^+$ Tregs represent a potent mechanism that negatively controls excessive immune responses to both self and foreign antigens without any distinction. The control of ''global pathogenic autoimmunity'' in 2–4 days' thymectomized mice and the surfin mice by the $Foxp3^+$ Tregs is consistent with the notion that these cells mediate a potent but nonspecific amelioration of the overwhelming systemic inflammatory process via controlling the magnitude and class of immune responses. In this regard, as discussed in Section 4.1.2, the commonly seen, organ-specific autoimmune diseases occur after normal thymic negative selection is completed and the control of organ-specific autoimmune diseases requires mechanisms that are capable of discriminating self from nonself. $Foxp3^+$ Tregs are, therefore, unlikely a ''specialized'' mechanisms that the immune system evolves to discriminate self from nonself in order to specifically control pathogenic autoimmunity. Instead, these cells represent a potent mechanism that controls the magnitude and class of immune responses to both self and foreign antigens, which is also important in ensuring an optimal function of the immune system, if it is understood correctly and put into the right perspective (Cohn, 2004; Jiang and Chess, 2006).

Lineage specificity defined by Foxp3 has been the hallmark of the ''specialized Tregs'' that makes them a unique regulatory T cell population distinct from any other $CD4^+$ T cells with regulatory potential, such as Th1 versus Th2 or Tr1 and Tr3 cells (Fontenot *et al.*, 2003, 2005). However, currently, it is unclear whether these ''specialized Tregs'' do commit to a stable ''lineage'' or whether the regulatory phenotype is a more plastic reversible state (Bluestone and Abbas, 2003; von Boehmer *et al.*, 2003), for example studies showed that naïve $CD4^+$ T cells can convert into ''antigen-specific'' Tregs triggered by foreign antigens under certain conditions (Kretschmer *et al.*, 2005). The conceptual dilemma is obvious. If the ''specialized Tregs'' are indeed a ''lineage''-specific subset of T cells, what defines it? Can Foxp3 function as a ''lineage-specific'' marker to define the ''specialized Tregs'' when itself

is an inducible gene which is dynamically regulated during effector T cell differentiation (Brunkow *et al.*, 2001; Chen *et al.*, 2003; Kasprowicz *et al.*, 2005; Schubert *et al.*, 2001) and is only linked to the functional stage of certain T cells (Fontenot *et al.*, 2003; Khattri *et al.*, 2003; Kretschmer *et al.*, 2005)? In this regard, increasing evidence indicates that Foxp3 is also expressed on ''nonregulatory'' effector and memory T cells (Bacchetta *et al.*, 2006; Vukmanovic-Stejic *et al.*, 2006; Wang *et al.*, 2007), as well as on nonlymphoid, normal mammary epithelial cells (Zuo *et al.*, 2007), seriously challenging the concept that Foxp3 is a ''lineage-specific'' marker for the Tregs (Fontenot *et al.*, 2003, 2005). If the ''specialized Tregs'' are not a ''lineage''-specific subset of T cells, what are the essential differences between the ''specialized Tregs'' and the conventional T cells, which exert the regulatory function (including Th1 vs Th2 or Tr1 and Tr3 cells)? The recent evidence that there are at least 28 ''flavors'' or phenotypically distinct subsets of different kinds of $Foxp3^+$ Tregs (Shevach, 2006) makes it more confusing and difficult to construct a conceptual framework to precisely understand the biology of these cells. Taken together, a conceptual framework that could uniformly explain the biological functions of the ''specialized $CD25^+Foxp3^+$ Tregs'' *in vivo* and clarify the most basic issues in T cell biology for these cells, at both theoretical and experimental levels, is certainly required in the field of immune regulation (Bach, 2003; Cohn, 2008; Ziegler, 2007).

To summarize, self–nonself discrimination and control of the magnitude and class of immune responses are two equally important but distinct peripheral regulatory mechanisms, that function in concert to ensure an optimal function of the immune system (Cohn, 2004; Jiang and Chess, 2006). The Qa-1/HLA-E-restricted $CD8^+$ T cell-mediated pathway represents one peripheral mechanism of self–nonself discrimination to maintain self-tolerance. Based on the overwhelming information currently available, $CD25^+Foxp3^+$ Treg is likely a potent regulatory mechanism that functions to control the magnitude and class of immune responses. Since $CD25^+Foxp3^+$ Tregs cannot discriminate overall immune responses to self versus to foreign antigens, the experimental evidence that $CD25^+Foxp3^+$ Tregs suppress autoimmunity is most likely a reflection of their capacity to suppress the magnitude and alter the class of immune responses to any antigens. As we go forward in the field of immune regulation, it becomes an intellectual necessity to classify the existing peripheral regulatory mechanisms, in the context of either self–nonself discrimination or control of magnitude and class of immune responses. It is necessary, not only because it is conceptually important but also because it is directly related to the development of precise and safe therapeutic approaches to solve clinical problems caused by different immune mechanisms.

ACKNOWLEDGMENTS

This manuscript has been supported by NIH grants RO1 AI065609 to HJ, U19 AI46132 to LC and HJ, and National Natural Science Foundation of China (NSF-30830093), National Key Program (973) for Basic Research of China (2009CB522409) to HJ.

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CHAPTER 3

Cellular and Molecular Mechanisms in Atopic Dermatitis

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Advances in Immunology, Volume 102
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)01203-6

Abstract Atopic dermatitis (AD) is a pruritic inflammatory skin disease associated with a personal or family history of allergy. The prevalence of AD is on the rise and estimated at ~17% in the USA. The fundamental lesion in AD is a defective skin barrier that results in dry itchy skin, and is aggravated by mechanical injury inflicted by scratching. This allows entry of antigens via the skin and creates a milieu that shapes the immune response to these antigens. This review discusses recent advances in our understanding of the abnormal skin barrier in AD, namely abnormalities in epidermal structural proteins, such as filaggrin, mutated in ~15% of patients with AD, epidermal lipids, and epidermal proteases and protease inhibitors. The review also dissects, based on information from mouse models of AD, the contributions of the innate and adaptive immune system to the pathogenesis of AD, including the effect of mechanical skin injury on the polarization of skin dendritic cells, mediated by keratinocyte-derived cytokines such as thymic stromal lymphopoietin (TSLP), IL-6, and IL-1, that results in a Th2-dominated immune response with a Th17 component in acute AD skin lesions and the progressive conversion to a Th1-dominated response in chronic AD skin lesions. Finally, we discuss the mechanisms of susceptibility of AD skin lesions to microbial infections and the role of microbial products in exacerbating skin inflammation in AD. Based on this information, we discuss current and future therapy of this common disease.

1. INTRODUCTION

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease often associated with a family and/or personal history of allergy. The prevalence of the disease is on the rise all over the world, but particularly in Western and industrialized societies (Leung and Bieber, 2003; Novak *et al.*, 2003a). Its prevalence is currently ~17% in US children (Laughter *et al.*, 2000). AD almost uniformly starts in infancy. Although it tends to resolve or remarkably improve by age 5, it can persist into adult life in ~15% of cases. The disease causes a tremendous physical, psychological, and financial burden to the patients and their families, manifested by loss

of school attendance in children, loss of productivity in adults and in substantial consumption of heath care resources.

The hallmarks of AD are skin barrier dysfunction, which results in dry itchy skin, which leads to scratching that inflicts mechanical injury and allergic sensitization to environmental antigens and allergic skin inflammation. Histopathology of AD skin lesions reveals an intense mononuclear cell infiltrate in the dermis with T cells, monocytes, macrophages, dendritic cells (DCs), mast cells, and eosiniphils or their products. In addition, there is fibrosis and collagen deposition in chronic skin lesions.

Two hypotheses have been proposed for the pathogenesis of AD. One hypothesis holds that the primary defect is intrinsic to skin epithelial cells and results in a defective skin barrier function with a secondary immune response to antigens that enter via damaged skin barrier. The other hypothesis holds that the primary abnormality is in the immune system and results in a Th2/IgE-dominated immune response that causes a secondary defect in barrier skin function. In this review we will argue, based on genetic defects in humans that are associated with AD or AD-like skin lesions and on mouse models of allergic skin inflammation, that both mechanisms are likely to contribute to the pathogenesis of AD. However, in the majority of AD cases, there is no predisposition to systemic infections, and no autoimmune manifestations, to suggest a primary immune defect. Thus, we argue that in the majority of AD patients, a primary defect in barrier function in the context of a genetically inherited immune predisposition to mount a Th2/IgE-dominated immune response which in turn aggravates the skin barrier defect initiating a vicious cycle that triggers and perpetuates AD skin lesions.

2. PHYSIOLOGY AND IMMUNOLOGY OF NORMAL SKIN

2.1. Barrier function of the skin

The skin is made of three layers, the epidermis, the dermis, and the hypodermis (Fig. 3.1). The epidermis consists primarily of stratified epithelial cells, or keratinocytes, organized in a basal layer, a spinous layer, a granular layer and the stratum corneum (SC) which consists of terminally differentiated lipid-depleted keratinocytes (corneocytes) that have replaced their plasma membrane with a tough insoluble macromolecular layer called the cornified envelope that are dying (inner SC) or dead (outer SC). The epidermis is interspersed with antigen-presenting hematopoietically derived Langerhans cells (LCs). The dermis is a vascularized layer that consists of fibroblasts and dense connective tissue with collagen and elastic fibers, populated by hematopoietically derived cells that include DCs, mast cells, macrophages, and few lymphocytes. The

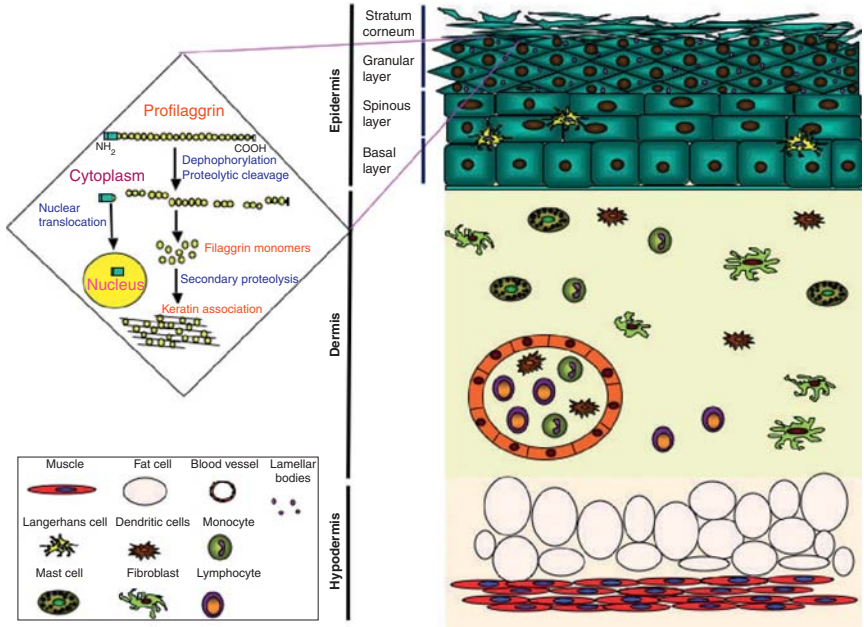

FIGURE 3.1 Schematic representation of normal skin layers. The inset summarizes the structure of filaggrin and its metabolism in keratinocytes.

hypodermis is a layer of fat cells and loose connective tissue in contact with underlying muscle. The main function of skin is to provide protection as a physical barrier to the entry of foreign materials that include irritants, allergens, and pathogens, and to regulate the loss of water. This barrier function is performed by the epidermis and particularly by the SC. The dermis plays a very small part in the overall barrier function. The barrier to water permeation is not absolute and the normal movement of water through the SC into the atmosphere is known as transepidermal water loss (TEWL) and constitutes part of insensible water loss.

The terminal differentiation of keratinocytes from granular cells to corneocytes is a critical step for the maintenance of skin barrier function. The formation of the cornified epithelium involves the sequential expression of several major structural proteins. These proteins are extensively cross-linked by transglutaminases and act as a scaffold for the attachment of a layer of lipids covalently bound to the extracellular surface, forming an outer lipid envelope. Many of the proteins involved in skin cornification are encoded for in a 1.62 megabase locus of ~70 genes on chromosome 1q21, termed the epidermal differentiation complex (EDC) (Mischke *et al.*, 1996). EDC genes are expressed during the late stages of terminal keratinocyte differentiation and encode for proteins such as loricrin,

involucrin, small proline-rich proteins, late envelope proteins, and the S100 calcium-binding proteins, of which filaggrin is a key member (Cookson, 2004). EDC proteins share significant sequence similarities. Furthermore, there is considerable redundancy in EDC genes, as absence of one EDC protein can be compensated for increased expression of others (Sevilla *et al.*, 2007). The EDC gene *filaggrin* (*FLG*) has been the focus of much attention recently following the discovery that it is mutated in ichthyosis vulgaris and in patients with AD in different ethnic populations (Nomura *et al.*, 2007; Palmer *et al.*, 2006; Smith *et al.*, 2006; Marenholz *et al.*, 2006; Weidinger *et al.*, 2006; Sandilands *et al.*, 2007). The giant inactive precursor, profilaggrin, is a large, complex, highly phosphorylated polypeptide that is the main constituent of the keratohyalin F granules that are visible in the granular cell layer of the epidermis. Profilaggrin is encoded by 3 exons, of which the third exon encodes the FLG protein repeats, which vary in number from 10 to 12 because of alternate splicing. During corneocyte differentiation, profilaggrin is dephosphorylated and proteolytically cleaved by serine proteases, into multiple filaggrin polypeptides. After cleavage, liberated filaggrin binds to keratin in a structure aligned parallel to the outer surface of the epidermis (Fig. 3.1, inset). The cleaved N-terminal S100-like calcium-binding domain of profilaggrin translocates to the nucleus, where it may regulate terminal differentiation. Within the SC, filaggrin peptides are further degraded into hydrophilic amino acids, including urocanic acid, pyrrolidone carboxylic acid, and alanine (Rawlings and Harding, 2004).

The lipid bilayer in the SC is essential for the barrier function. The three major SC lipids are ceramides, free fatty acids, and cholesterol. Lipid synthesis is believed to take place in the granular cell layer where small cytoplasmic inclusions (lamellar bodies) are formed and packed in multilayered stacks. At the SC interface, lamellar body exocytosis takes place to form the lipid lamellae. The SC has an unusual lipid composition consisting of a roughly equimolar mixture of ceramides (45–50% by weight), cholesterol (25%), and free fatty acids (10–15%) plus less than 5% each of several other lipids, the most important of which is cholesterol sulfate. The multiple layers of corneocytes in the SC contribute a tough and resilient framework for the intercellular lipid lamellae. The interior surface of the cornified envelope is composed of several structural proteins, notably involucrin and loricrin. On its exterior surface, the cornified envelope is a covalently bound layer of very long chain *o*-hydroxyceramides.

Skin barrier function is determined by structural proteins, lipids, proteases, and protease inhibitors. A balance between proteolytic and antiproteolytic activities is essential for normal homeostasis in the skin. Proteases, especially SC tryptic and chymotryptic enzymes, have been implicated in desmosome breakdown and corneocyte desquamation, and cholesterol sulfate has been shown to inhibit some of their activities

(Hansson *et al.*, 1994; Horikoshi *et al.*, 1998; Sato *et al.*, 1998; Simon *et al.*, 2001). Cholesterol sulfate also inhibits transglutaminase-mediated involucrin cross-linking as well as involucrin esterification to the *o*-hydroxyceramides of the lipid envelope (Nemes *et al.*, 2000). The enzymatic activity of skin proteases is controlled by protease inhibitors that include *SPINK5*, a gene which encodes a putative serine protease inhibitor, lymphoepithelial Kazal-type-related inhibitor (LEKTI), which is mutated in Netherton's syndrome, a severe inflammatory skin disease with features of AD (Shimomura *et al.*, 2005). Elafin is a product of skin fibroblasts that binds to elastic fibers by transglutaminase-mediated cross-linking and protects against their proteolytic breakdown (Schalkwijk, 2007). Alterations in structural proteins, lipids, proteases, and their inhibitors will result in altered SC. Alteration of the SC may be accompanied by an increase in the skin penetration of molecules and increased TEWL. Total elimination of SC by delamination with adhesive tape (stripping) leads to an increase in penetration by chemical agents (Bashir *et al.*, 2001; Kalia *et al.*, 2001). Deficiency in filaggrin in *flaky* mice and in patients with AD and *FLG* mutations are associated with increased TEWL (Kezic *et al.*, 2008; Presland *et al.*, 2000).

2.2. Innate immunity in skin

Pathogen associated molecular pattern (PAMP) molecules, the inflammasome, innate cytokines, and antimicrobial peptides (AMPs) expressed by skin keratinocytes play important roles in protection of the skin against infection. These innate defense mechanisms in the skin are discussed because some of them are altered in AD, and because patients with AD are susceptible to bacterial and viral infections of the skin particularly with *Staphylococcus aureus (S. aureus)*, Herpes simplex virus (HSV), and Vaccinia virus (VV).

PAMPs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NODs), and peptidoglycan recognition proteins (PGRPs). Originally identified in *Drosophila melangastor*, 11 different mammalian TLRs (TLR1-11) have, so far, been identified in humans (Akira *et al.*, 2006; Hashimoto *et al.*, 1988; Lemaitre *et al.*, 1996). These are expressed on many cells important for the innate immune system, including macrophages, DCs, PMNs, mucosal epithelial, and endothelial cells. Skin keratinocytes express mRNA for TLRs 1, 2, 3, 4, 5, 6, 9, and 10 (Kollisch *et al.*, 2005; McInturff *et al.*, 2005; Schiller *et al.*, 2006). Ligand recognition via TLRs induces a conserved host defense sequence of events via the nuclear factor κB (NFκB), leading to expression of inflammatory cytokines (TNF-α, IL-1, IL-12), endothelial adhesion molecules (E-selectin), costimulatory molecules, AMPs, and inducible nitric oxide synthase (iNOS). TLR2 is vital for the recognition of diverse bacteria, mycobacteria, protozoans and fungi, fungal zymosan, lipopolysaccharide

(LPS), and other PAMPs such as the staphylococcal cell-wall components lipoteichoic acid (LTA) and peptidoglycan (PGN). Mice deficient in TLR2 and MyD88, the adaptor proteins that participate in the TLR signaling cascade are highly susceptible to *S. aureus* infection (Takeuchi *et al.*, 2000). CD14 is a receptor for LPS and other bacterial wall components that associate with TLR4. It is expressed as a soluble or membrane-bound receptor predominantly on monocytes as well as on keratinocytes. CD14 induces cellular activation in response to LTA through a TLR2-dependent pathway and has binding activity for PGN.

The NOD family of proteins is shown to be responsible for the recognition of intracellular PAMPs, like PGN. This family is also known as Caspase recruitment domain (CARD) family. Members of this family include NOD2/CARD15 and the closely related NOD1/CARD4 protein. NOD1 identifies diaminopimelic acid-type PGN, which is produced by gram-negative bacteria. Stimulation of NOD1 and NOD2 expressing keratinocytes with PGN leads to the production of IL-6. NOD2 senses the muramyl dipeptide motif in PGN of all types of bacterial origin. Stimulation of keratinocytes with the muramyl dipeptide results in the expression of human β-defensin 2 (HBD-2), an important AMP (Voss *et al.*, 2004).

PGRPs are a family of pattern recognition molecules that were discovered first in insects and then in mammals. Up to 17 different PGRPs have been described in insects that recognize PGN and bacteria. They cause the activation of the prophenoloxidase cascade that generates antimicrobial melanin and reactive oxygen species (Kang *et al.*, 1998; Werner *et al.*, 2000; Yoshida *et al.*, 1996). They also activate the Toll and the immune deficiency (IMD) pathways (which induce production of AMPs), participate in phagocytosis of bacteria, and are PGN-lytic enzymes (Leulier *et al.*, 2003). Mammalian PGRPs do not function as cell-surface receptors, but more likely work as effector molecules (Liu *et al.*, 2001). So far, four different proteins have been identified in human. These are PGRP-S (PGRP1), PGRP-L (PGRP2), PGRP-1α (PGRP3), and PGRP-1β (PGRP4). Mammalian PGRP1 is present in granulocyte granules and has antibacterial properties, and mammalian PGRP2 is an *N*-acetylmuramoyl-L-alanine amidase constitutively produced in the liver and secreted into the bloodstream. PGRP3 and PGRP4 are found in epithelial cells; however, not much is known about their activity (Liu *et al.*, 2001). PGRP3 protein has been detected in human keratinocytes, and stimulation with the staphylococcal-specific PAMP LTA enhanced its expression (Liu *et al.*, 2001). PGRP2 is not expressed in healthy human skin; however, its expression was highly induced in keratinocytes upon exposure to gram-positive and gram-negative bacteria or cytokines (Wang *et al.*, 2005).

The inflammasome is a cytosolic protein complex regulating the activation of caspase-1 (CASP1), which cleaves the proinflammatory cytokines IL-1β and IL-18 into their active form (Lamkanfi and Dixit, 2009). The

inflammasome is composed of a NACHT-, LRR-, and pyrin (NALP) family member that acts as a sensor for danger signals, and the adaptor protein apoptosis-associated speck-like protein containing a CARD domain, which allows the recruitment of CASP1 in the complex. Danger signals that activate the inflammasome possibly converge to cause lysosomal damage and rupture (Hornung *et al.*, 2008). The key components of the inflammasome are present in human keratinocytes. In contact hypersensitivity to haptens, efficient priming of the adaptive immunity depends on the activation of the inflammasome in the skin (Watanabe *et al.*, 2007).

Keratinocytes express the innate immunity cytokines IL-1, IL-18, and IL-6. Expression of these cytokines is upregulated following skin mechanical injury or exposure to ultraviolet B (UVB) light (Shinoda *et al.*, 1998). Expression of IL-1α is upregulated in mouse skin during *S. aureus* infection (Miller *et al.*, 2006). IL-1α and IL-1β are known ligands of IL-1R. Signaling via IL-1R uses the adaptor protein MyD88. Both IL-1R and MyD88 deficient mice have impaired neutrophil (PMN) chemotaxis in response to a cutaneous *S. aureus* challenge. TLR2 also uses the adaptor MyD88, but TLR2 deficient mice do not show a similar defect to IL-1R deficient mice, thereby suggesting a unique and important role for IL-1R in immune response to cutaneous *S. aureus* infection (Miller *et al.*, 2006). AD has been shown to have an increased ratio of IL-1R antagonist to IL-1α in the SC, which would have an inhibitory effect on IL-1-mediated actions (Terui *et al.*, 1998). Corticotropin-releasing hormone (CRH) downregulates IL-18 (Park *et al.*, 2005). Since inflammatory skin diseases including AD are exacerbated by stress (see Section 3.4), it has been suggested that stress-induced release of CRH and the ensuing reduction of IL-18 levels might play a role in the susceptibility of AD patients to cutaneous infections. The release of IL-6 by keratinocytes following skin injury may play a critical role in shaping adaptive immunity to cutaneously introduced antigens in AD, as discussed below.

AMPs constitute a protective chemical shield on the surface of skin. AMPs are expressed by resident skin keratinocytes and cells that form the sebaceous and sweat glands, and mast cells. Circulating cells, which are recruited to the skin, such as PMNs and natural killer (NK) cells, also contribute to the total amount of AMPs present. AMPs show antimicrobial action against bacteria, viruses, and fungi. In mammalian skin, there are two major classes of AMPs, the cathelicidins and the defensins. Defensins are broad-spectrum antibiotics that kill a wide variety of bacterial and fungal pathogens. β-Defensins can also act as chemoattractants for immature DCs and memory T cells via the CC chemokine receptor CCR6. In addition to its bactericidal and fungicidal actions, human cathelicidin hCAP18/LL-37 displays antimicrobial activity against viral pathogens, neutralizes LPS bioactivity, and induces the expression of the inflammatory mediators by keratinocytes that are chemoattracts for PMNs, monocytes, mast cells, and T cells (Braff *et al.*, 2005; Howell *et al.*,

2006b). The combination of hCAP18/LL-37 and HBD-2 has been shown to achieve synergistic antimicrobial activity in killing *S. aureus* (Ong *et al.*, 2002). Healthy skin expresses only small amounts of AMP. However, synthesis of HBD-2, HBD-3, and hCAP18/LL-37 by keratinocytes increases in response to inflammatory cytokines. High amounts of AMP have been found in the skin of patients with chronic inflammatory skin diseases such as psoriasis or contact dermatitis. On the other hand, both lesional and nonlesional AD skin have low expression of HBD-2, 3 and hCAP18/LL-37 compared to psoriasis skin (Nomura *et al.*, 2003; Ong *et al.*, 2002). The Th2 cytokines IL-4 and IL-13 have been shown to downregulate TNF-α or IFN-γ induced HBD-2 and HBD-3 mRNA expression in keratinocytes and normal skin explants (Howell *et al.*, 2006a; Nomura *et al.*, 2003; Ong *et al.*, 2002), suggesting that the reduced levels of antimicrobial peptides in AD skin lesions may be explained by the predominance of Th2-type cytokines. Several studies suggest that AMP deficiency contributes to the susceptibility of atopic patients to skin infections. Clinical isolates of *S. aureus* from AD patients can be killed by a combination of HBD-2 and hCAP18/LL-37 at the concentrations found in psoriatic lesions, but the levels present in AD skin are too low to be effective (Menzies and Kenoyer, 2005; Schibli *et al.*, 2002). The cathelicidin hCAP18/LL-37 and the defensin HBD-3 exhibit antiviral activity against HSV and VV (Howell *et al.*, 2006c, 2007b). The hCAP18/LL-37 deficiency in AD skin might predispose to eczema vaccinatum (EV) caused by orthopox virus and eczema herpeticum (EH) caused by HSV. Lower levels of cathelicidin have been shown in skin lesions of AD patients with EH compared to those of AD patients who did not develop EH (Howell *et al.*, 2006c). Dermcidin (DCD) is another AMP with broad range of antibacterial and antimycotic properties. DCD is known to be expressed constitutively in human eccrine sweat glands and is secreted into sweat (Schittek *et al.*, 2001). The amount of several DCD-derived peptides in sweat of patients with AD was found to be significantly reduced compared to healthy controls, especially in patients with a history of bacterial and viral infections (Lai *et al.*, 2005; Rieg *et al.*, 2005).

3. CLINICAL ASPECTS OF AD

3.1. Definition

AD is a chronic, relapsing inflammatory skin disease that occurs most commonly during infancy and childhood. The diagnosis of AD is based on clinical presentation of skin erythematous plaques, eruption, and/or lichenification typically in flexural areas accompanied by intense pruritus and cutaneous hypersensitivity. AD can be divided into two distinct

forms based on the concentrations of total and allergen-specific IgE: ''Extrinsic'' AD (EAD), affecting 70–80% of AD patients is characterized by elevated concentrations of total and allergen-specific IgE in serum and skin, and ''Intrinsic'' AD (IAD) that affects 20–30% of AD patients is characterized by normal total IgE levels and negative serum allergen-specific IgE (Novak *et al.*, 2003c).

3.2. Clinical and pathological manifestations

AD frequently starts in early infancy and 45% of all cases of AD occur within the first 6 months of life. In about 85% of cases the disease becomes quiescent by age 5. The clinical manifestations of AD vary with age. In infancy, the skin lesions are located on the cheeks and scalp (Fig. 3.2A). In childhood, lesions are located on flexures and nape of the neck and on dorsal aspects of the limbs (Fig. 3.2B). In adolescents and

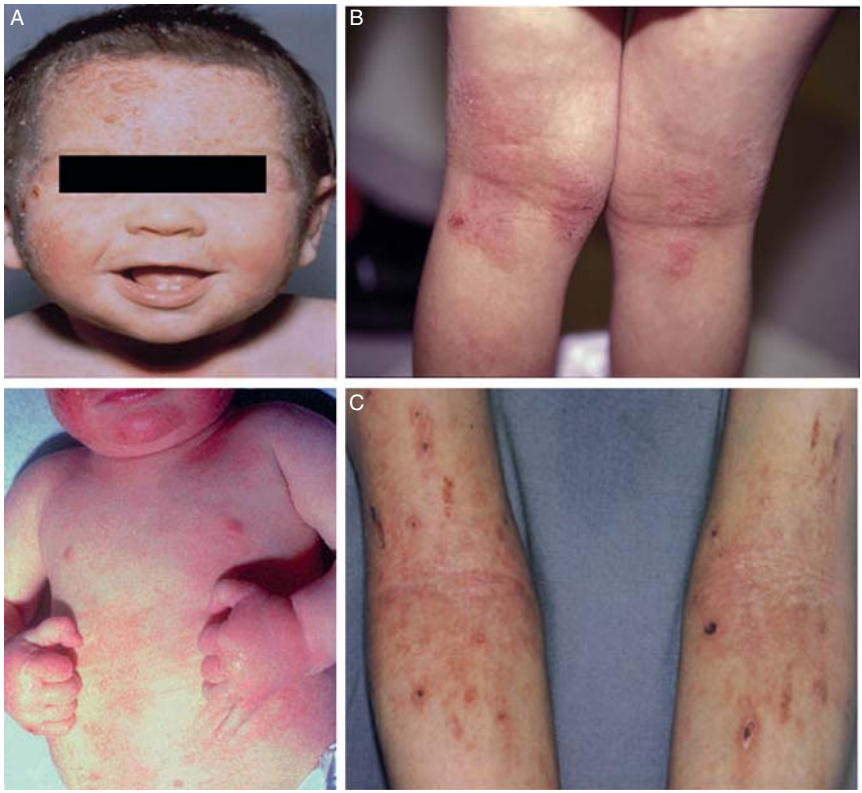

FIGURE 3.2 Gross appearance of AD skin lesions. AD skin lesions in infancy (A), childhood (B), and adolescence (C).

adults, lichenified plaques affect the flexures, head and neck, the result of fibrosis and increased collagen deposition in the skin (Fig. 3.2C). AD is associated with one or more typical atopic signs such as palmar hyperlinearity and infraorbital fold. AD is part of the atopic disease complex, along with asthma and allergic rhinitis, and AD patients frequently exhibit elevated serum IgE levels and blood eosinophilia. Most AD patients have personal or family history of allergies or asthma. Infants with AD have an increased tendency to develop asthma and allergic rhinitis later in life, a phenomenon known as the atopic march.

Acute AD skin lesions clinically show intensely pruritic, erythematous papules associated with excoriation and serous exudation (Fig. 3.2A and B). Pathological examination reveals spongiosis, that is intercellular epidermal edema, hyper-/parakeratosis, that is superficial epidermal hypertrophy and acantholysis, epidermal vesiculation/separation, with a marked infiltration of $CD4^+$ activated memory T cells, antigen-presenting cells (APCs), including LCs, inflammatory dendritic epidermal cells (IDECs), macrophages, and degranulated mast cell in acute lesional skin (Fig. 3.3A). Chronic AD skin lesions clinically show thickened plaques with increased lichenification (Fig. 3.2C). Pathological examination reveals marked epidermal hyperplasia, acanthosis with macrophage-dominated mononuclear cell infiltrate in the dermis, and perivascular accumulation of lymphocytes in smaller numbers than seen in acute AD (Fig. 3.3B) (Leung and Bieber, 2003). Clinically unaffected skin in AD patients is not normal. It is frequently dry and has a greater irritant skin response to chemicals or physical agents than normal healthy skin. There is a sparse perivascular T-cell infiltrate in unaffected AD skin that is not seen in normal healthy skin.

Dermal infiltration with T cells shows no difference between EAD and IAD (Akdis *et al.*, 1999) but dermal eosinophil infiltration is significantly increased in EAD, although both forms of AD have comparable blood eosinophilia (Jeong *et al.*, 2003; Kagi *et al.*, 1994). It has been reported that circulatory and skin APCs from EAD expressed higher levels of the high-affinity receptor for IgE (FcεRI) than those from IAD (Oppel *et al.*, 2000). The skin of EAD shows higher expression of Th2 cytokines compared to IAD, including IL-4 and IL-13 that are responsible for the induction of IgE synthesis by B cells, and IL-5 that is important for the recruitment and survival of eosinophils (Akdis *et al.*, 1999; Jeong *et al.*, 2003). The chronic skin lesions from EAD and IAD show no difference in the upregulation of IFN-γ that is important to induce epidermal keratinocyte apoptosis by upregulating Fas (CD95) on keratinocytes (Jeong *et al.*, 2003) and similar spongiosis in the epidermis.

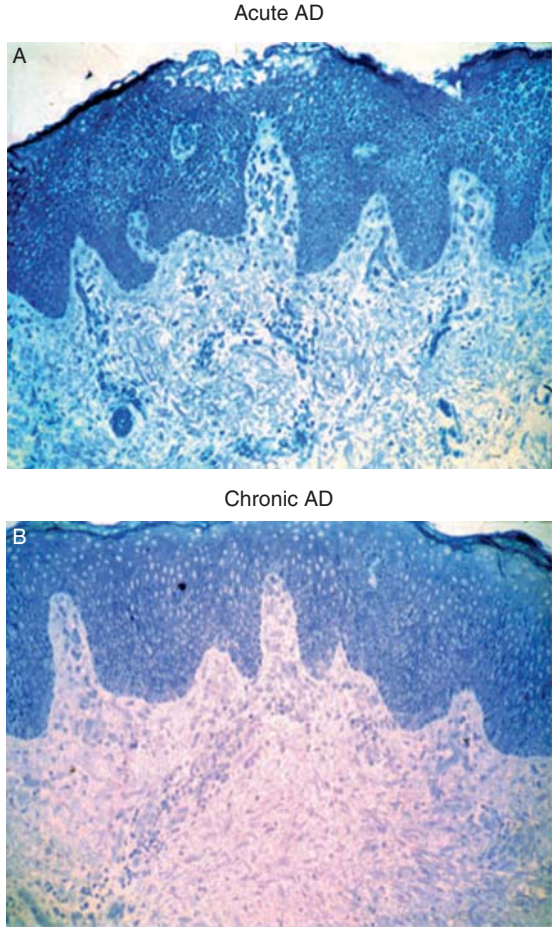

FIGURE 3.3 Histology of AD skin lesions. Photomicrographs of acute (A) and chronic (B) AD skin lesions.

3.3. Itching in AD

Itching is the hallmark of AD. The skin of AD patients is dry and demonstrates increased TEWL. Scratching of dry itchy skin causes mechanical skin injury that activates keratinocytes to release proinflammatory cytokines and chemokines that induce the expression of adhesion molecules on vascular endothelium and facilitate the extravasation of inflammatory cells into the skin. In addition, damage by scratching to an already abnormally permeable skin enhances the entry of allergens and infectious agents. Several mediators are associated with itching in AD, including histamine, IL-31, neuropeptides, opioids, and serine proteases.

Elevated concentrations of histamine have been found *in vivo* in the skin and in the plasma of patients with AD especially during exacerbation of the disease (Ring and Thomas, 1989). The major histamine receptor in the skin is the type-I receptor. IL-31 is a four-helix bundle cytokine that is preferentially produced by T helper type 2 (Th2) cells. IL-31 signals through a receptor composed of the IL-31 receptor A and oncostatin M receptor. Keratinocytes express both receptors constitutively. IL-31 is upregulated in AD skin lesions in humans (Sonkoly *et al.*, 2006). IL-31 expression is associated with T cells that express the skin homing receptor, cutaneous lymphocyte-associated antigen (CLA^+ T cells) in AD (Bilsborough *et al.*, 2006). In addition, transgenic mice overexpressing IL-31 developed severe AD (Dillon *et al.*, 2004) and IL-31 causes the itch-associated scratching behavior in conventional NC/Nga mice, an experimental animal model for AD. This is evidenced by the finding that intraperitoneal administration of IL-31 antibody reduced scratching behavior in NC/Nga mice with AD-like lesions (Grimstad *et al.*, 2009). These observations support a role for IL-31 in itching in AD skin.

Neuropeptides is a heterogeneous group of proteins that function as neurotransmitters, neuromodulators, and neurohormones. Neuropeptides such as substance P (SP), vasoactive intestinal polypeptide (VIP), somatostatin, and neurotensin provoke itching and neurogenic inflammation with erythema, wheal, and flare, the so-called triple response of neurogenic inflammation. Neuropeptides and neuropeptide-positive nerve fibers are prominently increased in AD skin (Raap and Kapp, 2005). The serum levels of several neuropeptides including SP are elevated in AD (Salomon and Baran, 2008). Stress was shown to worsen AD via SP-dependent neurogenic inflammation in mice (Pavlovic *et al.*, 2008). Furthermore, SP induces an itch response in humans via the release of histamine from mast cells, supporting a role of SP in mediating itch *in vivo*. These observations support a role of neuropeptides in the pathophysiology of itching in AD.

Opioid peptides play an important role in motivation, emotion, attachment behavior, response to stress and pain, and control of food intake. Endogenous opioid peptides and opiates transmit their pharmacological effects through membrane-bound opioid receptors, which belong to the family of G-protein-coupled receptors. There are three major types of opioid receptors, μ-type (MOR), κ-type (KOR), and δ-type (DOR). MOR, but not KOR or DOR agonists induce itching (Ko *et al.*, 2004). Opioid-induced itch is a well-known side effect of pain treatment with morphine and other MOR agonists in humans. Human keratinocytes express a functionally active MOR (Bigliardi *et al.*, 1998) (see also Bigliardi-Qi *et al.*, 2000). MOR expression is altered in the epidermis and nerve endings of AD (Bigliardi-Qi *et al.*, 2005). Internalization of the MOR in the epidermis of patients with AD may lead to the availability of free opioid ligands, which then induce chronic pruritogenic signals via altered unmyelinated nerve C-fibers.

Serine proteases such as tryptase and their respective proteinase-activating receptor 2 are founded in skin of patients with AD. Epidermal expression of SC chymotryptic enzyme (SCCE) is increased in chronic AD lesions (Hansson *et al.*, 2002). Transgenic mice expressing human SCCE in suprabasal epidermal keratinocytes develop AD-like lesions (see Section 7.2). Chymase, a chymotrypsin-like serine protease, stored in secretory granules of mast cells is thought to participate in allergic diseases. The chymase inhibitor SUN13834 improves dermatitis in Nc/Nga mice and decreases scratching behavior induced by skin inflammation (Terakawa *et al.*, 2008). These data suggest that serine proteases may play a role in itching in AD.

3.4. AD triggers

Environmental factors, including inhaled antigens, microbial antigens, food antigens, and contact sensitizers, as well as pruritus and stress contribute to the development of eczematous skin lesions in AD patients. Patients with EAD are sensitized to many allergens. This is evidenced by the presence of IgE antibodies to aeroallergens, food antigens and bacterial antigens such as staphylococcal enterotoxins. It is also evidenced by the presence of allergen-specific T cells in blood and skin lesions (Burks, 2003; Reekers *et al.*, 1996; van Reijsen *et al.*, 1998). Dust mite is the most common aeroallergen that results in allergic sensitization in children with AD (Hon *et al.*, 2008). Sensitization likely occurs via an abnormal skin that is made more permeable by mechanical injury inflicted by scratching. The prevalence of sensitization to common aeroallergens is generally higher in older children than in infants. A role for aeroallergens in AD is suggested by the observation that patch testing of uninvolved skin of patients with AD triggers Th2-dominated skin inflammation (Thepen *et al.*, 1996). Effective house dust mite (HDM)-reduction intervention can improve AD (Darsow *et al.*, 2004). Intranasal or bronchial inhalation challenge with specific aeroallergens, such as HDM or animal dander, in AD patients can aggravate pruritus and eczematous skin lesions and contribute to the flares of AD (Tupker *et al.*, 1996). This suggests that allergen-specific cells activated in the airways can home to skin.

The prevalence of food allergy in infants with AD is reported to range from 20% to 80% in various studies, and may be estimated at 30% (Sicherer and Sampson, 1999). Foods, such as cow's milk, hen's eggs, peanut, soy, and wheat account for about 90% of allergenic foods in children with AD. Sensitization may occur through the oral route, possibly because an intrinsic defect that affects epithelial cells in skin, gut, lung, and other tissues in AD. Alternatively, sensitization may occur via skin exposure to food antigens (Lack *et al.*, 2003). Ingestion of these foods has been shown in double-blinded placebo controlled challenges to provoke

AD flares and to induce skin lesions with evidence of eosinophil infiltration in children with moderate to severe AD (Ellman *et al.*, 2002; Sampson and McCaskill, 1985). Numerous studies have shown that elimination of relevant food allergens can lead to improvement in skin symptoms and that repeat challenge can lead to redevelopment of symptoms (Burks, 2003). However, not all children with AD who have evidence of IgE antibodies to food allergens experience flare following oral food challenge. There are to date no reliable markers for the identification of patients with food-responsive AD.

AD patients are highly susceptible to certain cutaneous bacterial, fungal, and viral infections. Bacterial colonization with *S. aureus* is the most common skin infection in AD patients and occurs in more than 90% of lesional skin and in more than 70% of nonlesional skin from AD patients compared with 5% in skin of normal individuals (Leyden *et al.*, 1974). The number of bacteria correlates with the degree of skin inflammation (Goodyear *et al.*, 1993), and AD lesions flare up with frank bacterial superinfection usually with *S. aureus* (Fig. 3.4A). Furthermore, there is a correlation between the presence of IgE antibodies specific for staphylococcal superantigens and both the severity of AD and total serum IgE levels (Mrabet-Dahbi *et al.*, 2005). Combination treatment with steroid and antibiotic shows more efficacy than treatment with steroids alone (Lever *et al.*, 1988). Various factors, including dysfunctional epidermal barrier and decreased innate immune responses, contribute to the altered *S. aureus* colonization in AD skin. Exacerbation of AD by colonization and infection with *S. aureus* is in part mediated by the release of Staphylococcal superantigens at the skin surface. Skin inflammation could be provoked or worsen by application of *S. aureus* or its enterotoxins on the skin of AD patients (Baker, 2006; Kim *et al.*, 2006; Leyden *et al.*, 1974). This mechanism of superantigenic aggravation could be mediated by expanding the polyclonal activation of superantigen-specific T cell receptor Vβ T cells and favoring a Th2-like cytokine profile (Remitz *et al.*, 2001; Skov *et al.*, 2000). In addition to T cells, superantigens can also act on other cell types such as eosinophils, LCs, and keratinocytes during flares of AD (Travers *et al.*, 2001; Wedi *et al.*, 2002). Staphylococcal superantigen also exacerbate AD by activating mast cells, basophils, and other Fcε-receptor bearing cells carrying the relevant antitoxin IgE antibody. In addition to bacteria superinfections, yeast Malassezia species is also often isolated from superinfected AD lesions. Patients with AD run a higher risk of developing severe skin superinfections with a number of viruses that includes HSV resulting in EH (Fig. 3.4B), *Molluscum contagiosum* virus resulting in eczema molluscatum (Fig. 3.4C), and VV resulting in EV (Fig. 3.4D). These conditions exacerbate AD skin lesions and can be life threatening as they may involve viral dissemination to internal organs in EH and EV.

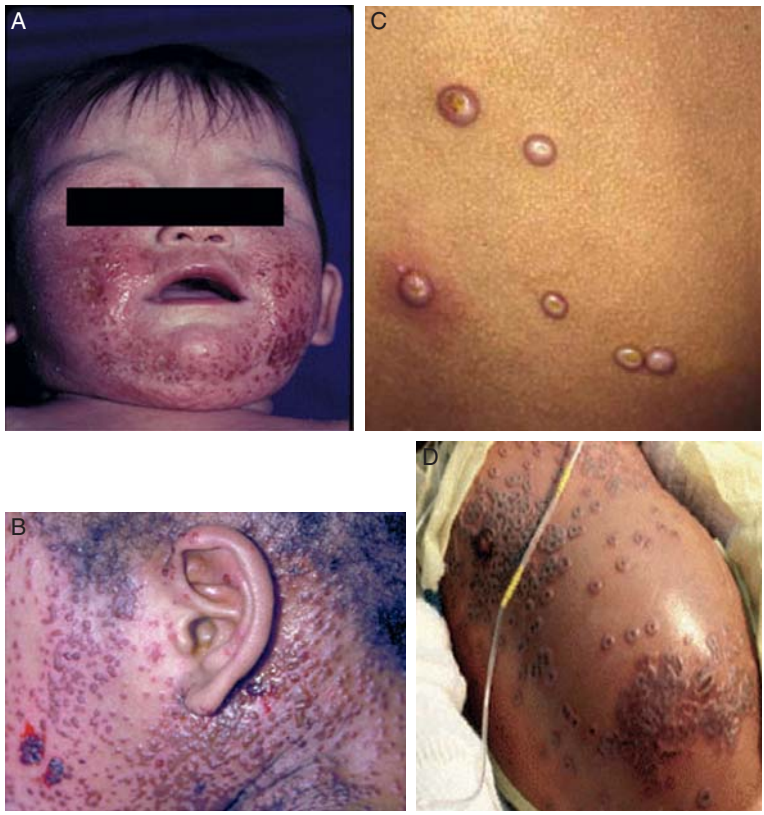

FIGURE 3.4 AD patients with microbial skin infection. (A) *S. aureus* infection, (B) Eczema hepeticum, (C) Eczema molluscatum, and (D) Eczema vaccinatum.

Pruritus causes an itch–scratch cycle that further damages epidermal barrier. Scratching induces skin mechanical injury that results in the release of a panel of proinflammatory cytokines and chemokines, which are believed to be important for initiating allergic skin inflammation. This is illustrated by several key observations. Development of dermatitis and elevation of the serum IgE level were significantly suppressed in Nc/Nga mice, in which scratching was suppressed following neonatal capsaicin treatment to ablate sensory nerves in the skin (Mihara *et al.*, 2004). Antigen application to mouse skin that has been tape stripped to mimic mechanical injury caused by scratching elicits a Th2-dominated immune response and Th2-dominated skin inflammation with many features of AD (Spergel *et al.*, 1998). The importance of itching in the perpetuation of AD is illustrated by the clinical observation that prevention of scratching on one side of

the body by more than 70-year-old observation that bandaging and restraining results in clearing of the skin lesions on the ipsilateral site, but not on the contralateral site (Engman *et al.*, 1936). Scratching also increased susceptibility to *S. aureus*, which aggravates AD.

Stress is an important trigger leading to the exacerbation of AD. Psychologic and stress-reduction interventions have been shown to lead to significant improvements in cutaneous manifestations of AD patient (Arndt *et al.*, 2008). The exact mechanisms by which stress exacerbates AD are not well understood. Neuroimmunologic factors are likely to play a role. Psychological stress alters levels of circulating lymphocyte subsets and eosinophils in AD patients (Schmid-Ott *et al.*, 2001). Eosinophils and Th2 cells could be major cellular sources of serum neurotrophic factors whose levels correlate positively with AD activity (Namura *et al.*, 2007). Video games-induced stress increased plasma levels of SP, VIP, and nerve growth factor (NGF), with a switch to a Th2 cytokine pattern in AD patients (Kimata, 2003). Scratching caused by psychogenic itch from emotional stress can stimulate the free nerve endings in the skin to release neuropeptides, especially SP and calcitonin gene-related peptide. Neuropeptides released in the skin may mediate neurogenic inflammation, including mast cell degranulation and the release of inflammatory mediators by keratinocytes (Jarvikallio *et al.*, 2003). The level of NGF in the horny layer of skin was found to reflect the severity of itching and eruptions in AD (Yamaguchi *et al.*, 2008).

4. EPIDEMIOLOGY

Multiple factors such as genetic background, age of onset, socioeconomic status, breastfeeding, food allergy, and endotoxin exposure influence the development of AD (Berth-Jones *et al.*, 1997). The most significant risk factor for a child developing AD is a parental history of AD (Purvis *et al.*, 2005). Maternal atopy is considered a greater risk for atopic disorders in offspring than paternal atopy (Harris *et al.*, 2004). Monozygotic twin pairs are more often concordant for AD than dizygotic twin pairs. In a Danish study monozygotic twins ran a risk of 0.86 of having AD if the twin partner has the disease, whereas the disease risk of 0.21 by dizygotic partners did not differ from the frequency seen in ordinary brothers and sisters (Larsen *et al.*, 1986). Patients with AD onset during the first year of life that was accompanied by asthma, hay fever, or both, and with living in an urban area, have more severe disease, independent of other potential risk factors (Ben-Gashir *et al.*, 2004).

The effect of breastfeeding on the development of AD remains controversial. Several studies comparing breast-fed to cow's milk formula-fed infants suggested a decreased incidence of AD and lower serum IgE in

infants who were nursed. A very large (17,046 infant–mother pairs) prospective, interventional study demonstrated a 46% reduction in the incidence of AD in infants whose mothers participated in a program designed to promote breast feeding compared with infants whose mothers did not have such support (Kramer *et al.*, 2001). On the other hand, other studies have suggested that breast-fed infants have an increased risk of developing allergies, including AD, compared with those fed cow's milk (Kay *et al.*, 1994) (Purvis *et al.*, 2005). Methodological and genetic differences, and differences in maternal diet and breast milk composition may affect whether breastfeeding is protective against the development of AD.

Allergic disorders have been on the increase in recent decades, especially among children living in developed countries. Most, but not all studies show that AD is more prevalent in groups with higher socioeconomic status (Kramer *et al.*, 1998; McNally *et al.*, 1998a; Suarez-Varela *et al.*, 1999). There is a marked increase in the prevalence of AD with increasing maternal education and in less crowded homes (Harris *et al.*, 2001). It has been suggested that cross infection from other siblings in large families may have a protective role in AD and that an increased risk of AD may result from decreases in helminthic infestation. Studies of migrant groups have shown large increases in disease prevalence compared with migrants' country of origin (Rottem *et al.*, 2005), suggesting clues as to the importance of socioeconomic and environmental changes such as those associated with industrialization.

Environmental factors such as endotoxin may modulate development of AD. A series of epidemiologic reports suggests that the frequency of allergic diseases is significantly decreased among children of farmers compared to children of nonfarmers in Western, industrialized countries (Weiss, 2002). Several studies showed an inverse relationship between endotoxin levels and AD at high levels of exposure at 1 year of age (Gehring *et al.*, 2001; Phipatanakul *et al.*, 2004). In another cross-sectional study with 812 children between 6 and 13 years of age from farming and nonfarming households in rural areas of central Europe, investigators found that endotoxin levels in samples of dust from the child's mattress are inversely related to the occurrence of hay fever, atopic asthma, and atopic sensitization (Braun-Fahrlander *et al.*, 2002). Association between endotoxin exposure and production of TNF-α, IFN-γ, IL-10, and IL-12, suggests that exposure to endotoxin may have a crucial role in the development of tolerance to allergens, that is, the hygiene hypothesis. The finding that dog exposure at birth is associated with decreased risk of AD may be related to increased exposure to endotoxin and other microbial products (Bufford *et al.*, 2008). This is supported by the observation that antibiotic use is associated with an increase in AD risk (Flohr *et al.*, 2005).

Infants born in autumn show the highest and those born in spring show the lowest prevalence of AD (Kusunoki *et al.*, 1999). There was no

such tendency for the prevalence of bronchial asthma and allergic rhinitis. This may be related to dryness of the skin due to indoor heated environments in winter months, which would promote itching and scratching in predisposed infants. Exposure to hard water has been associated with increased prevalence of AD (McNally *et al.*, 1998b). There is also a relationship between higher exposure to photochemical pollutants and high prevalence of AD, possibly because of the chemical effect of pollutants on skin permeability to antigens (Sole *et al.*, 2006).

5. GENETICS

The inheritance of AD is complex, since the development of the disease is affected by a multiplicity of genes and disease expression is influenced by environmental factors. Recent progress in genome-wide linkage studies has highlighted several possible AD related loci on chromosomes 3q21, 1q21, 17q25, 20p12, and 3p26. (Akdis *et al.*, 2006a; Cookson *et al.*, 2001; Haagerup *et al.*, 2004; Lee *et al.*, 2000). Genetic predisposition to AD and other allergic diseases could overlap because they share a systemic Th2 response evidenced by elevated number of eosinophils and elevated serum IgE levels. However, the loci associated with AD have generally not overlapped with regions linked to asthma, for example, 13q14 (Fig. 3.5) (Cookson, 2004) and this finding is consistent with epidemiologic data (Williams and Flohr, 2006). On the other hand, AD loci show a greater degree of association with psoriasis loci (1q21, 3q21, 17q25, and 20p12) (Fig. 3.5), although these two diseases are rarely linked. These results suggest that genes expressed in the skin play an important role in the development and expression of AD. In contrast to hypothesis-free linkage approaches, candidate gene studies are based on hypothesis made from pre-existing knowledge of biologic function and disease pathways. Candidate gene approaches are fundamentally dependent on the accuracy and completeness of existing data. Because of this nature, the approach is therefore limited in its potential for the discovery of novel targets and pathways. To date, several candidate genes have been identified in AD (Chien *et al.*, 2007; Hoffjan and Epplen, 2005; Morar *et al.*, 2006), which can be categorized into two groups: (1) genes involved in skin barrier function and (2) genes involved in the immune response.

5.1. Genes involved in skin barrier function

AD shows strong genetic linkage to Chromosome 1q21, which contains the human EDC (Cookson, 2004). Mutations in the *FLG* gene located on chromosome 1q21.3, have been identified in ichthyosis vulgaris, the most common autosomal dominant disorder of keratinization (Smith

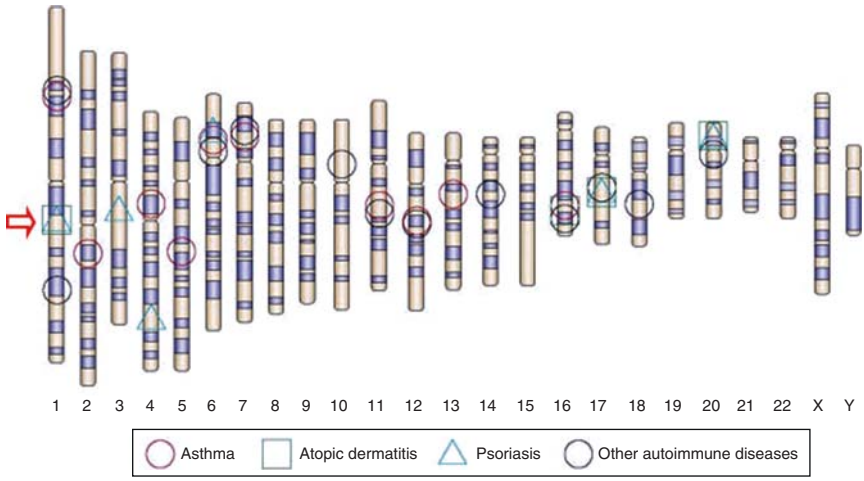

FIGURE 3.5 Susceptibility loci identified by genome screens in asthma, AD, and other immune disorders. Only loci with significant linkages are indicated. The 1q21 locus that contains the EDC gene complex is denoted by an arrow. Note that several loci for AD and psoriasis overlap, but that there is little overlap of AD and asthma associated loci. (From Cookson (2004). *Nat. Rev. Immunol.* **4**(12), 978–988.)

et al., 2006), and in patients with AD in different ethnic populations including several European countries and Japan (Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Sandilands *et al.*, 2007; Weidinger *et al.*, 2006; Nomura *et al.*, 2007). To date, at least 20 *FLG* mutations have been identified in European populations, and an additional 17 mutations in Asian population (Fig. 3.6) (Irvine and McLean, 2006; O'Regan *et al.*, 2008). Two loss-of-function mutations (R510X and 2282del4) of *FLG* gene account for the majority of *FLG* mutations in European patients with AD and are major risk factors for AD, and AD-associated asthma, but not for asthma alone (Palmer *et al.*, 2006; Weidinger *et al.*, 2006). The majority of *FLG* mutations in AD are heterozygous. Null mutations in the *FLG* gene are a predisposing factor for early-onset AD, which persists into adulthood (Barker *et al.*, 2007). Recent data demonstrate a strong association between *FLG* mutations and EAD (Weidinger *et al.*, 2006). Genetic variants of *FLG* in AD interfere with the processing of profilaggrin to filaggrin monomers (Palmer *et al.*, 2006). Decreased filaggrin expression has been observed in skin of AD patients with *FLG* mutations (Seguchi *et al.*, 1996). This likely causes increased TEWL and increased transepidermal entry of environmental antigen. Furthermore, the association of *FLG* mutations with AD suggests that the skin barrier defect precedes the development of AD. *FLG* expression is also reduced in AD patients with no *FLG* mutations. This may be due to local expression of the Th2 cytokines IL-4 and IL-13, which have been shown to downregulate *FLG* expression in keratinocytes (Howell *et al.*, 2007a).

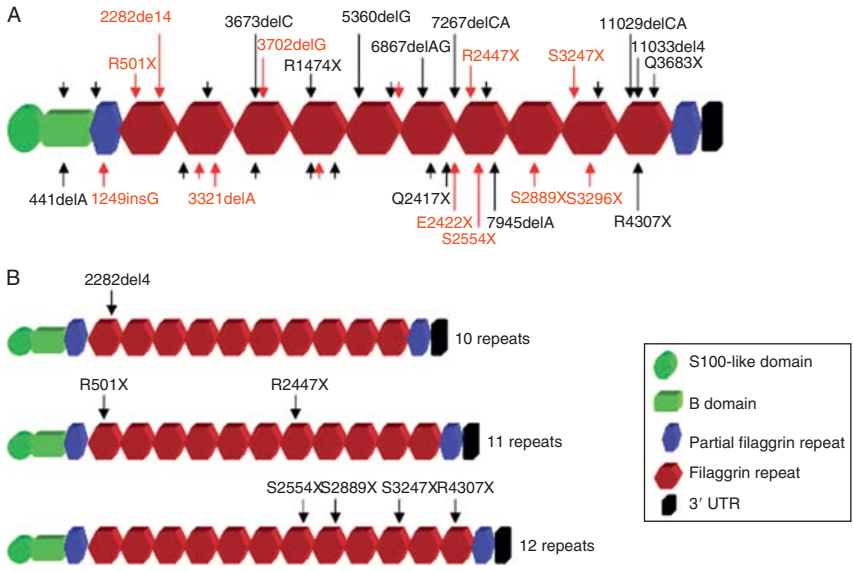

FIGURE 3.6 *Filaggrin* (*FLG*) gene structure and mutations in patients with AD. Protein organization and location of mutations. FLG is composed of a large transcript encoded by three exons, of which the third exon encodes the FLG protein repeats. The bulk of FLG protein sequences consist of a tandem array of repeating units of 35 kDa separated by a 7–10 amino acid linker peptide. There are 10 highly homologous FLG polypeptide units, with a variable number of FLG-repeat units consisting of 10, 11, or 12 units. The locations of 37 known mutations are shown. Reported mutations resulting in functional null alleles are numbered; positional locations of as-yet-unreported mutations are demonstrated by unmarked arrows. Prevalent mutations are indicated by red font, and family-specific mutations are in black (A). Mutations can occur on the background of the 10-repeat allele (A) or of the 11-, or 12-repeat alleles (B). UTR, untranslated region. (From O'Regan *et al.* (2008). *J. Allergy Clin. Immunol.* **122,** 689–93s.)

Since *FLG* mutations are identified in only a fraction of patients with AD, genetic variants of other genes involved in skin barrier function are likely to be important in the pathogenesis of AD. Indeed, mutations in two other genes involved in skin barrier function have been associated with AD, namely *SCCE* and *SPINK5*. *SCCE* is thought to play a central role in desquamation by cleaving proteins of the SC. The *SCCE* gene is located at 19q13. A 4-bp (AACC) insertion in the 3′-UTR of the *SCCE* gene is significantly associated with the subgroup of IAD patients (Vasilopoulos *et al.*, 2004). The *SPINK5* gene located at 5q32, encodes LEKTI, which regulates proteolysis in terminal keratinocyte differentiation. Mutations in *SPINK5* have been implicated in Netherton's syndrome, a syndrome with many features of AD including dermatitis, eosinophilia, and high IgE level, that causes early death in infancy due to dehydration

and infection via the skin (Chavanas *et al.*, 2000; Walley *et al.*, 2001). A E420K single nucleotide polymorphism (SNP) variant in the *SPINK5* gene has shown significant association with disease severity and with the presence of food allergy in children with AD (Kusunoki *et al.*, 2005). Other protease inhibitors with similar roles to SPINK5 are encoded by a cluster of genes on chromosome 20q12, close to a region that has been linked to AD. These proteins, which have homology to whey acidic protein, are produced at epithelial-cell surfaces and include elafin and secretory leukocyte protease inhibitor.

5.2. Genes involved in the immune response

A clustered family of Th2 cytokine genes that include *IL-4*, *IL-5*, and *IL-13* is located on chromosome 5q31-33 (Liu *et al.*, 2004). Two functional SNPs in *IL-13* have been associated with AD. These are −1112C/T in the promoter region associated with increased IL-13 production (van der Pouw Kraan *et al.*, 1999), and R130Q which increases IL-13 binding to the IL-13 receptor α2 chain, leading to increased IgE levels (Wang *et al.*, 2003). The T allele of the −590C/T SNP in *IL-4*, which is associated with increased promoter activity, is also associated with AD (Kawashima *et al.*, 1998). A −703C/T SNP in *IL-5* gene is associated with blood eosinophilia in Japanese patients with AD (Yamamoto *et al.*, 2003).

Variants of a number of other immune genes located in different loci have been found to be associated with AD. SNPs within the αchain of the IL-4 receptor gene located at 16q12 that include −3112C/T, −327C/A, I50V, Q375A, C406R, Q551R, and S761P, have been identified in patients with AD (Callard *et al.*, 2002; Hershey *et al.*, 1997; Hosomi *et al.*, 2004; Novak *et al.*, 2002; Oiso *et al.*, 2000; Tanaka *et al.*, 2001). These SNPs may influence the outcome of IL-4 receptor signaling (Kruse *et al.*, 1999).

Th1 cytokines, that is, IL-12 and IFN-γ suppress Th2 immune response and production of IgE antibodies. The A allele of the 1188A/C SNP in the IL-12β subunit (IL-12 p40) gene located at 5q31–33 is associated with increased mRNA expression levels and is inversely associated with AD, but increased in psoriasis, a Th1-type disease, suggesting that these associations may be due to alterations of Th1/Th2 balance (Tsunemi *et al.*, 2002). The inflammatory cytokine IL-18, which enhances IL-4 and IL-13 production, and whose overexpression in mouse skin results in AD-like lesions is located at chromosome 11q22.2–22.3, a region that has been linked to atopy-related traits (Koppelman *et al.*, 2002). The SNPs 113T/G and 127C/T in exon 1, −137G/C in promoter region 1, and −133C/G in promoter region 2 of the human *IL-18* gene are significantly associated with AD (Novak *et al.*, 2005). The *IL-10* gene, located at 1q31–32 is known to play various roles in immune regulation and anti-inflammatory responses. The T allele of the −819T/C SNP, and the A allele of the

−592A/C SNP have been shown to be associated with AD (Sohn *et al.*, 2007). Allergen complexed to IgE binds to the high affinity receptor for IgE (FcεRI) on the surface of epidermal LC, mast cells, and basophils, triggering the release of inflammatory mediators. Two SNPs (allele 2 of RsaI intron 2 and allele 1 of RsaI exon 7) within the β subunit of *FcεRI* gene located at 11q13 have been shown to associate with AD and asthma (Cox *et al.*, 1998). The Th2 cytokine IL-31, associated with itching, is located on 12q24. In three independent European populations comprising 690 affected families there was a significant association of the IL-31 −1066 G/A with the nonatopic-type of intrinsic AD (Schulz *et al.*, 2007).

SNPs within the genes *NOD2/CARD15*, *NOD1/CARD4*, and *TLR2* involved in the recognition of microbial products have been associated with AD. Polymorphisms in *CARD15* located at 16q12 gene including the T allele of 2104C/T, the C allele of 2722G/C, R702W and C insertion at position 3020 have been associated with AD (Kabesch *et al.*, 2003; Macaluso *et al.*, 2007). One *NOD1/CARD4* haplotype and three polymorphisms (rs2907748, rs2907749, rs2075822) were significantly associated with AD in a population-based cohort, case-control population, and/or family-based association analysis (Weidinger *et al.*, 2005). Furthermore, the combination of a G allele of CARD4_In1 and A allele of CARD4_Ex6 was significantly associated with AD (Macaluso *et al.*, 2007). The R753Q polymorphism of *TLR2* gene located at 4q32, that is associated with *S. aureus* infections (Lorenz *et al.*, 2000), has been reported to be associated with an AD phenotype, characterized by markedly elevated IgE antibody levels, increased disease severity, and susceptibility to colonization with *S. aureus* (Ahmad-Nejad *et al.*, 2004). Further study showed that the *TLR2* mutation R753Q reduces IL-8 production from monocytes of AD patients and TLR2 expression on activated T cells in response to stimuli, suggesting a possible role of TLR2 on the level of innate-adaptive immune cooperation (Mrabet-Dahbi *et al.*, 2008). A C-1237T SNP in the promoter region of the *TLR9* gene is significantly associated with AD, in particular the intrinsic type (Novak *et al.*, 2007). Toll-interacting protein (TOLLIP) is an inhibitory adaptor protein within the TLR pathway. There is an association of *TLR2* and *TOLLIP* SNPs with AD (Schimming *et al.*, 2007). Selective impairment of TLR2-mediated proinflammatory cytokine production by monocytes from patients with AD has also been reported (Hasannejad *et al.*, 2007). Another study failed to find any significant difference in the mRNA expression level for TLRs 1, 2, 3, 5, and 6 between skin biopsies of patients with AD and psoriasis (Nomura *et al.*, 2003). Children with AD have also been shown to have reduced levels of soluble CD14 (sCD14) compared with nonatopic children (Zdolsek and Jenmalm, 2004). In addition, a study also found that reduced levels of sCD14 in breast milk were associated with the development of AD at 6 months of age (Jones *et al.*, 2002). Exclusively breast-fed children whose mothers had

higher levels of sCD14 in their breast milk had a decreased likelihood of having AD. The lower levels of sCD14 observed in children with AD or their mothers might be due to genetic differences or a reduced capacity to respond to microbial signals or decreased exposure to microbial signals. However, no difference was observed in the expression of CD14 on keratinocytes derived from the nonlesional skin of subjects with AD, subjects with psoriasis, or nonatopic control subjects (McGirt and Beck, 2006). SNP within the *NALP1* gene has been associated with AD (Macaluso *et al.*, 2007).

6. PATHOPHYSIOLOGY OF AD

A likely scenario in AD is that a genetically permeable and dry itchy skin predisposes both to the entry of allergens and microbial products as well as to the development of a local milieu of cytokines and chemokines. This instructs skin DCs that migrate to draining lymph nodes (DLNs) to polarize the T cell response to cutaneously introduced antigens towards a Th2-dominated response (Fig. 3.7). The individual contributions of both skin defects and immune defects to AD is illustrated by diseases that present with allergic skin inflammation indistinguishable from AD skin lesions and that represent two extremes of the involvement of skin versus the immune system in the pathogenesis of the disease. One extreme is represented by Netherton's syndrome (Fig. 3.8A), which involves mutation in the skin specific gene *SPINK5*. The other extreme is illustrated by dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome (IPEX) (Fig. 3.8B), and Wiskott–Aldrich syndrome (WAS) (Fig. 3.8C), which involve mutations in the immune specific genes *Foxp3* and WAS protein (*WASP*) respectively. Foxp3 is expressed in regulatory T (Treg) cells and IPEX patients have low numbers and poor function of Treg cells (Bennett *et al.*, 2001; Chatila, 2005). Patients with WAS have T cell dysfunction and low numbers of platelets (Snapper and Rosen, 1999) (see also Ochs and Thrasher, 2006). In this section, we will review the factors in AD skin that underlie the pathogenesis of the disease, and will discuss the immunopathology of the disease. We will support the above hypothesis with data from animal models, which are discussed in detail in Section 7.

6.1. Altered skin barrier function

Accumulating evidence has shown that loss of skin barrier integrity is an important factor for the development of AD. AD patients exhibit impaired skin barrier function and abnormal structure and chemistry of the SC in both lesional and nonlesional skin. Impaired barrier function as an important factor in AD is suggested clinically by the observation that

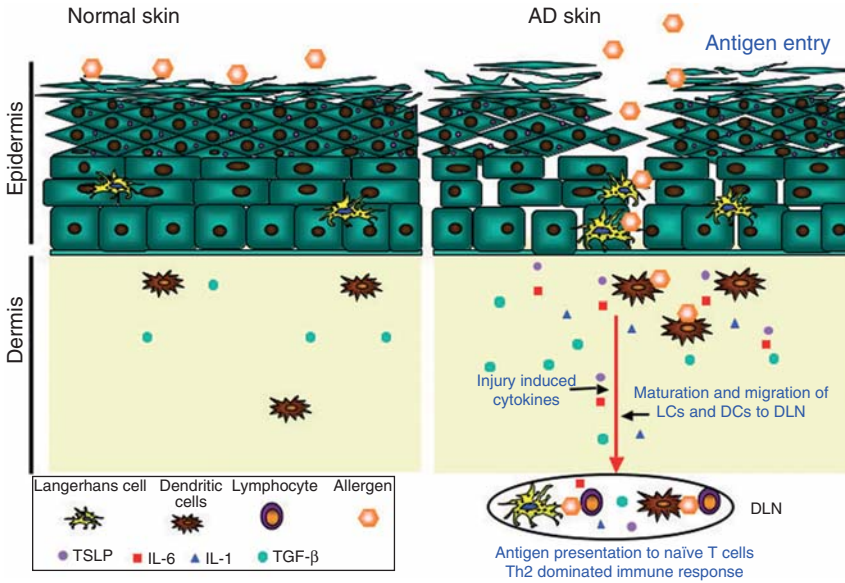

FIGURE 3.7 Skin barrier dysfunction in AD. An intact skin barrier (left panel) prevents allergens from entering normal skin. Damage to this barrier (right panel) allows allergens to penetrate into the subepidermal layer and interact with APCs, and induces cytokines by keratinocytes that includes TSLP, IL-1, IL-6, and TGF-β. This leads to maturation and migration of APCs to DLN where they present antigens to naïve T cells, resulting in Th2-dominated immune response.

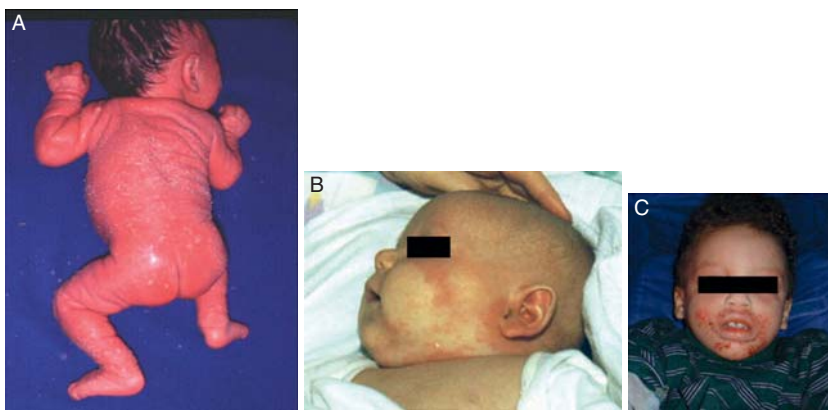

FIGURE 3.8 Human single gene defects associated with AD-like skin lesions. (A) Netherton syndrome (SPINK 5 deficiency). (B) IPEX syndrome (Foxp3 deficiency). (C) Wiskott–Aldrich syndrome (WASP deficiency).

AD skin lesions spare the relatively occluded and better hydrated axillary and diaper areas. Alteration of the skin barrier in AD patients is evidenced by the reduction in the water-content of the SC and by an increase in TEWL (Seidenari and Giusti, 1995; Werner and Lindberg, 1985). Barrier dysfunction with increased water loss is responsible for the dry skin (xerosis) that ultimately results in dry, scaly, rough, dull, slightly wrinkled skin, hallmarks of AD and is responsible for the pruritus that increases the penetration of allergens (Goerdt *et al.*, 1999; Krasteva *et al.*, 1999; Thestrup-Pedersen *et al.*, 1997). It is likely that the skin barrier dysfunction is at least partly a primary lesion in AD. This could be due to mutations in keratinocyte structural proteins such as filaggrin, mutations that increased protease activity or to decrease protease inhibitor activity, or mutations that results in abnormal lipid composition in lipid metabolizing enzymes. Altered activity of proteases and protease inhibitors and altered lipid composition of the SC have been observed in AD patients compared to healthy subjects (Descargues *et al.*, 2006; Hosomi *et al.*, 2004; Pilgram *et al.*, 2001; Vasilopoulos *et al.*, 2004). Furthermore, flaky tail mice argue in favor of the hypothesis that there is a primary skin barrier dysfunction in AD, at least in these AD patients with *FLG* mutations, as these mice have dry skin and altered composition of the epidermal ceramides at birth and in the absence of skin inflammation (Aioi *et al.*, 2001; Presland *et al.*, 2000). Mechanical injury inflicted by scratching, as well as skin inflammation in AD lesions are likely to worsen skin barrier dysfunction, as suggested by the observation that the defect in TEWL improves with controlled or spontaneous improvement of disease activity (Matsumoto *et al.*, 2000). In support of skin barrier dysfunction as an important determinant of allergic sensitization, children with AD who are exposed to topical creams and lotions containing peanut protein show a significantly increased risk of peanut sensitization, indicating a close relation between the integrity of the skin barrier and the development of skin-mediated systemic sensitizations (Morar *et al.*, 2007).

Lipid analysis of SC from uninvolved as well as involved skin of patients with AD show a significant reduction in ceramides 1 and 3 and a significant increase in cholesterol compared with healthy subjects (Di Nardo *et al.*, 1998; Imokawa *et al.*, 1991) (see also Bleck *et al.*, 1999; Yamamoto *et al.*, 1991). There is a significant negative correlation between TEWL and the quantity of ceramide 3 (Di Nardo *et al.*, 1998). Disturbed hydrolysis of sphingomyelin, as a cause of reduced ceramide levels in AD skin, is suggested the observation that the levels of acid and neutral sphingomyelinases is significantly decreased in AD skin (Jensen *et al.*, 2004). Electron microscopic analysis of lesional and nonlesional AD skin found lamellar bodies in much more superficial layers of the SC than in controls. This observation suggests that retention of lipids at the site of their synthesis occurs in AD skin, making them unavailable to the SC

(Fartasch, 1997). The predominant phase of the lipids in human SC is orthorhombic rather than hexagonal packing, and ceramide 1 is thought to contribute to this feature. Compared to normal, the ratio of hexagonal to orthorhombic packing in SC was increased in AD patients (Pilgram *et al.*, 2001). It has been suggested that UV light increases the amount of all three major SC lipids, hence its efficacy in AD.

MicroRNAs (miRNAs) are a group of recently discovered endogenously expressed small noncoding RNAs that are believed to be important in many biological processes by means of their ability to regulate gene expression. A number of miRNAs, for example, miR-203 are differentially expressed in keratinocytes and are thought to be involved in keratinocyte proliferation and differentiation. Using miRNA array comparison of healthy and lesional skin of AD patients, 12 miRNAs were found to be upregulated, and 9 were found to be downregulated in AD skin (Sonkoly *et al.*, 2007). It will be of interest to determine whether these alterations in expression of miRNAs are primary and what roles they may play in the pathogenesis of AD.

Intrinsic abnormalities in barrier function in AD lead to dry itchy skin. There is strong experimental evidence to suggest that mechanical injury inflicted by scratching the skin plays a major role in the polarization of the immune response to cutaneously introduced antigens. We have shown that introduction of antigen via normal mouse skin mechanically injured by tape stripping results in allergic skin inflammation that exhibits many features of AD skin inflammation including infiltration with $CD4^+$ T cells, and eosinophils and expression of Th2 cytokines and to a lesser extent of IL-17 (He *et al.*, 2007; Spergel *et al.*, 1998). The polarizing effect of skin injury on the Th response is exerted, at least in part, at the level of skin DCs that carry antigen to DLNs as discussed in detail below. An important cytokine released by injured keratinocytes is thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine that uses a heterodimeric receptor that consists of IL-7Rα and TSLPR chains (Pandey *et al.*, 2000; Park *et al.*, 2000). TSLP has been shown to skew the Th response by inducing OX40L expression on DCs and its overexpression in mouse skin causes allergic dermatitis (see Section 7.2). Interestingly, TSLP mediates the ability of active forms of vitamin D to cause allergic skin inflammation when applied topically (Li *et al.*, 2006, 2009). In this respect, the active vitamin D metabolite $1,25\text{-}(OH)_2D_3$ (Vit D3) is produced photochemically in skin exposed to UV light from 7-dehydrocholesterol, a derivative of cholesterol found in the epidermis. Vit D3 binds to the vitamin D receptor, forms a heterodimer with the retinoid receptor and binds to a vitamin D responsive element which controls the transcription of target genes which include TSLP. However, Vitamin D may also exert potential anti-inflammatory effects, such as suppressing the number and antigen-presenting function of LC (Dam *et al.*, 1996) and inducing the expression of AMPs (Hata *et al.*, 2008).

6.2. Immunopathology

Components of both adaptive and innate immunity contribute to the immunopathology of AD. AD skin lesions are characterized by a dermal infiltrate consisting of activated $CD4^+$ MHC class II^+ memory/effector T cells, eosinophils, mast cells, distinct DC subsets, and macrophages that may be coated with IgE (Bieber, 2008; Leung *et al.*, 1983, 1987). There is also both epidermal and dermal thickening. The dermis is characterized by increased numbers of LCs, presence of intercellular edema leading to spongiosis, and increased apoptosis of keratinocytes that express the death receptor (Trautmann *et al.*, 2000). There is also increased collagen deposition in the dermis.

Memory/effector $CD4^+$ T cells play a critical role in AD. This is illustrated by two observations. AD-like lesions of patients with WAS resolve upon successful reconstitution of the T cell compartment with allogeneic cells from HLA matched donors (Parkman *et al.*, 1978), and mice that lack TCRαβ T cells fail to develop skin inflammation following epicutaneous (EC) sensitization with antigen (Woodward *et al.*, 2001). There are estimated 2×10^{10} T cells in the skin of a normal individual, nearly twice the number of T cells in the circulation. In humans, these cells appear to be exclusively TCRαβ T cells, while in mice TCRγδ T cells contribute heavily. Following cutaneous introduction of antigen, skin LCs and DCs capture antigen, and migrate to local DLN where they present the antigen to recirculating naive T cells. This leads to T cell proliferation and differentiation into memory/effector cells that express skin homing receptors such as cutaneous lymphocyte antigen (CLA), CCR4, and CCR10. Antigen-specific effector $CD4^+$ T cells leave the DLN into the circulation and re-enter the skin, via their skin specific receptors, where they can be activated locally by specific antigen to proliferate and secrete cytokines. CLA, which represents an inducible carbohydrate modification of P-selectin glycoprotein ligand-1, is virtually absent on naive T cells, but is expressed by 30% of circulating memory T cells and by approximately 90% of infiltrating T cells in inflamed cutaneous sites (Santamaria-Babi, 2004). CLA^+ T cells coexpress the CCR4 receptor, the ligand for CCL17/TARC and CCL22/MDC. The selective homing of memory/effector $CD4^+$ T cells to skin represents an important immunological event in the development of allergic skin inflammation. There are higher proportions of CLA^+ T cells and of $CCR4^+CD4^+$ T cells circulating in the peripheral blood of AD patients (Santamaria Babi *et al.*, 1995). Moreover, AD skin lesions exhibit abundant infiltration of CLA^+ and $CCR4^+CD4^+$ T cells.

Th2 cells are believed to play a key pathogenic role in AD. This is supported by the presence of blood eosinophilia and elevated serum IgE levels in the majority of AD patients. Acute AD skin lesions exhibit

Th2-dominant inflammation characterized by dermal infiltration of $CD4^+$ T cells and eosinophils with deposition of eosinophil-derived products and increased skin expression of Th2 cytokines IL-4, IL-5, and IL-13, with only little IFN-γ expression. A pathogenic role of IL-4 in AD is suggested by the observation that keratinocyte-specific overexpression of IL-4 in transgenic mice results in AD-like lesions (Chan *et al.*, 2001). As AD lesions become more chronic, they exhibit increased expression of IFN-γ, IL-12, and GM-CSF (Hamid *et al.*, 1994), and tissue remodeling with increased deposition of collagen and dermal thickening. The mechanism behind this switch from Th2 to Th1 type is not well understood, but could be related to microbial products. In this regard, we have recently demonstrated that TLR2 is important for the Th1 response to cutaneously introduced antigen (Jin *et al.*, 2009a).

Recently, the novel 4-helix-bundle IL-31, preferentially expressed by Th2 cells has been shown to be highly expressed in AD skin lesions (Dillon *et al.*, 2004). IL-31 expression is upregulated in both pruritic AD skin lesions and nonlesional AD skin, but not in nonpruritic psoriatic lesions, compared with healthy skin (Bilsborough *et al.*, 2006; Sonkoly *et al.*, 2006). Skin-infiltrating cutaneous CLA^+ T cells and peripheral blood CD45RO CLA^+ T cells are the source for IL-31 in AD (Bilsborough *et al.*, 2006). There is a correlation between IL-31 serum levels with the severity of AD (Raap *et al.*, 2008). Interestingly, staphylococcal enterotoxin rapidly induces IL-31 expression in lymphocytes from AD patients, suggesting that skin microbial infection can lead to the exacerbation of pruritus via the induction of IL-31 (Sonkoly *et al.*, 2006). Both subunits of IL-31 receptor, IL-31 receptor A and oncostatin M receptor, are expressed by human keratinocytes. IL-31 induces the expression of the inflammatory T cell attracting chemokines CCL17/TARC and CCL22/MDC by cultured keratinocytes (Sonkoly *et al.*, 2006). A pathogenic role of IL-31 is suggested by the observation that T cell-specific overexpression of IL-31 in transgenic mice induces alopecia and chronic pruritus (Dillon *et al.*, 2004).

Immunohistochemical analysis has demonstrated the presence of IL-17-secreting cells infiltrating the dermis more markedly in acute than chronic AD lesions (Koga *et al.*, 2008; Toda *et al.*, 2003). Furthermore, the number of Th17 cells is increased in the peripheral blood of AD patients (Koga *et al.*, 2008), and IL-17 serum levels are elevated in these patients (R. He and R. S. Geha, unpuplished observations). IL-17 stimulates keratinocytes to produce GM-CSF, TNF-α, IL-8, CXCL10, and vascular endothelial growth factor (VEGF), and HBD-2 (Eyerich *et al.*, 2008; Koga *et al.*, 2008). A marked synergistic effect between IL-17 and IL-22, another cytokine produced by Th17 cells, was observed on IL-8 and AMP production while IL-4 and IL-13 inhibited IL-17 mediated HBD-2 induction (Eyerich *et al.*, 2008; Liang *et al.*, 2006). These studies suggest that Th17 cells may exaggerate AD. However, a recent report has failed to

detect elevated IL-17 expression in AD lesions using gene microarray (Guttman-Yassky *et al.*, 2008).

Cutaneous immune responses must be tightly controlled to prevent unwanted inflammation in response to innocuous antigens, while maintaining the ability to combat skin-tropic pathogens. Foxp3$^+$ Treg cells are potent immune regulators and are found at high frequency in both human and mouse skin. Accumulating evidence has shown a role for CD25$^+$CD4$^+$ Treg cells in suppressing T cell responses to allergens (Akdis *et al.*, 2006b). CD4$^+$CD25$^+$FoxP3$^+$ Treg cells were reported decreased in lesional and atopy patch tested skin of AD (Verhagen *et al.*, 2006). However, AD patients have significantly increased numbers of peripheral blood Treg cells with normal immunosuppressive activity, but SEB stimulation caused loss of immunosuppressive activity in these Treg cells (Ou *et al.*, 2004). Given the fact that more than 90% of patients AD have *S. aureus* colonization of their skin lesions, these data suggest that the enhanced effector T cell activation in AD patients could be due to the abrogation of the number and the immunosuppressive activity of Treg cells. A recent study showed the presence of subtype of circulating CD4$^+$CD25$^+$ T cells in AD patients with high IgE. This subtype is negative for CCR6 and exhibit Th2-promoting properties (Reefer *et al.*, 2008). Furthermore, SEB induces Th2 cytokine secretion by these cells. These results suggested a new mechanism by which Treg cells contribute to the pathogenesis of AD. A potential role of Treg cells in AD is suggested by the observation that restoring the Treg cell compartment in Foxp3-deficient scurfy (sf) mice with WT T cells, but not with α-1,3-fucosyltransferase VII deficient T cells or CCR4 deficient T cells, which cannot migrate to the skin, inhibits cutaneous inflammation (Dudda *et al.*, 2008; H. Oettgen, personal communication)

EC sensitization with allergens is thought to play an important role in the pathogenesis of AD. This is supported by the observation that application of allergen to the abraded uninvolved skin of patients with AD provokes an eczematous rash with eosinophilic infiltration (Mitchell *et al.*, 1982). DCs are essential for the generation of an immune response. In the skin there are two main types of immature DCs: LCs in the epidermis and interstitial (dermal) DCs in the dermis. LCs form a DC network in the epidermis where they sample antigens that get through the skin barrier of SC. Antigen that reaches the interstitial spaces is taken by interstitial (dermal) DCs. Following antigen uptake and in the presence of a danger signal generated by microbial antigens that include normal flora, and/or mechanical injury with its release of mediators such as IL-1 and TNF-α, immature DCs acquire the phenotype of professional APCs. They upregulate the expression of MHC class II molecules and of key costimulatory molecules such as CD80 and CD86, reduce their capacity to take up antigen and express chemokine receptors, particularly CCR7 the receptor

for the chemokines CCL19 and CCL21 expressed in DLN, that allow them to migrate towards DLNs where they present antigenic peptides to naïve T cells that continuously circulate through the DLN. Interaction between antigen-laden DCs and antigen specific T cells leads to T cell proliferation and differentiation. In the case of $CD4^+$ T cells, differentiation leads to the generation of Th1 cells that secreted IFN-γ, Th2 cells that secrete IL-4, IL-5, and IL-13 or Th17 cells that secrete IL-17 and IL-22. DCs play a critical role in the polarization of T cells into Th1, Th2, or Th17 cells because of their specific expression of cytokines and costimulatory molecules. This is influenced by the individual tissue milieu from which they originate. Loss of protection by the skin barrier will increase exposure of LCs and dermal DCs to environmental antigens. More importantly, cytokines released by resident skin cells in response to mechanical injury inflicted by scratching and to PAMPs engaged by microbial antigens, are likely to play an important role in causing the maturation of DCs and in determining their ability of DCs to polarize T cells in patients with AD (Fig. 3.7). Examples of skin cytokines that are upregulated in skin after mechanical injury and that exert powerful effects on skin DCs include keratinocyte-derived TSLP, IL-1, IL-6, and TGF-β (He *et al.*, 2007) (our unpublished observations). TSLP is thought to be important in polarizing DCs to promote an inflammatory Th2 response characterized by high production not only of Th2 cytokines, but also of TNF-α, with little production of IL-10 (Liu, 2007). TGF-β, IL-1, and IL-6 all play a role in the Th17 response in humans (McGeachy and Cua, 2008).

Compared with normal skin, AD skin lesions exhibit increased numbers of LCs in the epidermis and of DCs in the dermis (Zachary *et al.*, 1985). In addition, AD skin is characterized by the presence of IDECs characterized as $CD1a^{dim}CD1b^{dim}FceRI^{bright}CD23^{dim}CD32^{dim}HLA\text{-}DR^{bright}CD36^{bright}$ cells and devoid of Birbeck granules, opposed to normal LCs, characterized as $CD1a^{bright}CD1b^{neg}$ $FcεRI^{dim}CD23^{neg}CD32^{dim}HLA\text{-}DR^{bright}CD36^{dim}$ which contain Birbeck granules, which are found in both normal and AD skin (Wollenberg *et al.*, 2002). IDECs are detectable exclusively at inflammatory sites. AD skin lesions have decreased numbers of plasmacytoid DCs (pDCs), which are abundant in the lesional skin of patients with psoriasis or allergic contact dermatitis (Novak and Bieber, 2005). LCs, IDECs, and pDCs express the high affinity IgE receptor FcεRI as well as the low affinity IgE receptor FcεRII/CD23, (Novak *et al.*, 2003b; Wollenberg *et al.*, 1996). High FcεRI surface expression by skin DCs is selective to AD as it is not observed in other inflammatory skin diseases such as psoriasis, contact dermatitis, or in cutaneous T-cell lymphoma (Wollenberg *et al.*, 1999). FcεRI expression on LCs correlated significantly with the serum IgE level. FcεRI aggregation on pDCs from AD patient impaired surface expression of MHC class I and II, induced the production of IL-10, and enhanced the apoptosis of pDCs. Importantly, FcεRI preactivated pDC

produced less IFN-α and IFN-β after stimulation with CpG and enhanced the outcome of Th2 immune responses (Novak *et al.*, 2004). Given the important role of pDCs as a critical source for the antiviral type-I interferons (IFN-α and IFN-β) and in the defense against viral infections, decreased number and function of pDCs in AD skin lesions may contribute to the predisposition of AD skin lesions to viral skin infections. Expression of FcεRI on LCs and DCs in AD skin enhances antigen presentation, by facilitating the capture and internalization of antigen complexed with IgE antibody, which promotes antigen presentation to T cells in DLN or in the skin itself (Novak *et al.*, 2004). Concomitantly, ligation of FcεRI on the surface of LCs induces them to release chemokines such as IL-16, CCL22/MDC, CCL17/TARC, and CCL2 that contribute to the recruitment of precursor cells of IDECs and T cells *in vitro* (Novak and Bieber, 2005). An important role of $FcεRI^+/IgE^+$ LCs in allergic skin inflammation is suggested by the observation that their presence is required to provoke atopic skin lesions in response to patch application of aeroallergens in patients with AD (Bruynzeel-Koomen and Bruynzeel, 1988). Stimulation of FcεRI on IDECs leads to the release of high amounts of proinflammatory cytokines which contribute to the amplification of the allergic immune response by acting on T cells (Novak *et al.*, 2004). Furthermore, stimulation of FcεRI on the surface of IDEC also promotes T-cell switching into IFN-γ-producing Th1 cells by release of IL-12 and IL-18. This may contribute to the switch from the initial Th2 immune response in acute AD skin lesions to the Th1 phenotype in chronic AD skin lesions. Other factors that contribute to this switch may include microbial antigens, as we have found that TLR2 is important for the IFN-γ response to cutaneously introduced antigen and that concomitant application of antigen and the TLR9 ligand CpG promotes the expression of IFN-γ the skin in a mouse model of AD elicited by EC sensitization with antigen described below (Jin *et al.*, 2009a and our unpublished observations).

CCL18 is specifically expressed by APCs in the dermis and by LCs and IDECs in the epidermis and represents the most highly expressed ligand in AD skin. Some trigger factors of atopic skin inflammation, such as allergen exposure and staphylococcal superantigens, markedly induce CCL18 *in vitro* and *in vivo* (Gunther *et al.*, 2005; Pivarcsi *et al.*, 2004). Stronger CCL18 expression was observed in lesional skins of EAD patients compared with IAD patients (Park *et al.*, 2008). Although the receptor for CCL18 remains unknown, CCL18 binds to CLA^+ T cells and induces migration of memory T cells *in vitro* and *in vivo* (Gunther et al., 2005).

Eosinophils have been long closely associated with the pathogenesis of allergic diseases, including AD. Peripheral blood eosinophilia and

elevated serum levels of eosinophil granule proteins, including basic proteins eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and major basic protein (MBP), are common findings in AD patients and appear to correlate with disease activity (Kagi *et al.*, 1992). Similarly, the level of the eosinophil mobilizing and survival and the level of Th2 cytokine IL-5 are elevated in the serum of patients with AD and correlates with diseases severity (Kondo *et al.*, 2001). AD associated with respiratory allergic diseases shows more pronounced blood eosinophilia (Uehara *et al.*, 1990). A correlation between skin eosinophilia and the degree of spongiosis was observed in acute AD or acute exacerbations of chronic AD (Kiehl *et al.*, 2001). There is increased expression of the eosinophil chemotactic factor CCL11/eotaxin and of IL-5 and IL-5Rα in acute and chronic skin lesions as well as in the blood in AD patients (Taha *et al.*, 1998, 2000). Levels of mRNA for the CCL11/eotaxin receptor CCR3, and for IL-5Rα expressed on eosinophils are upregulated in AD skin (Yawalkar *et al.*, 1999). Eosinophilic infiltration of AD skin lesions is often difficult to demonstrate because of the fragility of human eosinophils. However, deposition of eosinophilic granule protein is readily detected in nearly all biopsies of skin lesions from both acute and chronic stages of AD and correlates with disease severity (Omoto *et al.*, 2000). Strong MBP deposition was shown to occur in acute lesions of AD patients who have a predisposition to respiratory allergy. In chronic AD, more pronounced eosinophilia was found in lesions with marked epidermal hyperplasia compared with those with no or slight hyperplasia. Pronounced tissue eosinophilia, especially ECP deposition in skin lesions of chronic AD, may be involved in the development and chronicity of AD (Kiehl *et al.*, 2001). Circulating levels of IL-5 and CCL11/eotaxin increase during flares of AD (Hossny *et al.*, 2001), and this might lead to eosinophil recruitment to the skin, where they are activated and undergo degranulation and cytolytic degeneration, the products of which promote inflammation and tissue damage (Leiferman *et al.*, 1985).

Mast cells reside in large numbers in tissues that interface the external environment, including the skin, where they play an important role in both adaptive and innate immunity. In early stage AD, mast cells are present in normal numbers but undergo degranulation in the affected skin. In late stage AD, mast cells are present in increased number, but without degranulation (Navi *et al.*, 2007). Aggregation of mast cell FcεRI receptors leads to degranulation and the release of mediators, such as histamine, eicosanoids, and proinflammatory cytokines. Mast cells are activated by other triggers such as anaphylatoxins, immunoglobulin-free light chains, superantigens, neuropeptides, TLRs, and cytokines leading to selective release of mediators without degranulation. Mast cell-derived histamine, the mast cell proteinases enzymes tryptase and chymase (MCC), and other inflammatory mediators contribute to pruritus and

inflammation in AD. Histamine upregulates the production of various inflammatory cytokines by keratinocytes. Increased proteinase activity of MCC in AD skin may also be involved in promoting a skin barrier defect in AD (Badertscher *et al.*, 2005). A role of mast cells in AD has also been suggested by studies of gene SNPs. Variants of the MCC gene located on chromosome 14q11.2 have been linked to AD, particularly to IAD (Mao *et al.*, 1996, 1998; Tanaka *et al.*, 1999). However, mast cells are not required in the AD mouse model elicited by EC sensitization (Alenius *et al.*, 2002).

Keratinocytes, the main constituents of the epidermis, play an important role in cutaneous immune responses. They actively participate in the skin immune response through the production of proinflammatory cytokines, chemokines, and AMPs in response to multiple stimuli. Itch-induced scratching leads to the release of proinflammatory cytokines and chemokines from keratinocytes. Atopic keratinocyte-derived GM-CSF can stimulate mononuclear cell proliferation and support the differentiation of peripheral blood monocytes into mature DCs in the presence of IL-4 (Pastore *et al.*, 1997). IL-1 and IL-18 are stored as biological inactive precursors in keratinocytes, and converted to their active forms by CASP1 enzyme after stimulation by danger signals. Furthermore, mechanical injury upregulates IL-1 and IL-6 mRNA expression in skin as determined by gene microarray (our unpublished observations). A potential role for IL-18 in AD has been suggested by several observations. The amount of active IL-18 in the sera of AD patients increases with exacerbation of their disease, and supernatants of PBMCs from AD patients show significantly higher amount of IL-18 after stimulation with SEB compared to HC subjects (Novak and Bieber, 2005). IL-18 SNPs are associated with AD (Novak *et al.*, 2005). Microbial products, physical injury, or inflammatory cytokines, including proinflammatory (TNF-α and IL-1α) and Th2 (IL-4 or IL-13) cytokines induce the production of TSLP in human skin explants (Allakhverdi *et al.*, 2007; Bogiatzi *et al.*, 2007). TSLP is highly expressed by keratinocytes from skin lesions of AD patients. As discussed above, TSLP polarizes human DCs to skew the T cell response to Th2. In addition, it contributes to the initiation of the allergic inflammation through induction of the migration of LCs into DLNs (Ebner *et al.*, 2007; Soumelis *et al.*, 2002). TSLP is also essential for local antigen-driven Th2 cytokine secretion by directly acting on skin infiltrating antigen-specific $CD4^+$ T cells (He *et al.*, 2008). Furthermore, TSLP, synergistically with IL-1 and TNF-α, stimulates the production of high levels of Th2 cytokines by human mast cells (Allakhverdi *et al.*, 2007). In addition to keratinocyte-derived mediators acting on T cells, activated skin-infiltrating T cell can upregulate Fas expression on keratinocytes and induce keratinocyte apoptosis, which leads to spongiosis, a key pathogenic event in AD (Trautmann *et al.*, 2000, 2001).

Keratinocytes from AD patients overexpress numerous chemokines that can contribute to the activation and recruitment of DCs, T cells, and

other leukocyte subsets to amplify and maintain skin inflammation response in the skin (Giustizieri *et al.*, 2001). These include CCL20/MIP-3α, CCL27, CCL17/TARC, and CCL22/MDC that can attract DCs and T cells. CCL20/MIP-3α is an important chemokine responsible for the recruitment of CCR6-expressing immature DCs and memory/effector T cells into the dermis of atopic skin (Nakayama *et al.*, 2001; Vanbervliet *et al.*, 2002). CCL20/MIP-3α is weakly expressed in healthy skin but markedly produced by keratinocytes in the lesional skin of AD patients (Schmuth *et al.*, 2002). AD patients show increased propensity toward EV. Insufficient expression and production of CCL20/MIP-3α in AD skin has been shown to increase the potential for disseminated VV infection after smallpox vaccination (Kim *et al.*, 2007a). CCL27 is exclusively expressed by keratinocytes and selectively attracts memory T cells to the skin by interacting with its receptor CCR10 (Homey *et al.*, 2000, 2002; Morales *et al.*, 1999). Lesions from acute and chronic AD patients exhibit strong CCL27 expression which correlates with the locally increased accumulation of $CCR10^+$ T cells (Homey *et al.*, 2002). $CCR10^+$ T cells are found in the skin of psoriasis, suggesting different Th1/Th2 profiles of $CCR10^+$ T cells. CCL17/TARC and CCL22/MDC are high-affinity ligands for CCR4. CCL17/TARC is highly expressed by keratinocytes and DCs in AD skin. Their receptor CCR4, an important skin homing receptor expressed preferentially on Th2 cells, was found only in AD skin lesions (Wakugawa *et al.*, 2002) (see also Vestergaard *et al.*, 2003). AD patients show higher $CCR4^+$ T cells in both peripheral blood and skin lesions than controls (Nakatani *et al.*, 2001). Serum CCL17/TARC levels are associated with disease activity in AD (Kakinuma *et al.*, 2001). Furthermore, serum CCL17/TARC and CCL27 levels significantly correlate with disease severity both in children with EAD and IAD (Song *et al.*, 2006).

Keratinocyte-derived AMPs are an important part of the skin innate immune response because of their broad-spectrum activity against a variety of pathogens. Injury or inflammation of the skin can trigger the production of the β-defensins HBD-2 and HBD-3, and the cathelicidin, hCAP18/LL-37 (Schauber *et al.*, 2008). Expression of the antimicrobial peptides HBD-2, HBD-3, and hCAP18/LL-37 is significantly decreased in acute and chronic AD skin lesions compared to psoriasis skin lesions (Nomura *et al.*, 2003; Ong *et al.*, 2002). Furthermore, IL-13 and IL-4 inhibit the production of HBD-3 by keratinocytes from AD patients, suggesting that the Th2 cytokine milieu in AD skin may suppress the innate immune response against bacterial and viral pathogens (Howell *et al.*, 2006a). Decreased AMPs in AD skin may contribute to its susceptibility to infections.

NK cells account for ~10% of peripheral blood lymphocytes, and are one of the important cell types of the innate immune system. NK cells lyse infected target cells by the release of perforin and granzyme from their cytoplasmic granules. NK cells are identified by the expression of CD56

but lack CD3, CD19, and TCR on their cell surface and may release a variety of proinflammatory cytokines such as IFN-γ, TNF-α, GM-CSF, IL-5, and IL-8, which help recruitment of other innate immune cells (Aktas *et al.*, 2005). Circulating $CD56^+CD16^+$ NK cells are notably reduced in AD patients; however, their number increases in lesional skin with severity of the disease (Hall *et al.*, 1985; Katsuta *et al.*, 2006). There is a significant negative correlation between the percentage of NK cells and total serum IgE levels and disease activity in AD (Lever *et al.*, 1985; Wehrmann *et al.*, 1990). NK cells have been shown to be functionally defective in AD as observed by the MHC-nonrestricted cytotoxicity against standard NK-sensitive target cells, and reduced release of IFN-γ (Katsuta *et al.*, 2006). Decreased NK activity in AD patients may contribute to their skin susceptibility to infection.

Invariant natural killer T (iNKT) cells are a unique subset of T cells that recognize glycolipid antigens in the context of the antigen-presenting molecule CD1d. iNKT cells display features of T cells and NK cells, use a limited set of *TCRα* and *TCRβ* genes, and rapidly release Th1 and Th2 cytokines following antigen recognition (Kronenberg, 2005). iNKT cells have been reported to play an important role in asthma (Meyer *et al.*, 2008). However, iNKT cells were not found to be important in the EC sensitization model of AD (Elkhal *et al.*, 2006). The percentages of the $Vα24^+CD161^+$ NKT cell subset, which produces large amounts of IL-4 and IFN-γ, but not of $CD3^+CD16^+CD56^+$ NKT cells are significantly lower in patients with AD than healthy individuals (Ilhan *et al.*, 2007; Takahashi *et al.*, 2003). The significance of this observation is not clear.

AD skin lesions are prominent for their lack of detectable PMNs, even in the setting of intense scratching or *S. aureus* colonization and/or infection. A PMN chemotactic defect in AD patients has been suggested and such a defect was found to correlate with markers of AD disease severity, serum IgE levels, and skin bacterial infection. The lack of PMNs may also be due to reduced production of PMN chemoattractants such as hCAP18/LL-37, which acts through the *N*-formyl-methyl-leucyl-phenylalanine (FMLP) receptor and reduced expression of IL-8 in AD skin. PMN functions are impaired in AD patients especially during the course of an infectious period (Ternowitz and Herlin, 1986). These include impaired phagocytosis, reduced capacity to produce reactive oxygen species, impaired release of β-glucuronidase, defective leukotriene B4 production and release, absent deposition of extracellular PMN granule proteins such as lactoferrin and PMN elastase in skin biopsies with normal serum elastase levels, and decreased CD11b-upregulation response to both activating stimulation (IL-8 and GRO-α) and priming (GM-CSF) stimuli (Bankova *et al.*, 2007). The inability to attract PMNs to the skin and the functional defects in PMNs may both contribute to the susceptibility of AD skin to infection.

7. ANIMAL MODELS OF AD

Our understanding of human diseases has been enormously expanded by the use of animal models, because these models allow in-depth investigation of cellular and molecular mechanisms underlying disease pathogenesis and provide invaluable tools for diagnostic and pharmaceutical purposes. Because there is no satisfactory therapy for AD, understanding its pathogenesis through the study of animal models is a pressing need in order to develop strategies for better prevention and treatment. Although species other than mouse, for example dogs and guinea pigs, can develop AD-like lesions, mouse models have been primarily used because of their ease of manipulation, low cost, and most importantly, because of the availability of genetically manipulated mice strains. Since the description of the Nc/Nga mouse as the first spontaneously occurring model of AD in 1997 (Matsuda *et al.*, 1997), a number of mouse models have been developed over the past two decades. These models can be categorized into three groups: (1) mice that spontaneously develop AD-like skin lesions, (2) genetically engineered models, and (3) models induced by EC application of sensitizers. These models display many features of human AD and their study has resulted in a better understanding of the pathogenesis of this disease.

7.1. Spontaneous mouse models of AD

7.1.1. Nc/Nga mice

Nc/Nga mice were established in 1957 as an inbred mouse strain in Japan and were the first reported mouse model of AD (Matsuda *et al.*, 1997). Nc/Nga mice kept under conventional conditions develop skin lesions that closely mimic those of human AD. Skin lesions fail to develop when mice are kept under specific pathogen-free (SPF) conditions. This strongly suggests that exposure to environmental aeroallergens is necessary to provoke skin inflammation. This is supported by the observation that the development of AD-like lesions in Nc/Nga mice is accelerated by application of superantigens and/or antigens to the skin (Fig. 3.9A). Scratching behavior in Nc/Nga mice kept under conventional conditions occurs at 6–8 weeks and is followed by rapidly developing erythematous, erosive lesions with edema and hemorrhage on the face, ears, neck, and back. Histological examination shows dermal infiltration with eosinophils and mononuclear cells (Fig. 3.9B). These changes are observed prior to the appearance of clinical skin manifestations. Hyper-/parakeratosis, hyperplasia, and spongiosis are observed in the skin lesions at the age of 17 weeks. Nc/Nga mice kept under conventional conditions, but not under SPF conditions, show skin barrier abnormalities with increased

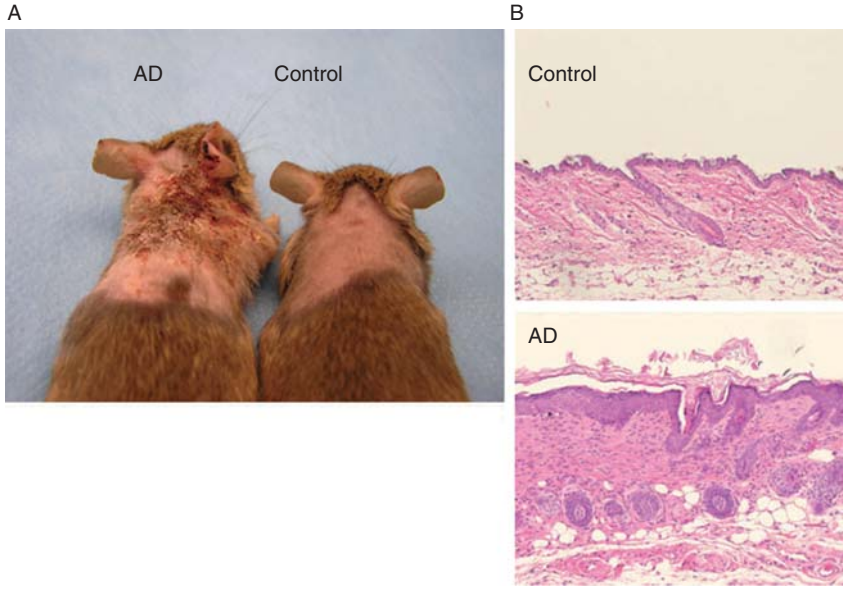

FIGURE 3.9 Nc/Nga mice. (A) Gross appearance. Comparison of skin lesions between AD-induced (left) and noninduced control mice (right). (B) Hematoxylin and eosin (H&E) staining of AD induced skin and control skin (magnification ×100).

TEWL and impaired ceramide metabolism, suggesting that abnormal skin barrier function is secondary to the development of skin inflammation (Aioi *et al.*, 2001). As in human AD, scratching plays an important role in determining the severity of dermatitis in Nc/Nga mice (Hashimoto *et al.*, 2004). Skin severity score, TEWL, number of mast cells, and serum IgE concentration statistically increased in Nc/Nga mice with toenails but not in Nc/Nga mice with clipped toenails after dermatitis induction (Hashimoto *et al.*, 2004). Furthermore, ablation of sensory fibers delays and reduces the severity of the skin disease in Nc/Nga mice (Mihara *et al.*, 2004).

Genetic analysis of Nc/Nga mice has demonstrated that the mutation responsible for the Nc/Nga phenotype is on chromosome 9 at the derm1 locus. Several functional candidate genes are located near this locus. These candidates are *Thy1*, *CD3δ*, *CD3ε*, *CD3γ*, *IL-10Rα*, *IL-18*, and *Csk*, all of which could be involved in allergic responses through effects on T-cell function. Of these candidates, *Csk* is the strongest, since its position is the most tightly linked to the derm1 locus (Kohara *et al.*, 2001).

Along with the skin changes, Nc/Nga mice exhibit preferential Th differentiation toward Th2 cells in the spleen, dense accumulation of eosinophils and mast cells in the skin lesions, and an increased serum

level of total IgE (Kohara *et al.*, 2001) (see also Vestergaard *et al.*, 1999). Moreover, the Th2-specific chemokines, CCL17/TARC and CCL22/MDC, and their receptor, CCR4, are highly expressed in the lesions of the Nc/Nga mice (Vestergaard *et al.*, 1999). These findings strongly suggest the possible involvement of Th2 cells in the development of AD-like skin lesions in the Nc/Nga mice. Constitutive and enhanced tyrosine phosphorylation of Janus kinase 3, a tyrosine kinase involved in IL-4R and CD40 signaling, is thought to be involved in the enhanced sensitivity of B cells from Nc/Nga mice to IL-4 and CD40L, leading to elevation of total IgE levels (Matsumoto *et al.*, 2004). However, STAT6-deficient Nc/Nga mice exhibit comparable skin changes with infiltration by T cells and eosinophils as STAT6-positive Nc/Nga littermates, but with undetectable serum level of IgE, suggesting that AD-like skin changes in Nc/Nga mice can develop independently of Th2 cells and IgE (Yagi *et al.*, 2002). The DLNs of lesional skin in STAT6-deficient Nc/Nga mice exhibit massive enlargement elicited by the accumulation of activated IFN-γ secreting T cells. Moreover, CASP1, IL-18, IL-12, and IFN-γ are found to be highly expressed in the skin lesions of STAT6-deficient Nc/Nga mice. There is also elevation of eotaxin 2 and CCR3 expression, possibly driven by IFN-γ. IFN-γ production in lesions of STAT6-deficient Nc/Nga mice could be driven by a defect in the innate immunity of Nc/Nga mice. In this regard, the *IL-18* gene near the locus is responsible for skin disease in Nc/Nga mice on chromosome 9. Given the fact that expression of skin specific IL-18, IL-33, or TSLP transgenes results in the development of AD skin lesions (see Section 7.2), it is possible that an abnormal innate reaction with increased CASP1, IL-18, and IL-12 expression in the skin microenvironment of Nc/Nga mice may result in increased IFN-γ production, leading to the induction of eotaxin 2 expression that acts as a chemoattractant for CCR3 expressing eosinophils. Generation of Rag2-, CASP1-, IL-18-, and IFN-γ deficient Nc/Nga mice is needed to answer these questions.

7.1.2. DS-nonhair (DS-Nh) mice

The DS-Nh mice is a spontaneous hairless inbred strain mutant of the DS mouse established in 1976. DS-Nh mice develop spontaneous dermatitis and elevated serum IgE levels under conventional conditions, and less commonly under SPF conditions (Watanabe *et al.*, 2003). Histopathological examination of the skin lesions reveals inflammatory cells consisting of mast cells, eosinophils, $CD4^+$ T cells and $CD11b^+$ macrophages, and a Th2 cytokine expression (Hikita *et al.*, 2002). The severity of the dermatitis correlates with the serum level of total IgE (Hikita *et al.*, 2002). IgE hyperproduction is regulated by the Nh mutation, while other genetic factor(s) are also involved in the development of dermatitis. Interestingly, heavy colonization of *S. aureus* was found in the skin lesions. Moreover, skin

application of heat-killed *S. aureus* to DS-Nh mice induced similar dermatitis, suggesting that these mice could be a good model for *S. aureus* associated AD (Haraguchi *et al.*, 1997). However, no data was obtained on specific IgE sensitization. In addition, total IgE levels and TEWL were detected only after development of skin lesions, suggesting that, like in Nc/Nga mice, these features are secondary responses rather than primary pathogenic factors.

7.1.3. Naruto Research Institute Otsuka (NOA) mice

Naruto Research Institute Otsuka (NOA) mice exhibit hair loss and pruritic ulcerative dermatitis with mast cell accumulation in the dermis, and high serum level of IgE; however, they lack lymphocyte infiltration in the skin and classical histological characteristics of human AD (Watanabe *et al.*, 1999). Because NOA mice display only specific aspects of cutaneous inflammation found in AD, they may serve as useful model to study these specific aspects but not as an ideal model of AD.

7.1.4. Flaky tail (*ft*) mice

Flaky tail (*ft*) is a spontaneously arising autosomal recessive mutation in mice located at chromosome 3, within the EDC, and is in close linkage with another mutation (*ma*) that causes hair matting (Fig. 3.10A). ft/ft

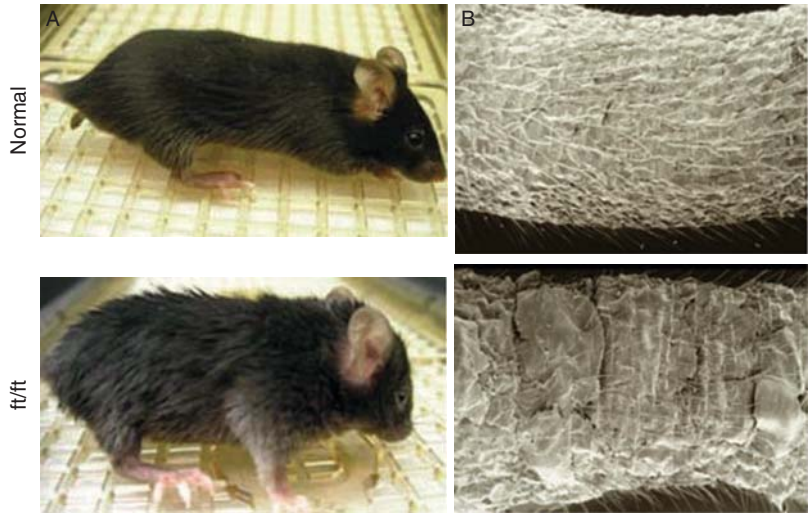

FIGURE 3.10 Flaky tail (*ft*) mice. (A) Gross appearance of normal and ft/ft mice (B) Electron micrographs from the tails of normal and *ft/ft* mice. Control mice (C57BL6) has regularly organized scales, whereas ft/ft mice have disorganized, desquamating scales with some lifting off the epidermis. (B; modified from Presland *et al.* (2000). *J. Invest. Dermatol.* **115,** 1072–1081.)

mice have dry, flaky skin (Fig. 3.10B), annular tail, and paw constrictions in the neonatal period. The skin of ft/ft mice exhibits marked attenuation of the epidermal granular layer, mild acanthosis, and hyperkeratosis (Presland *et al.*, 2000). The skin of ft/ft mice expresses an abnormal profilaggrin that is not processed to filaggrin monomers, resulting in the lack of normal keratohyalin F-granules. Filaggrin is absent from the cornified layers of ft/ft epidermis; however, the expression of epidermal keratins is unchanged and the cornified envelope proteins involucrin and loricrin are increased in ft/ft epidermis. Keratinocytes from ft/ft mice express reduced amounts of profilaggrin mRNA and protein, suggesting that the defect in profilaggrin expression is intrinsic to epidermal cells. These results suggest that absence of filaggrin underlies the dry, scaly skin characteristic of ft/ft mice. As loss-of-function mutations in *FLG* gene is a major risk factor for AD, ft mice may provide an important tool for understanding the role of filaggrin in human skin barrier dysfunction including ichthyosis vulgaris and AD.

7.2. Genetically engineered mouse models of AD

7.2.1. IL-4 transgenic mice

Transgenic mice overexpressing an IL-4 transgene driven by the keratin-14 (K14) promoter in skin keratinocytes develop spontaneous pruritus and chronic progressive dermatitis at the age of 4 months (Chan *et al.*, 2001). The disease in these mice reproduces all key features of human AD, including xerosis, conjunctivitis, inflammatory skin lesions, *S. aureus* infection, histopathology of chronic dermatitis with T cell, mast cell, macrophage-like mononuclear cell, and eosinophil infiltration, and elevation of total serum IgE and IgG1. The onset and early progression of skin inflammation was found to correlate with elevation of serum IgE and IgG1 levels. Early skin lesions are characterized by prominent infiltration of T cells in the epidermis and dermis, whereas chronic skin lesions show T cell accumulation in the dermis. As the skin disease evolves, skin DLN cells from IL-4-transgenic mice show spontaneous proliferation and progressively enhanced proliferative response to stimulants including anti-CD3, ConA, PHA, SEA, and SEB. Furthermore, the percent of lymphoid organ T cells expressing activation molecules (CD44 and CD69) and costimulatory molecules (ICOS and PD-1) progressively increases. In addition, the percent and total number of T cells become reduced in the secondary lymphoid organs while the number of T cells infiltrating the skin increases (Chen *et al.*, 2005). There is also a progressive increase in blood vessel number and diameter and in mRNA and protein expression of mRNAs for the angiogenic factor VEGF-A, but not VEGF-B, VEGF-C, or VEGF-D in the skin of transgenic mice (Chen *et al.*, 2008a).

In an attempt to develop an AD mouse model that has a skin structure closer to that of the human skin, which is relatively hairless in the flexural areas where AD lesions predominate, IL-4-transgenic mice were crossed with SKH1 hairless mice. The resulting hairless IL-4-transgenic mice developed an inflammatory skin disease like that of the normally haired IL-4-transgenic mice; however, total serum IgE and IgG1 levels remained normal compared with nontransgenic littermates. Further examinations indicated impaired CD40L upregulation on helper T cells as the cause for the inability to increase IgE production. The SKH1 hairless IL-4-trasngenic mouse line could be a useful model to study IAD (Chen *et al.*, 2008b).

7.2.2. IL-13 transgenic mice

IL-13 has been implicated in the pathogenesis of AD and its expression is increased in the patients with AD (Hamid *et al.*, 1996). Transgenic mice were made to overexpress a tetracycline-inducible, skin-specific IL-13 by the K5 promoter (Zheng *et al.*, 2008). These mice exhibit signs of dermatitis including xerosis, pruritus, loss of hair, erythema, crusting, excoriation, bacterial pyoderma, and erosions in the skin at 6–8 weeks after doxycycline (dox) treatment, and progress as IL-13 induction continued. Histological examination of skin lesions showed dermal infiltration of $CD4^+$ T cells, mast cells, eosinophils, macrophages, and LC and skin remodeling with fibrosis and increased vasculature. Skin lesions of IL-13 transgenic mice exhibit a Th2 cell profile with upregulation of chemokine and cytokine genes, including TSLP, CCL11/Eotaxin, CCL17/TARC, CCL27, MCP-1, CCL22/MDC, RANTES, and TGFβ-1. These mice also show elevated serum total IgE and IgG1 and increased production of IL-4 and IL-13 by $CD4^+$ T cells from lymphoid tissues and peripheral blood mononuclear cells. These findings indicate that dermal expression of IL-13 is sufficient to cause a chronic inflammatory response in the skin and systemic evidence of Th2 bias that remarkably resembles human AD.

7.2.3. IL-31 transgenic mice

Transgenic mice overexpressing IL-31 driven by the lymphocyte-specific promoter Lck or the ubiquitous elongation factor-1α promoter, exhibit signs of dermatitis at age of 2 months, including pruritus, mild to moderate hair loss, and considerable thickening of ear skin (Dillon *et al.*, 2004). These symptoms progress with age, and reach a peak at age of 6 month. Histological examination of skin lesions revealed hyperkeratosis, acanthosis, inflammatory cell infiltration, and an increase in number of mast cells, which resemble the skin lesions of human AD. However, these mice exhibit normal serum concentrations of IgE.

7.2.4. CASP1 and IL-18 transgenic mice

IL-18 is a unique proinflammatory cytokine capable of strongly stimulating both IFN-γ and IL-4 production when it acts on freshly isolated T cells with IL-12 and IL-2, even in the absence of T cell antigen receptor engagement. IL-18 in the presence of IL-3 directly stimulates basophils and mast cells to produce IL-4, IL-5, and IL-13 cytokines in an IgE-independent manner *in vitro* (Nakanishi *et al.*, 2001). Administration of IL-18 to normal BALB/c or C57BL/6 mice induces polyclonal IgE production in a $CD4^+$ T cell-, STAT6-, and IL-4-dependent manner (Okamura *et al.*, 1995). IL-18 is expressed in AD skin and SNPs in the *IL-18* gene are associated with AD (Kim *et al.*, 2007b). Like IL-1β, IL-18 is stored as a biologically inactive precursor in various cell types, including macrophages and keratinocytes, and becomes active after cleavage with CASP1. Transgenic mice overexpressing the human CASP1 precursor gene in epidermal keratinocytes under the control of the human K14 promoter (CASP1Tg mice) showed by age 8 weeks elevated serum levels of IgE and IgG1, mild pruritic dermatitis around the eyes and ears at 16 weeks (Yamanaka *et al.*, 2000). Histological examination showed acanthosis, papillomatosis, hyperparakeratosis, and intracellular edema with dense infiltration of lymphocytes, PMNs and mast cells, but not eosinophils in the skin lesion. CASP1Tg mice on STAT6 deficient background still suffered from chronic dermatitis similar to that observed in CASP1Tg littermates, but with no detectable IgE production, suggesting a dispensable role of IgE in the development of the AD-like dermatitis in CASP1Tg mice (Konishi *et al.*, 2002). High concentration of mature IL-18 was found in the serum of CASP1Tg mice. IL-18-deficient CASP1Tg mice evaded the dermatitis, confirming the critical role of IL-18 in the CASP1 mouse model of AD and suggesting that IL-18 causes the skin changes in the absence of IgE and STAT6 (Konishi *et al.*, 2002). Because of this and there is no obvious need for allergen exposure to develop the dermatitis in this model, it has been proposed that the CASP1 mouse model may mimic intrinsic AD in which also elevated levels of serum IL-18 can be found.

Transgenic mice overexpressing murine mature IL-18 under the control of the human K14 promoter exhibited similar skin changes, but a delayed disease onset when compared to CASP1Tg mice (Konishi *et al.*, 2002). IL-1α/β deficient CASP1Tg mice exhibited similar dermatitis but at a later stage of around 6 months, suggesting a role for IL-1α/β in accelerating the dermatitis initiated by IL-18 locally released in the skin (Konishi *et al.*, 2002). Both CASP1Tg and IL-18Tg mice had spontaneous deviation of splenic T cells to Th2 and away from Th1, with increased production of IL-4 and IL-5 and decreased IFN-γ production in response to anti-CD3 stimulation. In addition, both Tg mice had elevated serum IgG1 as well as IgE levels. As importantly, the number of skin mast cells,

the levels of histamine in the plasma and the frequency of skin scratching were elevated in CASP1Tg and IL-18Tg mice as observed in AD.

7.2.5. Cathepsin E (Cat E) knockout mice

The aspartic proteinase Cat E is a major intracellular aspartic protease which is predominantly present in the cells of the immune system and is implicated in antigen processing via the MHC class II pathway (Zaidi and Kalbacher, 2008). Cat E is localized mainly in the endosomal structures of APCs and has been implicated in a variety of immune responses. Under conventional conditions, but not under SPF conditions, Cat $E^{-/-}$ mice develop pruritic and erosive skin lesions, from which *S. aureus* is cultured (Tsukuba *et al.*, 2003). Serum level of total IgE is elevated and secretion of Th2 cytokines by splenocyte *in vitro* is increased, whereas the production of IFN-γ and IL-2 is normal. Histological examination shows epidermal hyperplasia and dermal infiltration with eosinophils, lymphocytes, and macrophages. The relative rates of degradation of IL-18 and IL-1β are significantly lower in Cat $E^{-/-}$ mice than WT mice, suggesting the development of AD in Cat $E^{-/-}$ mice may be initiated by systemic accumulation of IL-18 and IL-1β, due to their reduced turnover rates, leading to stimulation of Th2 responses (Tsukuba *et al.*, 2003). Cat E deficiency markedly decreases the ability of macrophages to present intact ovalbumin (OVA) to cognate T cells, while that of DCs was enhanced due to increased phagocytic activity and increased expression of the costimulatory molecules CD86, CD80, and CD40 (Kakehashi *et al.*, 2007). In addition, Cat E deficiency downregulates the expression of TLRs, resulting in increased predisposition to infection (Tsukuba *et al.*, 2006). Increased microbial load and enhanced presentation of antigens by skin DCs may contribute to the development of allergic skin inflammation in Cat $E^{-/-}$ mice.

7.2.6. RelB knockout mice

RelB belongs to the NFκB/Rel family of transcription factors, which play critical roles in stress-induced, immune, and inflammatory responses. In adult mice, RelB expression is restricted to lymphoid tissues. $RelB^{-/-}$ mice exhibit hematopoietic abnormalities and mixed inflammatory cell infiltration in several organs, including skin (Barton *et al.*, 2000) (see also Weih *et al.*, 1997). These mice develop spontaneous dermatitis, hyperkeratosis, skin infiltration with $CD4^+$ T cells and eosinophils and elevated serum IgE, all features of human AD, although pruritus was not reported. When crossed with nur77 transgenic mice in which peripheral T cells are absent, $RelB^{-/-}$ mice exhibited attenuated dermatitis with reduced epidermal hyperplasia and keratinocyte proliferation, suggesting that skin inflammation in these mice is T cell dependent. RelB plays an important role in negative selection by controlling the generation of $AIRE^+$

medullary epithelial cells (Heino *et al.*, 2000). Thus, autoreactive T cells may play a role in the dermatitis in RelB$^{-/-}$ mice as they do in the dermatitis of Foxp3$^{-/-}$ mice (see below) and of patients with Foxp3 deficiency (IPEX).

7.2.7. Foxp3 knockout mice

$CD4^+Foxp3^+$ Treg cells play a critical role in maintaining tolerance against self-antigens and harmless environmental antigens and maintain immune homeostasis in the normal skin (Akdis *et al.*, 2006b; Dudda *et al.*, 2008). The number of $CD4^+CD25^+FoxP3^+$ Treg cells is decreased in lesional skin of patients with AD or psoriasis (Verhagen *et al.*, 2006), suggesting that impaired infiltration with $CD4^+CD25^+FoxP3^+$ Treg cells may cause dysregulation of effector T cells in lesion of AD patients. Scurfy (*sf*) is a spontaneous, recessive mutation that involves a 2-bp insertion in the *Foxp3* gene (Brunkow *et al.*, 2001; Godfrey *et al.*, 1991), which causes a frame-shift and premature translational termination. Consequently, the differentiation and function of Treg cell are blocked. *Sf* mice spontaneously develop multiorgan autoinflammatory disease that is fatal by 3–4 weeks of age. Gross lesions include marked splenomegaly, hepatomegaly, enlarged lymph nodes, and dermatitis with variable thickening of the ears. The characteristic histologic lesion is a lymphohistiocytic proliferation and infiltration of peripheral lymph nodes, spleen, liver, and skin (Godfrey *et al.*, 1991). Importantly, the *sf* phenotype was reproduced by target mutagenesis in *Foxp3* gene (Fontenot *et al.*, 2003; Lin *et al.*, 2005). Foxp3 deficient mice developed an intense multiorgan inflammatory response associated with allergic airway inflammation, a striking hyperimmunoglobulinemia E, eosinophilia, and dysregulated Th1 and Th2 cytokine production as well as scaly and inflamed skin (Fig. 3.11A). The skin is severely affected, displaying lymphohistiocytic infiltration of the dermis, along with epidermal thickening and erosion (Fig. 3.11B). Raising Foxp3 deficient mice in SPF condition does not alter disease expression, suggesting that disease may be the result of immune dysfunction caused by the lack of immune regulation by Treg cells.

7.2.8. TSLP transgenic mice

TSLP is highly expressed in the skin lesions of patients with AD (Soumelis *et al.*, 2002). Mice on BALB/c background were made to overexpress a tetracycline-inducible, skin-specific TSLP under the control of the K5 promoter (Yoo *et al.*, 2005). Skin erythema occurred at ~2–3 week of dox treatment, and progressed to AD-like changes, including erythema, mild xerosis, crusting, and erosions at 3–4 weeks. Histological examination of skin lesions showed changes similar to those observed in human AD, including hyperkeratosis, spongiosis, and dermal infiltration with lymphocytes and macrophage and an abundance of mast cells and

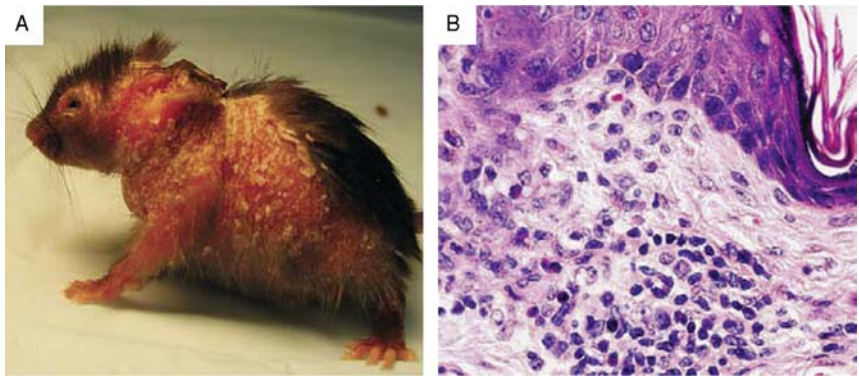

FIGURE 3.11 Foxp3 deficient mice. (A) A 5-week-old Foxp3$^{-/-}$ mouse on the C57/BL6 background shows intense dermatitis with scaling and fur loss. (B) H&E staining of the skin of Foxp3$^{-/-}$ mice shows inflammation with a prominent eosinophilic component (original magnification ×400). (Modified from *Lin et al.* (2005). *J. Allergy Clin. Immunol.* **116,** 1106–1115.)

eosinophils. Skin lesions of TSLP transgenic mice exhibited a Th2 cell profile with upregulation of IL-4, IL-5, and TNF-α. These mice also showed elevated serum levels of IgE and IgG1, and decreased IgG2a. When crossed with TCRβ$^{-/-}$ mice that lack T cells, TSLP transgenic mice still developed AD-like skin changes with a dense accumulation of mast cells and eosinophils in the dermis, suggesting that skin inflammation in these mice is T cell independent.

7.2.9. MAIL/IκBζ knockout mice

A molecule-possessing ankyrin repeats induced by LPS (MAIL) is a nuclear IκB protein that is also termed inhibitor of NFκB zeta (IκBζ). MAIL mRNA and protein is constitutively expressed in keratinocytes. Mail$^{-/-}$ mice develop by 4–8 weeks of age lesions in the skin of the periocular region, face, and neck and elevated serum IgE (Shiina *et al.*, 2004). Mail$^{-/-}$ skin lesions exhibit inflammatory cell infiltration and elevated expression of the chemokine similar to skin lesions of patients with AD. MAIL-deficient keratinocytes might be activated to produce chemokines and TSLP to induce intraepidermal filtration of inflammatory cells, resulting in the onset of the AD-like disease.

7.2.10. SCCE transgenic mice

SCCE, which belongs to the kallikrein group of serine protease, is preferentially expressed in cornifying epithelia. SCCE activity is increased in patients with Netherton's syndrome (SPINK5 deficiency) (Descargues *et al.*, 2006), suggesting that SCCE participates in the premature

degradation of corneodesmosomal cadherins in the upper most living layers of the epidermis. SCCE expression is increased in chronic lesions of AD as well as in psoriasis lesions (Ekholm and Egelrud, 1999). Overexpression of a human SCCE transgene in suprabasal epidermal keratinocytes of mice led to the development of AD-like skin inflammation characterized by increased epidermal thickness, hyperkeratosis, and dermal inflammation starting at the age of 7–8 weeks or older (Hansson *et al.*, 2002). This inflammation may be due to increased access of environmental antigens into a hypermeable skin due to disrupted cadherin mediated tight junctions. Itching develops later at age of 10–11 weeks in SCCE transgenic mice, suggesting that pruritus is secondary to the inflammatory changes in the skin, rather than a direct effect of SCCE. Histamine antagonists fail to alleviate the scratching behavior in SSCE transgenic mice suggesting that histamine is not the cause of pruritus in these mice.

7.2.11. Apolipoprotein C1 (APOC1) transgenic mice

APOC1 is an apolipoprotein involved in lipoprotein metabolism (Jong *et al.*, 1998). In healthy individuals, APOC1 is predominantly expressed in liver, skin, and brain tissue with macrophages and keratinocytes as major cell types. The protein is highly conserved and a high degree of homology exists between APOC1 in mice and man. Mice transgenic for human APOC1 (APOC1Tg mice) in liver and skin have increased levels of free fatty acids, cholesterol, and triglycerides, but show complete absence of subcutaneous fat and atrophic sebaceous glands. The composition of the SC is dependent on lipid homeostasis. APOC1Tg mice have not only have disturbed serum levels of lipids but display a disturbed skin barrier function, evident from increased TEWL. These mice spontaneously develop with age severe dermatitis with moderate epidermal hyperplasia and hyper-/parakeratosis, scaling, lichenification, excoriations, and pruritus. Histological analysis shows increased epidermal thickening and spongiosis in conjunction with elevated numbers of inflammatory cells including eosinophils, PMNs, mast cells, macrophages, and $CD4^+$ T cells, in the dermis (Nagelkerken *et al.*, 2008). In addition, affected mice have increased serum levels of IgE and show abundant mast cells in the dermis. Partial inhibition of disease could be achieved by restoration of the skin barrier function with topical application of a lipophilic ointment. Furthermore, the development of AD in these mice was suppressed by topical treatment with corticosteroids or calcineurin inhibitors, for example, tacrolimus. These findings underscore the role of skin barrier integrity in the pathogenesis of AD.

Relevant findings from genetically engineered mouse models of AD described above are summarized in Table 3.1.

TABLE 3.1 Genetically engineered mouse models of AD

Strain	Gene expression	Age develop AD	Skin histology	Skin infiltration	Skin cytokines/ chemokines	Systemic response	Others	References
IL-4 transgenic	Keratinocyte-specific (K14 promoter)	4 months	Xerosis Conjunctivitis Inflammatory skin lesions *S. aureus* infection*	Eosinophils Macrophages Mast cells T cells	IL-4 CCL27	Increased serum IgE, IgG1 Decreased serum IgG2a	Skin lesions develop both under SPF conditions and conventional conditions	Chan *et al.* (2001)
IL-13 transgenic	Keratinocytes-specific (Tetracycline-inducible K5 promoter)	6–8 weeks after doxycycline treatment	Xerosis Pruritus Hair loss Erythema Crusting Excoriation Bacterial pyoderma Erosions Lichenified skin lesions Skin remodeling (fibrosis and increased vasculature)	$CD4^+$ T cells Mast cells Eosinophils Macrophages LCs	TSLP Eotaxin CCL17/TARC CCL27 MCP-1 CCL22/MDC RANTES TGFβ-1	Increased serum IgE, IgG1 Increased production of IL-4 and IL-13 by $CD4^+$ T cells (from lymphoid tissue and PBMCs)		Hamid *et al.* (1996)
IL-31 transgenic	Lymphocyte-specific (Eμ-Lck promoter) or ubiquitous (EF1α promoter)	2 months	Pruritus Thickening Hair loss Conjunctivitis Hyper-/ parakeratosis Acanthosis	Mast cells		Normal levels of IgE, IgG		Dillon *et al.* (2004)

Caspase-1 (CASP1) transgenic	Keratinocyte-specific (Human K14 promoter)	8 weeks	Pruritus Erosive skin Acanthosis Papillomatosis Hyper-/ parakeratosis Spongiosis	Lymphocytes PMNs Mast cells	IL-18 IL-1β	Increased serum IgE, IgG1 Increased neutorophils in spleen Increased spleen IL-4, IL-5 Increased serum IL-18 Increased serum IL-1β Increased plasma histamin		Yamanaka *et al.* (2000)
IL-18 transgenic	Keratinocyte-specific (Human K14 promoter)	6 months under SPF conditions	Similar to CASP1 transgenics with marked lichenification	Lymphocytes Neutrophils Mast cells		Increased serum IgE, IgG1 Increased neutorophils in spleen Increased serum IL-18 Increased plasma histamine Increased IL-4, IL-5	Delayed disease onset compared to CASP1 transgenic mice	Konishi *et al.* (2002)
Cathepsin E knockout		14 weeks under conventional conditions	Pruritus Erosion Erythema Crusting Alopecia Epidermal hyperplasia after *S. aureus* infection	Lymphocytes Eosinophils Macrophages		Increased serum IgE Increased spleen IL-4 and IL-5 Increased serum IL-18 Increased serum IL-1β		Tsukuba *et al.* (2003)

(*continued*)

TABLE 3.1 (*continued*)

Strain	Gene expression	Age develop AD	Skin histology	Skin infiltration	Skin cytokines/ chemokines	Systemic response	Others	References
RelB knockout		4–10 weeks	Epidermal hyperplasia Hyper/para-keratosis Acanthosis Thickening Hair loss	$CD4^+$ T cells Eosinophils Granulocytes Mast cells Eosinophils	IL-4 IL-5 IFN-γ CCL11/ Eotaxin CCR3 TGF-β IL-1β	Increased serum IgE	Skin lesions are T cell dependent	Weih *et al.* (1997) Barton *et al.* (2000)
Foxp3 knockout		~2 weeks	Thickening Erosion Scaring Hair loss			Increased serum IgE, IgG1 Decreased serum IgG2a Increased serum IFN-γ Increased serum TNF-α Increased serum IL-4, IL-5, IL-10 Increased spleen IL-4, IL-5, IL-13	Intense multiorgan inflammatory response associated with allergic airway inflammation, hyper IgE, eosinophilia, and dysregulated Th1 and Th2 cytokine production	Fontenot *et al.* (2003) Lin *et al.* (2005)
TSLP transgenic	Keratinocyte-specific (tetracycline-inducible K5 promoter)	At 2–3 weeks of doxycycline treatment	Erythema Xerosis Crusting Erosion Hyper-/ parakeratosis Spongiosis Acanthosis	$CD4^+$ T cells Mast cells Eosinophils	IL-4 IL-5 TNF-α	Increased serum IgE, IgG1 Decreased serum IgG2a IL-4-producing CD4 T cells	Skin lesions are T cell independent	Yoo *et al.* (2005)

MAIL/IκBζ knockout		4–8 weeks	Scaring Thickening Alopecia Pruritus Erosion Hyper-/parakeratosis Acanthosis Partial spongiosis	Lymphocytes Melanophages PMNs Mast cells Eosinophils	Increased serum IgE	Shiina *et al.* (2004)
SCCE transgenic	Keratinocytes-specific (SV40 enhancer and promoter)	7–8 weeks	Epidermal hyperplasia Thickening Hyper-/parakeratosis Acanthosis	Lymphocytes Mast cells		Hansson *et al.* (2002)
APOC1 transgenic	Liver and skin-specific (APOC-1 promoter)	2 months	Epidermal hyperplasia Thickening Hyper-/parakeratosis Hyperlipidemia Scaring Hair loss Spongiosis TEWL	Eosinophils PMNs Mast cells Macrophages $CD4^+$ T cells	Increased serum IgE Increased serum cholesterol, triglycerides, free fatty acids No subcutaneous fat No atrophic sebaceous glands	Jong *et al.* (1998) Nagelkerken *et al.* (2008)

* Only under conventional conditions.

SCCE, stratum corneum chymotryptic enzyme; APOC1, apolipoprotein C1, TSLP, thymic stromal lymphopoietin; PBMC, peripheral blood mononuclear cells; LCs, Langerhans cells; DCs, dendritic cells.

7.3. AD models induced by EC sensitization with antigen hapten or superantigen

7.3.1. AD model induced by skin injury and EC sensitization with antigen

Our laboratory has developed a mouse model of AD induced by repeated EC sensitization of tape-stripped skin with OVA (Fig. 3.12) (Spergel *et al.*, 1998). EC sensitized mice develop increased scratching behavior and skin lesions characterized by epidermal and dermal thickening, infiltration of $CD4^+$ T cells, and eosinophils (Fig. 3.13A) and upregulated expression of the Th2 cytokines IL-4, IL-5, and IL-13 (Fig. 3.13B) with little or no change in the expression of IFN-γ. There is enhanced expression of CCL11/eotaxin and CCL17/TARC, the chemokines that respectively attract $CCR3^+$ eosinophils and skin homing $CCR4^+CD4^+$ T cells. There is also increased deposition of collagen. Systemically, serum OVA-specific IgG1, IgE, and IgG2a are elevated, and splenocytes from OVA-sensitized mice produce increased level of IL-4, IL-5, IL-13, and IFN-γ in response to OVA stimulation (Fig. 3.13C) (Spergel *et al.*, 1998). The fact that antigen specific IFN-γ producing cells are present in the spleen, with no detectable upregulation of IFN-γ expression in sensitized skin sites suggest that local factors at the site of sensitization promote selectively the activation of Th2 cells. In this respect, TSLP promotes the secretion of Th2 cytokines with no detectable effect on the secretion of Th1 cytokines by TCR-OVA

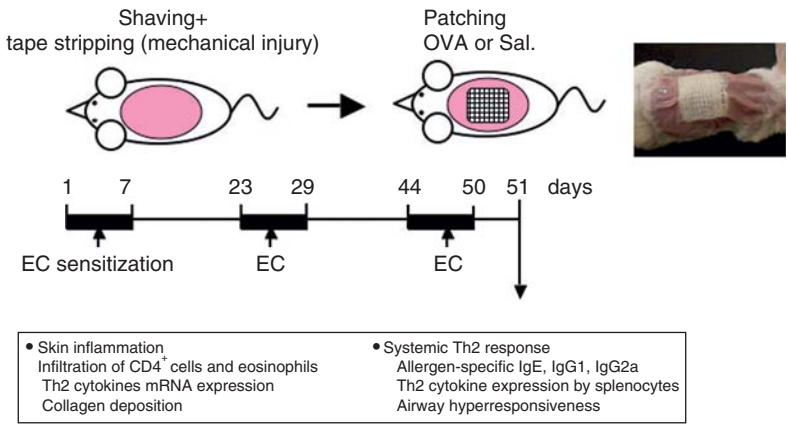

FIGURE 3.12 EC sensitization protocol to elicit allergic skin inflammation. A mouse model of AD. Mice are sensitized with OVA (100 μg) or saline applied in 100 μl to a sterile patch. The patch is placed for a 1-week period and then removed. Two weeks later, an identical patch is reapplied to the same skin site. Each mouse has a total of three 1-week exposures to the OVA patch separated from each other by 2-week intervals. All experiments are performed at the end of the third sensitization.

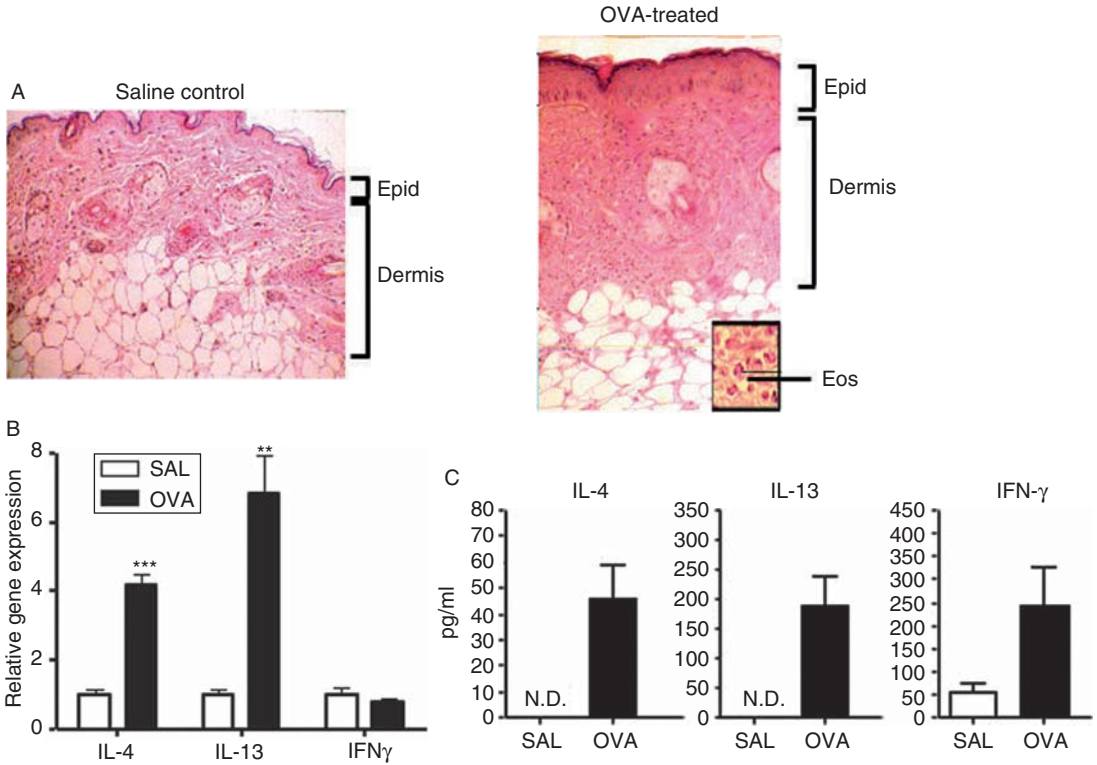

FIGURE 3.13 Characteristics of EC sensitization model of allergic skin inflammation: (A) Histological features of OVA and saline-sensitized skin sites in BALB/c mice. EC sensitization induces allergic skin inflammation. Skin sections were stained with H&E and examined at original magnifications ×200 and ×400. There is marked hyperplasia of the epidermis, a dermal infiltrate, and mild spongiosis. The cellular infiltrate consists of PMNs, eosinophils, and lymphocytes. Further magnification in the inset (bold-bordered box) shows the presence of the multiple eosinophils. (B) Th2-dominated skin mRNA cytokine expression. (C) Spleen cytokine production in response to *in vitro* stimulation with OVA.

transgenic T cells stimulated *in vitro* with OVA peptide (He *et al.*, 2008). In addition, OVA-sensitized mice develop increased airway hyperresponsiveness (AHR) following inhalation challenge with OVA, a feature observed in asthmatic patients with AD history (Spergel *et al.*, 1998). Decreasing the cycles of sensitization from three to two or compressing the duration of the sensitization protocol by decreasing the interval between the cycles of sensitization leads to suboptimal development of allergic skin inflammation. The requirement for a 7-week protocol of EC sensitization appears to mimic the exacerbation of AD over time. Withdrawal of antigen sensitization at the end of the 7-week protocol results in decreased skin inflammation with IL-4 mRNA levels returning to baseline within 7–10 days; but IL-13 mRNA levels decrease over a longer period of time.

Using RAG2$^{-/-}$ mice, which lack both T and B cells, B cell-deficient IgH$^{-/-}$ mice, T-cell receptor $\alpha^{-/-}$ mice, and CD40 deficient mice, we demonstrated that TCR$\alpha\beta^{+}$ T cells, but not $\gamma\delta^{+}$ T cells, B cells, or CD40L-CD40 interactions, are critical for skin inflammation and the Th2 response in AD (Woodward *et al.*, 2001). The study of mast cell deficient mice indicated that mast cells are not important for the development of Th2 mediated skin inflammation; however, they regulate IFN-γ expression in the skin (Alenius *et al.*, 2002). This is important, given the role of IFN-γ in upregulating Fas expression on keratinocytes targeting them for killing by activated FasL^{+} T cells (Trautmann *et al.*, 2000) and given the role of IgE-mediated reactions in exacerbating AD (Milgrom, 2002). iNKT cells are not required for allergic skin inflammation in this model (Elkhal *et al.*, 2006), suggested by the observation that skin infiltration by eosinophils and CD4^{+} cells and expression of mRNA encoding IL-4 and IL-13 in OVA-sensitized skin were similar in WT and CD1d$^{-/-}$ mice. Furthermore, no significant increase in iNKT cells was detectable in epicutaneously sensitized skin. In contrast, iNKT cells were found in the bronchoalveolar lavage (BAL) fluid from OVA-challenged epicutaneously sensitized WT mice, but not CD1d$^{-/-}$ mice, and EC sensitized CD1d$^{-/-}$ mice had decreased expression of IL-4, IL-5, and IL-13 mRNA in the lung and impaired AHR in response to airway challenge with OVA (Elkhal *et al.*, 2006).

A number of cytokines and chemokines play important roles in the development of allergic skin inflammation elicited by EC exposure to allergens (Kawamoto *et al.*, 2004; Laouini *et al.*, 2005; Ma *et al.*, 2002) (see also Spergel *et al.*, 1999). Eosinophils are virtually absent in OVA-sensitized skin sites of IL-5$^{-/-}$ mice, OVA-sensitized skin sites of IL-4$^{-/-}$ mice have increased inflammatory cells but decreased eosinophils, and those of IFN-$\gamma^{-/-}$ mice have decreased thickening of the dermal layer (Spergel *et al.*, 1999). IL-10 plays an important role in the Th2 response to antigen and in the development of skin eosinophilia (Laouini *et al.*, 2003a).

Skin infiltration by eosinophils and expression of CCL11/eotaxin, IL-4, and IL-5 mRNA in OVA-sensitized skin sites were all severely diminished in IL-$10^{-/-}$ mice. Following *in vitro* stimulation with OVA, splenocytes from EC-sensitized IL-$10^{-/-}$ mice secreted significantly less IL-4, but significantly more IFN-γ than splenocytes from WT controls. IL-$10^{-/-}$ APCs skewed the *in vitro* response of OVA TCR transgenic T cells towards Th1. Examination of the Th response of WT and IL-$10^{-/-}$ mice immunized with OVA-pulsed WT or IL-$10^{-/-}$ DCs revealed that both DCs and T cells participate in IL-10-mediated skewing of the Th2 response *in vivo*. Recent results support the hypothesis that IL-10 released by keratinocytes following mechanical injury might promote the Th2 response to EC sensitization by polarizing skin DCs to support Th2 differentiation (our unpublished observations).

CCR3 is expressed on eosinophils, mast cells, and Th2 cells. Its ligand CCL11/eotaxin is induced in fibroblasts and keratinocytes by IL-4 and IL-13 (Hoeck and Woisetschlager, 2001; Kagami *et al.*, 2005) and is highly expressed in OVA sensitized skin (Spergel *et al.*, 1999). Recruitment of eosinophils to OVA-sensitized skin was severely impaired in CCR$3^{-/-}$ mice. These mice also have impaired recruitment of eosinophils in their lung parenchyma and BAL fluid and fail to develop AHR to methacholine following antigen inhalation. These results suggest that CCR3 plays an essential role in eosinophil recruitment to the skin and the lung and in the development of AHR (Ma *et al.*, 2002). Skin homing T cells express the chemokine receptor CCR4. The CCR4 ligand CCL17/TARC is highly expressed in AD skin lesions. Furthermore, we have found that mechanical injury induces rapid expression in the skin of CCL17/TARC (our unpublished observations). Experiments with CCR$4^{-/-}$ mice have revealed decreased $CD4^{+}$ cell infiltration in OVA sensitized sites as well as decreased expression of IL-4 and IL-13 mRNA levels (our unpublished observations). CCR10 is also expressed on a subset of skin homing cells. Anti-CCR10 was reported to inhibit skin inflammation in response to EC sensitization with OVA (Homey *et al.*, 2006). However, we have found that CCR$10^{-/-}$ mice develop normal allergic skin inflammation. Figure 3.14 summarizes the steps involved in the development of allergic skin inflammation following EC sensitization in our model. These include (1) entry of antigen through mechanically injured skin, capture of antigen by skin DCs and mobilization of these DCs to DLN, (2) expansion and polarization of circulating naïve antigen specific T cells into Th memory cells that express skin homing receptors (e.g., CCR4) upon their encounter with skin derived DCs in DLN, (3) exit of memory T cells into the circulation and their localization in secondary lymphoid organs (e.g., spleen), and (4) homing of antigen specific memory cells to cutaneous sites of antigen re-entry.

Mouse skin is colonized with bacteria, a source of TLR2 ligands. In response to EC sensitization with OVA, TLR$2^{-/-}$ mice developed

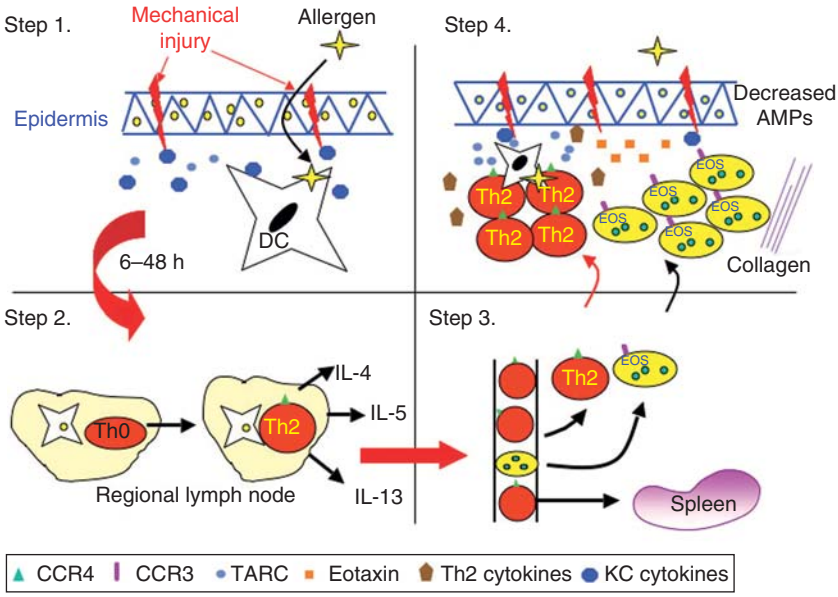

FIGURE 3.14 Steps in the induction of allergic skin inflammation in EC sensitized mice. (1) Following entry of antigen through mechanically injured skin, skin DCs capture antigen are exposed to keratinocyte-derived cytokines released following injury and migrate to DLN within 6–48 h. (2) Antigen-loaded mature DCs instruct naïve T cells to develop into polarized Th memory cells that express skin homing receptors (e.g., CCR4). (3) Memory T cells exit from DLN into the circulation and localize in secondary lymphoid organs (e.g., spleen). (4) Antigen specific T cells and eosinophils home to skin sites of antigen re-entry in response to increased production of CCL17/TARC released after skin injury and to CCL11/eotaxins released by keratinocytes and fibroblasts in response to Th2 cytokines. These cells are activated by antigen and cause allergic inflammation that aggravates skin barrier dysfunction, resulting in increased antigen entry, inhibition of AMP synthesis by Th2 cytokines with resulting increased susceptibility to infection, and cytokine- and eosniphil dependent collagen deposition.

skin infiltration with eosinophils and $CD4^+$ cells as well as upregulation of Th2 cytokine mRNAs that were comparable to WT littermates. In contrast, epidermal thickening, IFN-γ expression in the skin, IFN-γ production by splenocytes and IgG2a anti-OVA antibody levels were impaired in $TLR2^{-/-}$ mice (Jin *et al.*, 2009a). These findings suggest that the decreased IFN-γ response in $TLR2^{-/-}$ mice underlies their failure to develop epidermal thickening following EC sensitization. DCs from $TLR2^{-/-}$ mice induced significantly lower production of IFN-γ, but normal IL-4 and IL-13 production, in naïve T cells, suggesting that the regulatory effect of TLR2 may be exerted, at least in part, at the level of antigen-presenting DCs.

Allergic skin inflammation was severely impaired in TSLPR$^{-/-}$ mice EC sensitized with OVA, with decreased number of eosinophils and decreased local expression of Th2 cytokines in the skin. TSLPR$^{-/-}$ splenocytes produced Th2 cytokines normally. Skin DCs from TSLPR$^{-/-}$ migrated normally to DLNs, expressed activation markers normally, and induced normal proliferation and Th2 cytokine production by T cells. TSLPR$^{-/-}$ CD4^{+} T cells homed to the skin normally, but failed to transfer allergic skin inflammation to WT recipients. Intradermal injection of anti-TSLP antibody blocked the development of allergic skin inflammation. These findings suggest that TSLP is essential for antigen driven Th2 cytokine secretion by skin infiltrating effector T cells and could be a therapeutic target in allergic skin inflammation (He *et al.*, 2008).

The IL-21 receptor (IL-21R) is expressed on T cells, NK cells, NKT cells, B cells, DCs, and macrophages, as well as on nonhematopoietic cells, including keratinocytes and fibroblasts. IL-21 and IL-21R expression was upregulated in acute skin lesions of AD patients (Fig. 3.15) and in mouse skin by tape stripping, a surrogate for scratching (Jin *et al.*, 2009b). Both IL-21R$^{-/-}$ mice and WT mice treated with soluble IL-21R-IgG2a Fc fusion protein failed to develop skin inflammation after OVA-EC sensitization (Jin *et al.*, 2009b). Adoptively transferred OVA-specific WT CD4^{+} T cells accumulated poorly in DLNs of IL-21R$^{-/-}$ mice. Trafficking of skin DCs to DLNs was defective in IL-21R$^{-/-}$ mice and, to a lesser extent, in WT mice reconstituted with IL-21R$^{-/-}$ mice bone-marrow chimera. Skin DCs from tape-stripped IL-21R$^{-/-}$ mice failed to upregulate CCR7 and migrated toward CCR7 ligands. Treatment of epidermal and dermal cells with IL-21 activated metalloproteinases 2 (MMP2), which has been implicated in trafficking of skin DCs. These results suggest an important role for IL-21R in the mobilization of skin DCs to DLNs and the subsequent allergic response to EC introduced antigen.

EC sensitization with OVA drives the generation of IL-17-producing T cells in DLNs and spleen and a local and systemic Th17 response (He *et al.*, 2007). OVA inhalation by EC-sensitized mice induced IL-17 and CXCL2 expression and PMN influx in the lung along with bronchial hyperreactivity, which were reversed by IL-17 blockade. This is in contrast to the eosinophil-dominated response to airway challenge of intraperitoneally immunized mice. Although IL-17 was expressed in EC sensitized skin, there was little expression of CXCL2 and little infiltration of PMNs at EC sensitized skin sites. However, mechanical injury upregulated the expression of IL-6 and IL-23 in skin. IL-6, like TGF-β is an inducer of Th17 cells (Veldhoen *et al.*, 2006), while IL-23 promotes the growth of these cells (Langrish *et al.*, 2005). DCs trafficking from skin to lymph nodes expressed more IL-23 and induced more IL-17 secretion by naïve T cells than splenic DCs. This was inhibited by neutralizing IL-23 *in vitro* and by intradermal injection of anti-TGF-β neutralizing antibody

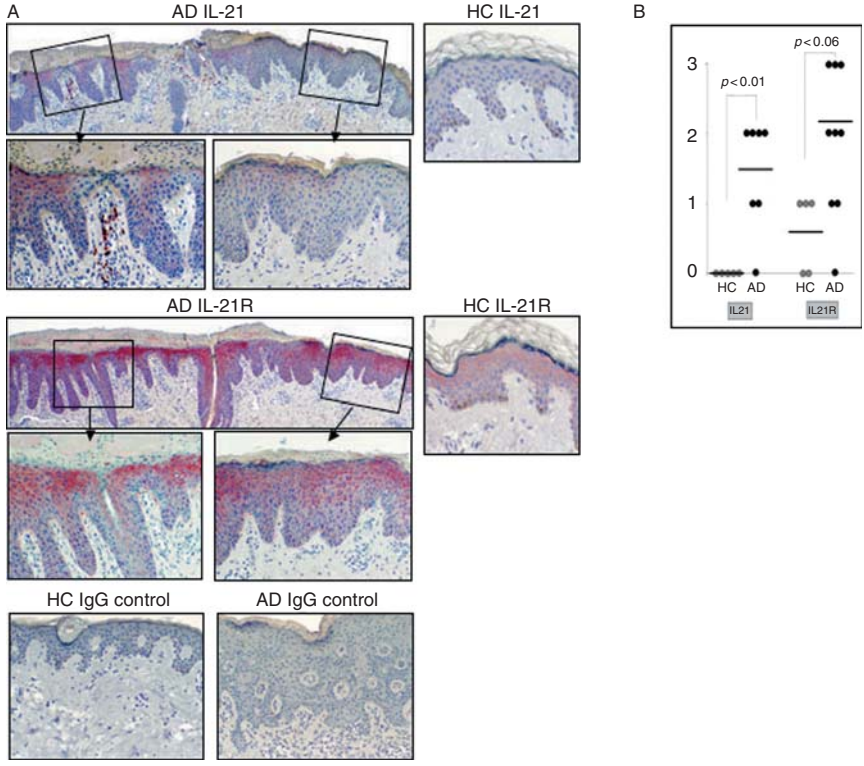

FIGURE 3.15 IL-21 and IL-21R expression is upregulated in skin lesions of acute AD and following mechanical skin injury. (A) IL-21 and IL-21R protein expression in skin from a healthy control (HC) and in an acute skin lesion from a patient with AD. Magnification is ×200, and ×400 for the enlarged panels. For the patient, the left side of the biopsy represents a more active skin lesion than on the right side. (B) Cutaneous expression of IL-21 and IL-21R in 7–9 AD patients and five controls was scored semi quantitatively (0 = no expression; 1 = weak expression; 2 = moderate expression; 3 = strong expression) and depicted in the graph. (From Jin *et al.* (2009b). *J. Clin. Invest.* **119**(1), 47–60.)

in vivo. These findings suggest that initial cutaneous exposure to antigens in patients with AD may selectively induce the generation of IL-17 producing cells. Upon antigen inhalation, these cells are recruited to the lungs where they are activated to secrete IL-17, which drives a PMN rich inflammation in the airways. These findings should prompt a search for airway and lung PMNs in AD patients who develop asthma. This would have important therapeutic implications.

Our model of AD induced by repeated EC sensitization with OVA involves mechanical injury inflicted by of tape-stripping the shaved skin prior to OVA application (Spergel *et al.*, 1998). This mimics skin injury

inflicted by scratching in patients with AD. Mechanical injury is critical in our model, because application of OVA to the skin of hairless mice does not result in the development of an immune response to OVA (our unpublished observation). Recently, we have begun to test the hypothesis that mechanical injury allows not only the breaching of the skin barrier and the introduction of antigen which is then captured by skin DCs, but also releases mediators that may play critical roles in polarizing the DCs to drive the differentiation of Th2 cells in DLNs. Gene array analysis of mouse skin 12 h after skin injury reveals the upregulation of a number of cytokines with a remarkable increase in IL-6, a cytokine which is important for both Th2 and Th17 differentiation differentiation. There is also increase in IL-23, IL-1, and IL-10 gene expression. In addition, a number of chemokine genes, as well as genes for MMPs and kallikeins are highly upregulated. These injury-induced are likely to play an important role in determining the polarity of the immune response to EC sensitization. In this regard, blocking IL-23 inhibits the Th17 response (Chen *et al.*, 2006) (see also Langrish *et al.*, 2005) and blocking IL-10 impairs the Th2 response (Oh *et al.*, 2002). We are using FITC painting of shaved versus shaved and tape stripped skin to track DCs that have emigrated from skin to DLN in order to test the hypothesis that this polarization effect is exerted at the level of the DCs that carry antigen from skin to DLN (Fig. 3.16). Preliminary data suggests that injury by itself upregulates CCR7 on skin DCs (Fig. 3.17A) and allows skin DCs to migrate to DLN (Fig. 3.17B). Furthermore, $FITC^{hi}$ DCs isolated from DLN of shaved tape stripped skin induce significantly more Th2 cytokine secretion in TCR-OVA transgenic DO11.10 cells than $FITC^{hi}$ DCs isolated from DLN of shaved skin that has not been tape stripped. Comparative analysis of the genes differentially expressed by these two populations of DCs may help elucidate the nature of the ''danger signal'' elicited by mechanical skin injury that results in the generation of a predominantly Th2 response to EC sensitization.

We have identified a number of negative regulators of allergic skin inflammation in our model. $C3aR^{-/-}$ mice exhibited an exaggerated Th2 response to EC sensitization with OVA. Presentation of OVA peptide by $C3aR^{-/-}$ APCs caused significantly more IL-4 and IL-5 secretion by OVA-specific T cells from DO11.10 transgenic mice compared with presentation by WT APCs. C3a inhibited the ability of splenocytes, but not of highly purified T cells, to secrete Th2 cytokines in response to TCR ligation. This inhibition was mediated by IL-12 secreted by APCs in response to C3a. These results suggest that C3a–C3aR interactions inhibit the ability of APCs to drive Th2 cell differentiation in response to epicutaneously introduced antigen (Kawamoto *et al.*, 2004). COX-2 was also shown to limit the Th2 response to EC sensitization. Infiltration by eosinophils and expression of IL-4 mRNA in OVA-sensitized skin sites,

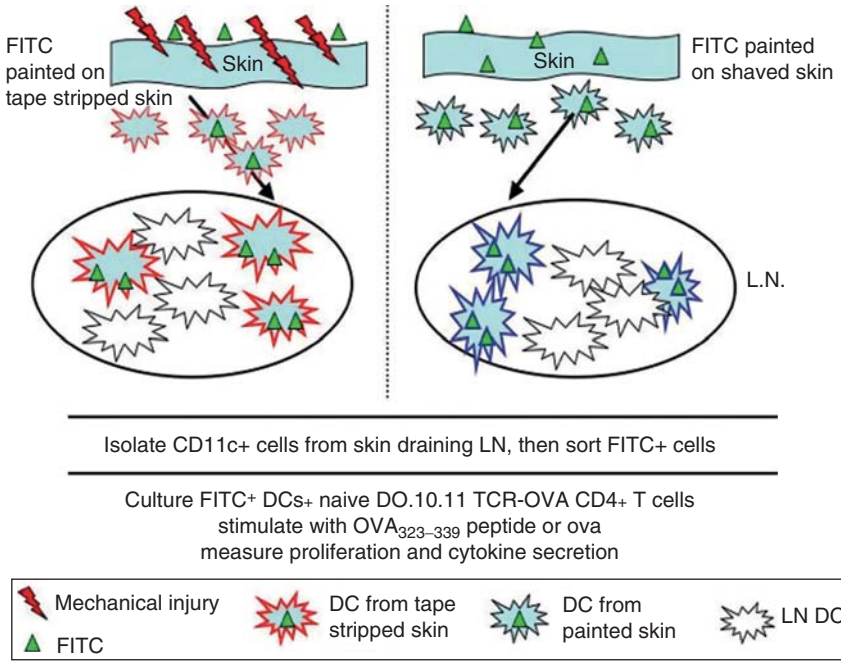

FIGURE 3.16 Scheme for testing the effect of tape stripping on DC polarity.

OVA-specific IgE and IgG1 antibody responses, and IL-4 secretion by splenocytes after OVA stimulation were all significantly increased in EC mice that received NS-398, a COX-2 inhibitor (Laouini *et al.*, 2005). In contrast, OVA-specific IgG2a antibody response and IFN-γ secretion by splenocytes after OVA stimulation were significantly decreased in these mice. COX-2-deficient mice also exhibited an enhanced systemic Th2 response to EC sensitization. These findings are important as they suggest that COX inhibitors may worsen allergic skin inflammation in patients with AD (Laouini *et al.*, 2005). Complement component C3 is synthesized by keratinocytes and is activated after skin injury (Hammerberg *et al.*, 1998). Skin Infiltration by eosinophils and expression of Th2 cytokines in OVA-sensitized skin sites was impaired in $C3^{-/-}$ mice. Splenocytes from EC sensitized $C3^{-/-}$ mice secreted less IL-4, IL-5, IL-13, and IFN-γ in response to OVA stimulation than splenocytes from WT control animals. $C3^{-/-}$ mice also had impaired IgG1, IgG2a, and IgE antibody responses after both EC immunization (Yalcindag *et al.*, 2006). These results suggest that C3 plays an important role in both the Th1 and Th2 response to antigen in AD. The opposing consequences of C3aR and C3 deficiency suggests that C3 degradation products other than C3a may promote allergic skin inflammation. C3b is a good candidate as its receptor is expressed on DCs.

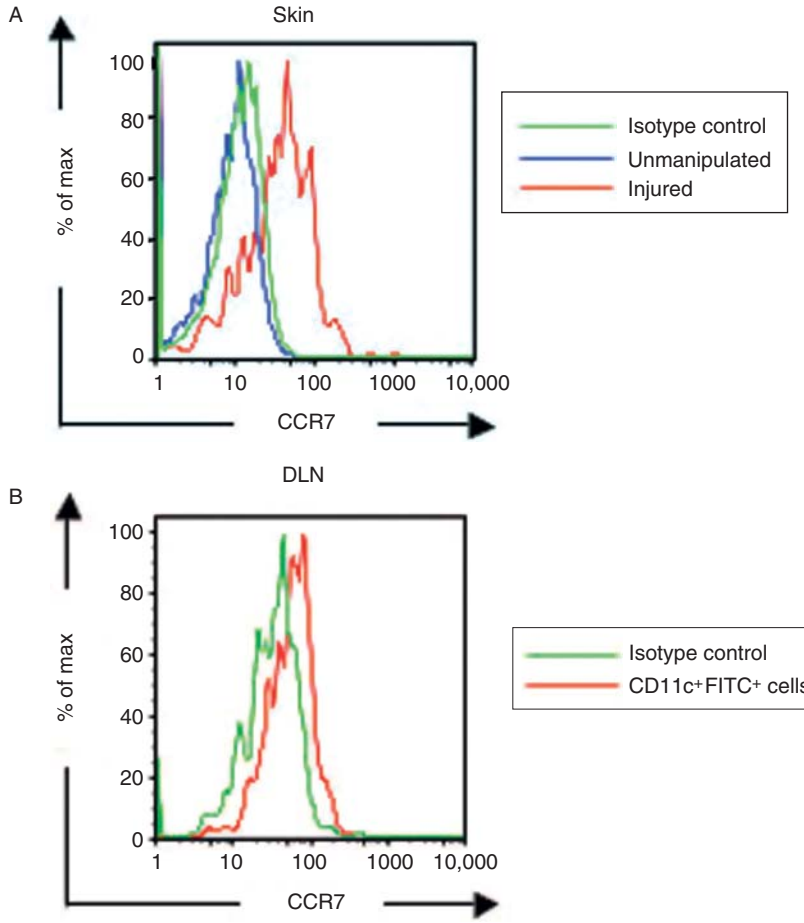

FIGURE 3.17 Induction of CCR7 expression on DCs following mechanical injury. (A) CCR7 expression by $CD11c^+$ cells from ear skin 6–8 h following tape stripping. (B) CCR7 expression by $CD11c^+FITC^+$ DCs from DLN of shaved skin 24 h after FITC painting.

These effects of gene knockouts on skin inflammation in mice EC sensitized with OVA described above are summarized in Table 3.2.

Clinical studies have provided evidence that HDM allergen is associated with human AD (Kimura *et al.*, 1998). BALB/c mice subjected to EC application of the recombinant mite allergen Derp8 exhibited features of dermatitis with epidermal hyperplasia and spongiosis, skin infiltraion with $CD4^+$ and $CD8^+$ cells, and a skewed Th2 response locally and systemically (Huang *et al.*, 2003). These findings are similar to those observed in our model of EC sensitization with OVA. Immunohistochemistry revealed the expression of neuropeptides only in Derp8 treated

TABLE 3.2 Effect of gene knockouts on skin inflammation in mice EC sensitized with OVA

		Skin inflammation				Systemic response				
	Strain	Eosinophils	$CD4^+$	Thickening	Cytokines	Cytokines	OVA-IgE	OVA-IgG1	OVA-IgG2a	References
Allergic skin Inflammation model in WT mice	WT	↑	↑	↑	IL-4 IL-5 IL-13	IL-4 IL-5 IL-13 IFN-γ	↑	↑	↑	Spergel *et al.* (1998)
Exaggerated skin inflammation	$C3aR^{-/-}$	↑	↔	n.a.	IL-4 ↔	IL-4↑ IL-5↑ IL-10↑	↔	↑	↔	Kawamoto *et al.* (2004)
	$COX2^{-/-}$	↑	↑	n.a.	IL-4↑	IL-4↑ IFN-γ↓	↑	↑	↓	Laouini *et al.* (2005)
Decreased skin inflammation	$IL\text{-}4^{-/-}$	↓	↑	↔	IL-4 n.d. IL-5↓ IL-2↑ IFN-γ↑	IL-4 n.d IL-13↓ IFN-γ↑	↓	↓	↑	Spergel *et al.* (1999)
	$IL\text{-}5^{-/-}$	↓	↔	↓	IL-5 n.d.	IL-5 n.d	↔	↔	↔	Spergel *et al.* (1999)
	$IL\text{-}10^{-/-}$	↓	↔	n.a.	IL-4↓ IL-5↓ Eotaxin↓ IFN-γ↑	IL-4↓ IFN-γ↑	↓	↓	↑	Laouini *et al.* (2003a)
	$IFN\text{-}\gamma^{-/-}$	↔	↔	↓	IL-4↑ IL-5 ↔ IL-2 ↔ IFN-γ n.d.	IL-4↑ IFN-γ n.d	↑	↑	↓	Spergel *et al.* (1999)
	$IL\text{-}21R^{-/-}$	↓	↓	↓	IL-4↓ IL-13↓ Eotaxin↓ TARC↓	IL-4↓ IL-13↓ IFN-γ↓	↓	↓	↓	Jin *et al.* (2009b)

	TSLPR$^{-/-}$	↓	↔	↓	IL-4↓ IL-13↓	IL-4 ↔ IL-13 ↔ IFN-γ ↔	↓	↓	↔	He *et al.* (2008)
	CCR3$^{-/-}$	↓	↔	↓	IL-4↔	IL-4 ↔ IL-5 ↔	↔	↔	↔	Ma *et al.* (2002)
	C3$^{-/-}$	↓	↔	n.a.	IL-4↓ IL-5↓	IL-4↓ IL-5↓ IL-13↓ IFN-γ ↓	↓	↓	↓	Yalcindag *et al.* (2006)
	TCRα$^{-/-}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Woodward *et al.* (2001)
	Rag 2$^{-/-}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Woodward *et al.* (2001)
No effect on skin inflammation	IgH$^{-/-}$	↔	↔	↔	IL-4 ↔	↔	n.d.	n.d.	n.d.	Woodward *et al.* (2001)
	IgE$^{-/-}$	↔	↔	↔	IL-4 ↔	↔	n.d.	↔	↔	Spergel *et al.* (1999)
	TCRδ$^{-/-}$	↔	↔	↔	IL-4 ↔	↔	↔	↔	↔	Woodward *et al.* (2001)
	CD40$^{-/-}$	↔	↔	↔	IL-4 ↔	↔	↓	↓	↓	Woodward *et al.* (2001)
	J-Kit^{W}/ Kit$^{W\text{-}v}$	↔	↔	n.a.	IL-4 ↔ IFN-γ↑	↔	↓	↔	↔	Alenius *et al.* (2002)
	CD1d$^{-/-}$	↔	↔	n.a.	IL-4 ↔ IL-13 ↔	↔	↔	↔	↔	ElKhal *et al.* (2006)
	TLR2$^{-/-}$	↔	↔	↓	IL-4 ↔ IL-13 ↔ IFN-γ↓	IL-4 ↔ IL-13 ↔ IFN-γ↓	↔	↔	↓	Jin *et al.* (2009a)

Comparison to WT control: ↑, increased; ↓, decreased; ↔, no change; n.a., not available; n.d., not detected.

skin. Nerve fibers were observed in close proximity of mast cells in the dermis. These findings may suggest an interaction between the nervous and immune systems in the skin lesion of AD.

7.3.2. Hapten induced mouse models of AD

Haptens such as oxazolone (OXZ) and trinitrochlorobenzene (TNCB) are commonly used to induce allergic contact dermatitis and have been thought to evoke primarily a Th1-dominated response. However, it has been recently reported that multiple challenges with OXZ or TNCB to the skin of hairless mice over a extended period causes the skin inflammation to shift from a typical Th1-dominated delayed-type hypersensitivity response to a chronic Th2-dominated inflammatory response that is similar to human AD (Man *et al.*, 2008; Matsumoto *et al.*, 2004). Indeed, 9–10 challenges with OXZ to hairless mice produced a chronic Th2-like skin inflammation. The inflammation was characterized by dermal infiltration of Th2 lymphocytes that express the prostaglandin 2 receptor CRTH2, mast cells and eosinophils, increased expression of IL-4 in the dermis, and highly elevated IgE levels. Repeated challenge with OXZ led to increased epidermal hyperplasia and decreased expression of the skin differentiation proteins filaggrin, loricrin, and involucrin. A skin barrier abnormality became evident and was associated with decreased SC ceramide content, decreased SC hydration and TEWL, and impaired lamellar body secretion, resulting in reduced lamellar membranes, as observed in AD patients. Furthermore, as in human AD, epidermal serine protease activity in the SC increased and expression of two lamellar body-derived antimicrobial peptides, CRAMP and mBD3, declined after OXZ challenges, paralleling the decrease of their human homologues in AD skin lesions. These changes were not observed after a single challenge with hapten, the classical way to elicit hapten delayed hypersensitivity reaction.

Although hapten-induced model is not a genetically driven model, like in the case of the allergen driven model, many of its aspects may be applicable to extrinsic allergen driven AD. Indeed, it particularly illustrates the notion that once inflammation sets it, which in the case of human AD may be due to increased allergen entry because of a barrier dysfunction, the resulting inflammation in response to allergens in turn causes changes in skin barrier that mimic those that may be found in conditions genetically inherited skin barrier dysfunction. This amplificatory cycle may play an important role in the perpetuation and exacerbation of human AD. The repeated hapten sensitization model needs to be compared head to head with the protein (OVA and HDM) repeated sensitization models. Because of its reproducibility, predictability, low cost, and relative rapidity the hapten repeated sensitization model could prove useful for evaluating pathogenic mechanisms and potential therapies for AD.

7.3.3. Superantigen induced mouse models of AD

Of all *S. aureus* strains isolated from lesional skin, up to 65% produce exotoxins with superantigenic properties. We have shown that application of SEB instead of OVA by repeated EC sensitization to tape-stripped skin was able to elicit Th2-dominated allergic skin inflammation accompanied by a systemic Th2 response to the superantigen (Laouini *et al.*, 2003b).

7.4. AD model in orally immunized mice epicutaneously challenged with antigen

The pathogenic role of food allergy in a subset of AD patients has been supported by clinical studies (Sampson and McCaskill, 1985). Repeated intragastric sensitization of C3H/HeJ mice with cow's milk, or peanut, with cholera toxin as adjuvant caused hair loss, scratching, and chronic relapsing AD-like skin lesions in up to 35% mice. This was accompanied with elevated serum level of specific IgE and blood eosinophils (Li *et al.*, 2001). Our recent findings show that mice orally sensitized with OVA and cholera toxin as an adjuvant develop allergic skin inflammation at skin sites challenged with OVA (Oyoshi *et al.*, unpublished observations). These results raise the possibility that flare-ups of AD lesions may occur in orally sensitized individual following introduction of food allergen into the skin.

7.5. Mouse models of EV

Smallpox is a disease caused by the Variola major virus. World-wide immunization with VV stopped the spread of smallpox three decades ago. Routine smallpox vaccinations in the U.S. terminated in 1972. Since then, smallpox vaccinations have been limited to military and other high-risk groups. However, recent concerns about the use of smallpox as a bioterror agent have led to consideration of the use of mass smallpox vaccination. AD patients exposed to the smallpox vaccine, VV, occasionally develop EV. EV is a rare and severe complication of smallpox vaccination, either local or systemic dissemination of vaccinia among individuals with eczema (Copeman and Wallace, 1964). It occurs in roughly 10–40 per million vaccinees with an approximately equal number of secondary cases in contacts of vaccinees (Lane *et al.*, 1969) (see also Center for Disease Control and Prevention, 2001; Neff *et al.*, 1967). EV occurs almost exclusively in subjects who have a history of AD, even if the skin conditions are not active at the time. While EV is usually confined to the skin and self-limited, it can progress to systemic disease. Mortality is ~5% in exposed AD cases, even with treatment of vaccinia immune globulin (VIG) treatment.

The reason that individuals with eczema are at risk for EV and other skin infections is not known. Breakdown in the barrier function of skin is

one obvious predisposing factor. However, the fact that the patients with a history of eczema, but no active lesions are at risk of EV suggests this is not the only cause. Other possibilities include decreased innate immunity in the skin, and changes in the Th1/Th2 polarization of both the local and systemic immune response. The current smallpox vaccine is an old formulation made with live VV. Establishment of animal models of EV and subsequent use of these models to design of a safer smallpox vaccine is extremely important. For this purpose, mice models of AD have been used to establish murine models of EV.

7.5.1. RelB$^{-/-}$ mice model

VV-infected RelB$^{-/-}$ mice via skin scarification but not WT mice, exhibited weight loss, markedly impaired systemic clearance of the virus and increased contiguous propagation from the inoculation site (Freyschmidt *et al.*, 2007). This was associated with lower number of IFN-γ-producing CD8^{+} vaccinia-specific T cells and decreased secretion of VV-specific IFN-γ by splenocytes. The Th2 cytokines-IL-4, IL-5, IL-13, and IL-10 were overproduced. When infected intraperitoneally, RelB$^{-/-}$ mice generated robust T cell responses with good IFN-γ production. These results suggest that allergic inflammation in RelB$^{-/-}$ mice is associated with dysregulated immunity to VV encountered via the skin. This may suggest that susceptibility of AD patients to overwhelming VV infection is similarly related to ineffective T cell responses (Freyschmidt *et al.*, 2007).

7.5.2. EC sensitization model

Mice inoculated with an attenuated thymidine kinase deficient strain of VV by skin scarification at sites of EC sensitization with OVA developed satellite pox lesions and had impaired secretion of Th1 cytokines in response to VV, decreased VV specific serum IgG2a, increased VV specific serum IgG1, and impaired upregulation of IFN-α, but not the cathelicidin-related antimicrobial peptide, at the infection site. The VV immune response of OVA-sensitized mice inoculated with VV at distant skin sites or intraperitoneally was normal (Scott *et al.*, 2007). These results suggest that local immune dysregulation at sites of allergic skin inflammation underlies the impaired Th1 immune response to VV introduced at these sites and the increased susceptibility to develop satellite pox lesions, a characteristic of EV in patients with AD (Scott *et al.*, 2007). Recent studies with a thymidine kinase sufficient strain of VV indicate that inoculation of this strain at sites of allergic skin inflammation also results in a Th2 skewed immune response to the virus, but also in increased virus spread in skin and internal organs and in a vigorous systemic and local IL-17 response with neutrophilic infiltration at the site of VV scarification (our unpublished observations).

8. TREATMENT OF AD

There is no cure for AD. However, the disease manifestations cease or improve remarkably in ~85% of the patients by age 5. Treatment of AD is predicated on restoring skin barrier function and inhibiting the skin inflammatory response. Other measures include avoidance of triggers, appropriate use of anti-infective agents, anti-histamines, and patient education.

A number of excellent recent reviews go into the details of AD treatment (Jung and Stingl, 2008; Krakowski *et al.*, 2008). We will only discuss the rational basis of AD therapies and the general strategies that must be followed. The goals in restoring barrier function is to hydrate the skin, prevent water loss, and suppress itching which leads to scratching and mechanical injury. This necessitates daily treatment applied to the entire skin. In contrast, the use of anti-inflammatory therapy is needed at the very first sign of skin disease activity, and is applied only to clinically affected skin areas. Hydration is achieved by daily soaking in a bath for 20–30 min. Addition of a small amount of disinfectant, such as Chlorox, is recommended to decrease bacterial skin colonization and helps to prevent superinfection. Hydration must be followed by application of a sealer such as petrolatum-based cream, or mineral oil, to prevent water loss (Loden, 2003). Newly available emollients, for example, Cetaphil and Aquaphor have both hydration and sealing functions (Krakowski *et al.*, 2008). Antihistamines have been used for decades to inhibit itching, to break the cycle of scratching, mechanical injury and barrier disruption. However, their efficacy in that regard is controversial (Sidbury and Hanifin, 2000). This is most likely because a number of mediators in addition to histamine contribute to itching in AD. Beneficial effects of antihistamines are more likely explained by their sedative effects via their action on the central nervous system, rather than by simply antagonizing the effect of histamine released in AD skin lesions.

Anti-inflammatory therapy in AD improves AD skin lesions by several mechanisms. These include inhibition of T cell activation, inhibition of cytokine release by T cells, mast cells, and other cells, and decreased numbers and activation of LCs (Hoetzenecker *et al.*, 2005). Inhibition of inflammation has also the secondary benefit of improving barrier function. This decreases skin permeability to antigens and antigen driven immune cell activation, and breaks the vicious cycle of itching/scratching/mechanical injury. The mainstay of anti-inflammatory therapy is topical application of corticosteroids, calcineurin inhibitors, or both. The reader is referred to excellent recent reviews for details of topical regimens of steroid treatment which should be used daily to treat active lesions and intermittently, for example twice weekly, to prevent flares in areas prone to develop AD lesions (Berth-Jones *et al.*, 2003; Krakowski

et al., 2008). Topical calcineurin inhibitors (TCIs), that is, pimecrolimus and tacrolimus are an extremely useful adjunct to topical corticosteroids. TCIs treatment results in sustained benefit and is well tolerated, particularly on delicate areas such as the face and neck, where the use of topical corticosteroids can be associated with a risk of skin atrophy (Langley *et al.*, 2008; Queille-Roussel *et al.*, 2001). TCIs are licensed for use only in children above age 2 and for adults. The theoretical risk for lymphoma and other malignancies, and for cutaneous infection have raised concerns and prompted an FDA mandated black box warning on the TCIs packages, There is to date no evidence of increased incidence of lymphoma or skin cancer in patients treated with TCIs, although longer term follow up is necessary. Although viral infections, such as EH has been observed during TCI treatment (Wollenberg *et al.*, 2003), it still remains unclear whether there is a correlation between increased frequency of viral superinfections and the use of TCIs (Suh *et al.*, 2008).

Patients with severe AD refractory to topical treatment have been treated systemically with oral immunosuppresants that include coticosteroids cyclosporine, mycophenylate motefil methotrexate, azathioprine, and phototherapy with Psoralent+UVA (PUVA) and narrow band UVB (Heller *et al.*, 2007; Meggitt *et al.*, 2006; Weatherhead *et al.*, 2007). Oral corticosteroids should be avoided, because the disease will flare when they are stopped and because of side effects. Oral corticosteroid treatment is very rarely justified; this may be in extremely severe cases that have failed to respond to topical therapy and/or who have such severe excoriated and infected skin lesions that bathing and topical application are difficult. In these cases, a short (1–2 weeks) of tapering course steroid may be used, under its cover topical treatment is introduced as the skin heals.

In conjunction with therapies aimed at restoring barrier function and reducing skin inflammation, efforts should be made to avoid exposure to irritants such a rough clothing, chemicals, that include preservatives often added to topical cream formulations, and inhaled and food allergens that may trigger disease flares. Most importantly, it is critical to treat microbial superinfections. This includes appropriate antibacterial therapy against *S. aureus*, ganciclovir at the first evidence of activation of HSV, administration of VIG in case of exposure to recently vaccinated individuals, and topical use of the TLR7 agonist imiquomod or the TLR7/8 agonist TLR7/8 agonist resiquimod for *M. contagiosum*. There is strong data to suggest that treatment of bacterial superinfection is beneficial in AD (Leung and Barber, 2003).

The increasing knowledge of the cellular and molecular mechanisms involved in AD should facilitate the development of novel, targeted therapeutic approaches. Biologicals aimed at restoring barrier skin function include the use of creams that aim to restore skin lipids, skin proteases, and AMPs. Benefit has been reported from application of creams that

contain ceramide or incorporate bacterial extracts rich in neutral sphingomyelinase to restore ceramide levels (Chamlin *et al.*, 2002; Di Marzio *et al.*, 2008). Creams containing AMP analogs are in clinical trials. The use of oral Vit D3 to promote AMP synthesis and as an anti-inflammatory agent (Hata *et al.*, 2008; Katayama *et al.*, 1996; Sidbury *et al.*, 2008) that inhibits antigen presentation has shown some initial benefit in limited open trials and is currently undergoing rigorous clinical evaluation.

Biologicals that target the adaptive immune response are also being assessed in AD. Preliminary beneficial results have been reported with the use of anti-CD11a monoclonal antibodies (mAb), but not anti-TNF-α mAb (Buka *et al.*, 2005; Jacobi *et al.*, 2005). The latter is not surprising, given the fact that skin TNF-α levels are low in AD compared to psoriasis. Other therapies that are being assessed with no definitive results to date include anti-CD20 mAb and anti-IgE mAb (Belloni *et al.*, 2007; Sediva *et al.*, 2008). There is data to suggest that HDM specific immunotherapy may be beneficial in AD (Bussmann *et al.*, 2006). Therapies that target the Th2 cytokines IL-4, IL-13, and IL-31 and the Th2 promoting keratinocyte cytokine TSLP are under consideration. Probiotics are cultures of potentially beneficial bacteria that positively affects host by enhancing the microbial balance. Studies based on the hygiene hypothesis have suggested immunomodulatory and anti-inflammatory properties for probiotics (Liu and Leung, 2006). There is, so far, no clear benefit from probiotics in AD (Lee *et al.*, 2008).

It has been suggested that early recognition and intervention may improve the clinical course of AD and influence the subsequent development of asthma and allergic rhinitis, that is, halt the atopic march. A double-blinded, parallel-group, randomized trial of the H1-antihistamine cetirizine given to infants between 1 and 2 years of age with AD revealed no effect on the prevalence of asthma 18 months later (Warner, 2001). However, cetirizine delayed or, in some cases, prevented the development of asthma in a subgroup of infants with AD sensitized to grass pollen and, to a lesser extent, HDM. There are current ongoing trials to evaluate the effect of early institution of TCIs and of oral probiotics in preventing the atopic march.

9. CONCLUSION

There is gathering evidence that a genetically defective skin barrier function is an important predisposing factor for AD, as illustrated by the observation that ~15% of AD patients have a defective *FLG* gene which is important for skin barrier function. Candidate genes that affect barrier function may do so by exerting an effect on the structural proteins of the SC, for example, filaggrin, claudin1, involucrin, on lipid composition of

the SC, for example, ceramide generating enzymes such as neural sphingomyelinase, or on protease and protease inhibitor activity, for example, SSCE, elafin, and SPINK5. In addition, genes of the innate immune system, for example, PAMP receptors, AMPs, and TSLP, selectively expressed in the skin and genes of the adaptive system, for example, Th cytokines, Foxp3, and WASP, also are important predisposing factors to AD. These may act by causing skin inflammation, which can disrupt barrier function. Finally environmental allergens, chemicals, and pollutants play an important role in triggering AD.

Much useful information on the pathogenesis of AD has been obtained by the study of transgenic mice and by the EC mechanical injury/EC sensitization model of AD. However, mice that have a genetic defect in barrier function will most likely provide a model of AD closer to the human disease than these models. A major caveat is that some of the genes involved in skin barrier function may have redundant functions. Based on the observations that barrier disruption by skin injury results in Th2-dominated skin inflammation and on the observation that chronic inflammation in normal mouse skin repeatedly sensitized with antigen disrupts barrier function, we predict that mice with genetically defective barrier function, will be highly sensitive to the development of Th2 skewed skin inflammation in response to environmental antigens and that this inflammation will further exacerbate the skin barrier defect and results in the downregulation of the expression of antimicrobial genes in the skin and in predisposition to bacterial growth and superinfection, all features of human AD. The insight into the mechanisms of AD that will be provided by these models will hopefully lead to a wider array of therapeutic interventions in this common and potentially debilitating disease.

ACKNOWLEDGMENTS

This work is supported by the National Institute of Allergy and Infectious Diseases under contract number N01 AI 40030. The authors are grateful to Drs. Robert Sidbury, Donald Leung, and Talal Chatila for providing pictures of patients and to Dr. Toshiaki Kawakami for pictures of Nc/Nga mice.

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CHAPTER 4

Micromanagers of Immune Cell Fate and Function

Fabio Petrocca and **Judy Lieberman**

Contents

Abstract

MicroRNAs (miRNAs) regulate cell fate during development and in response to environmental cues. Here, we review the emerging story of how miRNAs regulate immune cell development and function.

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Advances in Immunology, Volume 102
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)01204-8

1. INTRODUCTION

RNA interference (RNAi) is a powerful, highly specific and ubiquitous gene silencing mechanism that regulates the expression of important genes that control cell differentiation and survival (Ambros, 2004; Bartel, 2004; Dykxhoorn and Lieberman, 2005). RNAi was originally discovered in lower organisms, where it plays an important role in regulating development and cell differentiation and protecting the genome from viruses and transposable elements that can insert into and damage chromatin (Ambros, 2004; Du and Zamore, 2005; Dykxhoorn *et al.*, 2003). Seminal papers by Lee and Ambros describing a noncoding stem-loop RNA (lin-4) that regulates larval development in *Caenorhabditis elegans* (Lee *et al.*, 1993) and by Fire and Mello describing unanticipated gene silencing by small double-stranded RNAs in *C. elegans* triggered an explosion of research to understand how cells use small RNAs to regulate gene expression (Fire *et al.*, 1998). RNAi was only shown to operate in mammalian cells in 2001 (Elbashir *et al.*, 2001). Since then, this endogenous mechanism of gene silencing has become a powerful research tool for manipulating gene expression to understand the function of individual genes or to perform unbiased genetic screens in mammalian systems. In addition, researchers in academia and industry are actively investigating how to harness this endogenous and ubiquitous process to manipulate gene expression to treat a diverse array of diseases, including inflammatory and autoimmune diseases and infection (de Fougerolles *et al.*, 2007).

2. miRNA BIOGENESIS

Although there are multiple types of small double-stranded RNAs in cells (Ghildiyal and Zamore, 2009; Seto *et al.*, 2007), some of which are just being discovered by deep sequencing, the dominant class of endogenous small RNAs in somatic cells is the microRNA (miRNA) (Ambros, 2004; Bushati and Cohen, 2007; Du and Zamore, 2005; Dykxhoorn *et al.*, 2003). miRNAs are transcribed as stem-loop structures, either on their own or within miRNA clusters in longer primary transcripts (pri-miRNA) that are typically encoded within intergenic regions or introns. The expression of miRNA primary transcripts appears to be regulated via enhancers and promoters much like coding genes. In fact, transcription factors and miRNAs can sometimes coregulate each other (Chang and Mendell, 2007; Petrocca *et al.*, 2008). The promoters for intergenic miRNAs, however, have mostly not yet been defined. Through a series of sequential nuclear and cytoplasmic steps, the pri-miRNAs are processed by a family of related RNase enzymes into small imperfectly paired ~22 nt double-stranded

miRNAs. pri-miRNAs are first cleaved by the RNase Drosha in the nucleus, which converts them to shorter stem-loops (pre-miRNA) that are exported to the cytoplasm by Exportin V. In the cytoplasm, the loop of the pre-miRNA is removed by the RNase Dicer to yield a small imperfectly paired double-stranded RNA of ~22 nt in length, which is handed to the RNA-induced silencing complex (RISC) (Bartel, 2004). Recent studies suggest that these processing steps may also in some cases be highly regulated (Newman *et al.*, 2008; Thomson *et al.*, 2006; Viswanathan *et al.*, 2008). The RISC localizes to perinuclear sites of RNA storage and processing (P bodies) (Bhattacharyya *et al.*, 2006). One strand of the miRNA (the antisense or guide strand) buries its 5′-end into a pocket of the RISC RNase Ago and the other strand is removed by an unclear mechanism. Removing the passenger strand activates the RISC, which can now bind mRNAs bearing partially complementary sequences to the guide strand.

3. miRNA REGULATION OF GENE EXPRESSION

In mammalian cells, miRNAs primarily regulate gene expression by blocking translation, but some targeted mRNAs may also be cleaved by the RISC or undergo accelerated degradation (Bagga *et al.*, 2005; Giraldez *et al.*, 2006; Mathonnet *et al.*, 2007, Olsen and Ambros, 1999; Pillai *et al.*, 2005, 2007; Yekta *et al.*, 2004). Therefore, reduced protein, with or without reduced mRNA, is the hallmark of miRNA targeting. The extent of gene silencing by a given miRNA on a particular protein product may be modest (Baek *et al.*, 2008; Selbach *et al.*, 2008). However, subtle changes in protein concentration can have profound physiological effects as demonstrated by the pathologies arising from haploinsufficiency. Moreover, the targeting of several components of a functional network by a single miRNA may further enhance its biological impact. Although some miRNAs may act as fine tuning rheostats to adjust gene expression subtly, there is increasing evidence that others, such as let-7 (Yu *et al.*, 2007), act as master regulators of differentiation and of the cell's response to environmental change and stress. miRNAs are estimated to regulate expression of anywhere from a third to 90% of genes (Friedman *et al.*, 2009; Lim *et al.*, 2005).

The first example of a mammalian miRNA with functional consequences, published in 2004, showed that miR-181a regulates lymphocyte development (Chen *et al.*, 2004). miRNA effects can be experimentally enhanced in cells by transfection of small double-strand miRNA mimics that resemble the Dicer cleavage product. They can also be inhibited by transfection of antisense oligonucleotides (ASO) (Krutzfeldt *et al.*, 2005). Viral vectors can also be used to express miRNAs within cells (Hannon and Conklin, 2004; Paddison *et al.*, 2004). Other approaches to antagonize

an miRNA include overexpression of transcripts with multiple miRNA recognition sites to act as a ''sponge'' and compete with endogenous mRNA binding (Ebert *et al.*, 2007).

4. miRNA TARGET GENES

Each miRNA may regulate hundreds of genes, but identifying the critical genes that are regulated by an miRNA is not straightforward and is a major stumbling block to figuring out the biological role of any individual miRNA. Nucleotides 2–9 in the active strand form a ''seed'' region; pairing of a target mRNA to this seed is an important determinant of binding, but other factors, including downstream complementarity and RNA secondary structure, are also important (Brennecke *et al.*, 2005; Hammell *et al.*, 2008; Lewis *et al.*, 2003). Although most known miRNA recognition sites in regulated gene transcripts are in the 3′UTR, recent studies suggest that sequences in the 5′UTR or coding sequences can also be important for miRNA gene regulation (Lytle *et al.*, 2007; Tay *et al.*, 2008). Current approaches to identify miRNA targets fall short of the task. The difficulty researchers face in identifying target genes, which are only partially complementary to the miRNA 22-mer active strand, is a major obstacle for understanding how miRNAs regulate cellular outcomes. The major tools that have been used are (1) bioinformatic algorithms that predict potential target genes that contain conserved complementary sequences in their 3′UTR to a seed region at the 5′-end of the miRNA active strand (Brennecke *et al.*, 2005; Doench and Sharp, 2004; Lewis *et al.*, 2003) and (2) analysis of mRNAs that are downregulated when an miRNA is overexpressed (Johnson *et al.*, 2007; Lim *et al.*, 2005). The bioinformatic approach is hampered by the fact that the existing algorithms have a high margin of error (the majority of predicted genes are not real targets and some of the key targets, such as RAS for let-7, are not predicted (Johnson *et al.*, 2007)). For many miRNAs, current algorithms predict hundreds or even thousands of potential targets, making it difficult to identify the most important targets. Gene expression array analysis does not readily distinguish direct mRNA targets from mRNAs downregulated through secondary effects and misses most target genes that are regulated by blocking translation rather than by mRNA degradation. Moreover, even when mRNA degradation occurs, changes in mRNA levels may be small (often less than twofold) and may be difficult to distinguish from background fluctuations, especially in genome-wide surveys. Combining these two approaches may work better than either approach alone, but still is not helpful in many situations. Recently, mRNA targets of miRNAs have been identified by their enrichment in coimmunoprecipitates with tagged Argonaute proteins in *Drosophila* and

human cell lines overexpressing the miRNA of interest (Beitzinger *et al.*, 2007; Easow *et al.*, 2007; Hammell *et al.*, 2008; Hendrickson *et al.*, 2008; Karginov *et al.*, 2007; Zhang *et al.*, 2007). However, these studies have not yet been shown to identify new miRNA targets. Argonaute overexpression globally increases miRNA levels, perhaps obscuring the effect of an individual overexpressed miRNA (Diederichs and Haber, 2007). Another recent approach, differential labeling with stable isotopes (SILAC), identifies proteins that are differentially expressed when an miRNA is overexpressed or antagonized (Baek *et al.*, 2008; Selbach *et al.*, 2008). This approach complements and improves upon mRNA profiling, since miRNAs have a greater effect on protein expression in mammalian cells than on mRNA. However, it does not distinguish between direct gene silencing and secondary effects. Moreover, it is a costly technology not readily available to most laboratories.

5. miRNAs REGULATE HEMATOPOIETIC DIFFERENTIATION AND IMMUNE FUNCTION

In the past 2 years, miRNAs have emerged as important regulatory elements in the control of immune cell differentiation and homeostasis and immune response (Baltimore *et al.*, 2008; Lodish *et al.*, 2008; Merkenschlager and Wilson, 2008) (Fig. 4.1). Genetic ablation, as well as ectopic or overexpression of individual miRNAs, has significant physiological consequences in the immune system, ranging from cell death and impairment of immune function to autoimmunity, lymphoproliferation, and cancer. Some pathogens, especially viruses, also encode their own miRNAs or modulate the expression of host cell miRNAs to regulate their replication or latency or to manipulate or evade host immune responses (Gottwein and Cullen, 2008).

Early evidence that miRNAs might play an important role in regulating the ordered and well-studied programs of cell differentiation that occur during immune cell development and response to antigen came from genetic manipulation of miRNA expression in mice. Infection of hematopoietic stem cells (HSC) with a lentivirus expressing a miRNA that is highly expressed in the thymus and lymphoid tissue (miR-181a) led to preferential expansion of B cells when transplanted into irradiated mice (Chen *et al.*, 2004). Conditional genetic ablation of Dicer, the RNase required to generate mature miRNAs from precursor transcripts, in immature DN3 thymocytes by use of Cre recombinase expressed from an lck promoter (lck-cre) to generate Dicer$^{fl/fl}$lck-cre mice profoundly reduced thymocyte numbers (~10-fold) and there was a virtual absence of T cells in the periphery. If Dicer was deleted somewhat later in thymocyte development in double positive (DP) thymocytes via CD4-cre then

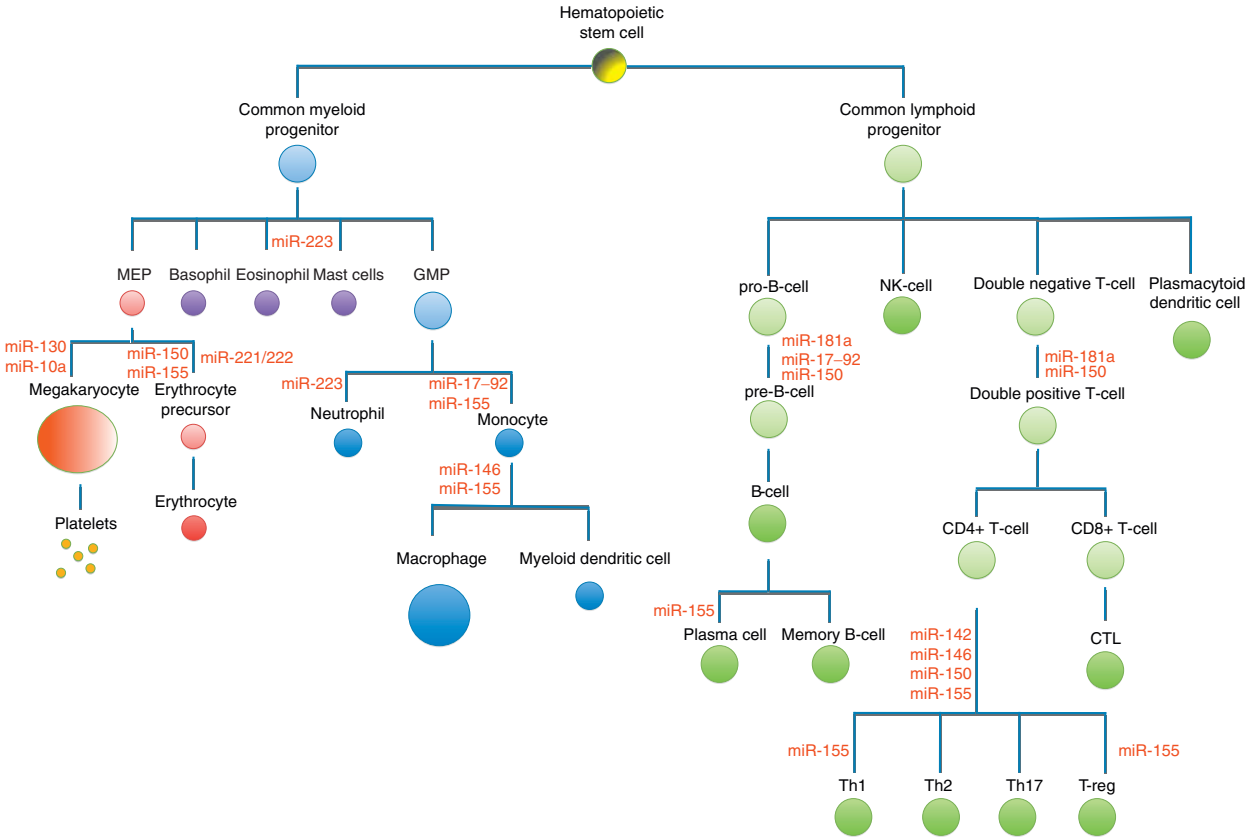

FIGURE 4.1 miRNAs implicated in hematopoiesis. Figure has been adapted from Baltimore *et al.* (2008).

peripheral T cell numbers were only moderately reduced but CD4 T cells developed aberrantly, favoring differentiation into T_H1 cells (Muljo *et al.*, 2005). There was also a substantial reduction in T_{REG}, which resulted in autoimmune colitis, splenomegaly, and lymphadenopathy. In a group of recent studies, Dicer or Drosha, another miRNA-processing enzyme, were ablated specifically in T_{REG} by use of FoxP3-cre. miRNAs were found to be essential to maintain the T_{REG} phenotype and Dicer deficient T_{REG} began to express effector molecules associated with other lineages (granzymes, IL-10, IFN-γ, IL-4) and were prone to apoptosis (Chong *et al.*, 2008; Liston *et al.*, 2008; Zhou *et al.*, 2008). The effect of Dicer depletion in this lineage was uniformly fatal, resulting in autoimmune inflammation and tissue damage that was indistinguishable from the pathology that develops in the FoxP3$^{-/-}$ *scurfy* mouse. miR-155 whose transcription is upregulated by FoxP3, plays an important role in promoting T_{REG} proliferation in nonlymphopenic settings in part by suppressing Socs1 expression and thereby enhancing IL-2 signaling (Lu *et al.*, 2009). However, other miRNAs that remain to be defined are likely important in maintaining T_{REG} function, since miR-155$^{-/-}$ mice, although they have reduced T_{REG} numbers, do not develop the severe autoimmunity of mice conditionally depleted of Dicer in FoxP3^{+} T cells. These studies suggest that miRNAs may be required to maintain lineage commitment of immune cells. The miRNAs and their regulated genes that mediate the effects of Dicer knockout on T cell development and lineage commitment are largely not known.

miRNAs are needed not only for T cell development but also for B lymphocyte development. Ablation of Dicer in the earliest stages of B cell development by use of an mb-1-cre allele leads to a nearly complete block in development at the pro-B cell stage (Koralov *et al.*, 2008). The K Rajewsky and Jacks labs were able to implicate the miR-17–92 cluster family, upregulated in pro-B cells, in this process. While mice depleted of this cluster have a defect in B cell development (Koralov *et al.*, 2008; Ventura *et al.*, 2008), mice expressing a miR-17–92 transgene develop lymphoproliferative disease and autoimmunity through a defect in B cell apoptosis (He *et al.*, 2005; Xiao *et al.*, 2008). miRNAs in these gene families prevent pre-B cell apoptosis, at least in part, by suppressing the proapoptotic bcl-2 family member Bim. The Dicer knockout B cells that do develop also have altered N additions and distinct patterns of D_H gene usage during Ig gene rearrangement, suggesting an intriguing potential role of Dicer-modified small RNAs at the Ig locus affecting gene accessibility and rearrangement (Koralov *et al.*, 2008).

Recently, a group of papers have highlighted the role of individual miRNAs in both innate and adaptive immunity. These studies are likely to be the first of many examples of a substantial role of miRNAs in regulating immune cell development and response to pathogens. Although these studies clearly implicate certain miRNAs in regulating

immunity, identification of relevant miRNA-regulated immune genes is just beginning. Most of the algorithms that predict miRNA regulated target genes rely heavily on evolutionary conservation going as far back as chickens. Since many of the features of adaptive immunity have evolved more recently, many features of miRNA regulation of immune gene expression may not be evident if conservation is used to filter examination of evolving regulatory networks. This may be especially relevant for pathogen-specific responses, because many of the key human pathogenic viruses do not infect other species.

6. miRNA REGULATION OF LYMPHOCYTE DEVELOPMENT AND FUNCTION

miR-181a acts as a rheostat to regulate the sensitivity of T cells to TCR stimulation (Li *et al.*, 2007). By downmodulating the expression of multiple inhibitory phosphatases involved in TCR signaling, miR-181a enhances phosphorylation of key signaling molecules and consequently sensitivity to antigen. Modulating miR-181a expression is even able to convert an antagonistic peptide antigen to an agonistic peptide. miR-181a is highly upregulated in DP thymocytes, relative to more mature SP cells, and also enhances their sensitivity to antigen. As a consequence, manipulating miR-181a in thymocyte cultures has a profound effect on both positive and negative selection *in vitro*. Interestingly, although miR-181c has an almost identical active strand sequence to miR-181a, only pre-miR-181a is processed in thymocyte progenitor cells (Liu *et al.*, 2008). Differences in the pre-miRNA loop sequence appear to control differential processing of these two related miRNA family members.

miR-150 and miR-155, mostly expressed in hematopoietic cells, also have profound immune effects (Landgraf *et al.*, 2007; Monticelli *et al.*, 2005). miR-150 is expressed in mature B and T cells, but not earlier in development (Xiao *et al.*, 2007). When miR-150 is expressed ectopically in B cell progenitor cells, B cell development is partially blocked. Ablation of miR-155, which is highly expressed in activated B and T lymphocytes and activated macrophages and dendritic cells, leads to profound defects in a variety of immune responses. miR-155 KO mice develop autoimmune pulmonary and enteric pathology and have impaired cellular and antibody responses to pathogens and immunization (Rodriguez *et al.*, 2007; Thai *et al.*, 2007; Vigorito *et al.*, 2007). The T cell response is skewed toward T_H2 cells and c-maf, IL-10, and IL-4 gene expression is increased. The germinal center reaction is suppressed as is production of both IgM and class-switched high affinity specific antibodies. An important gene regulated by miR-155 is AID, responsible for both somatic hypermutation and class switch recombination (Dorsett *et al.*, 2008).

7. miRNA REGULATION OF INFLAMMATORY RESPONSES

miR-155 also has a profound effect on myeloid cells and is upregulated, together with miR-132 and miR-146, during the inflammatory response to endotoxin (O'Connell *et al.*, 2008; Taganov *et al.*, 2006; Tili *et al.*, 2007). miR-155 can also be induced by Type-I IFNs. miR-155 induction by both TLRs and Type-I IFN induction is dependent on activating the JNK pathway. Transducing HSC with miR-155 also leads to preferential expansion of myeloid cells, mimicking the proinflammatory effect of LPS. Some of the likely important targets for miR-155 in the response to inflammatory stress are genes that regulate transcriptional activation in myeloid cells (PU.1, Cepbb), cytokine receptors (Csfr1), and the stress response transcription factor HIF1α.

miR-146, another important miRNA family upregulated in response to endotoxin and IL-1β in monocytes and other cells, including pulmonary epithelial cells, in response to NF-κB signaling, targets downstream genes involved in cytokine and TLR signaling, including the TNF receptor-associated protein (TRAF6) and IL-1 receptor-associated kinase (IRAK1) (Perry *et al.*, 2008; Taganov *et al.*, 2006). It may also regulate expression of the chemokine receptor CXCR4 (Labbaye *et al.*, 2008). Therefore, it serves to dampen the proinflammatory response to TLR signaling. miR-146a expression is greatly increased in macrophages and some lymphocytes of synovial tissues of rheumatoid arthritis patients (Nakasa *et al.*, 2008).

8. VIRUSES HARNESS THE miRNA MACHINERY

When pathogens invade host cells, they can take advantage of the endogenous miRNA machinery to advance their own replication or modulate host immune responses to their own benefit. (A detailed description of miRNA regulation of viral infection is beyond the scope of this review; for a comprehensive review see Gottwein and Cullen (2008).) This has been studied for viruses, which can express their own miRNAs (some of which mimic host miRNAs) or modulate the expression of host miRNAs (Fig. 4.2). Viral miRNAs have been described for a variety of DNA viruses, including herpesviruses, polyomaviruses, and adenoviruses. Like eukaryotic small RNAs, these molecules are transcribed mostly from Pol II promoters, processed through the host RNAi machinery and incorporated into the host RISC. Although some studies have suggested that RNA viruses, including HIV, encode for miRNAs (Bennasser *et al.*, 2004; Omoto *et al.*, 2004), this is controversial. Studies, which identified viral miRNAs in DNA virus-infected cells, failed to detect viral small RNAs by deep sequencing small RNAs from cells infected with HIV-1

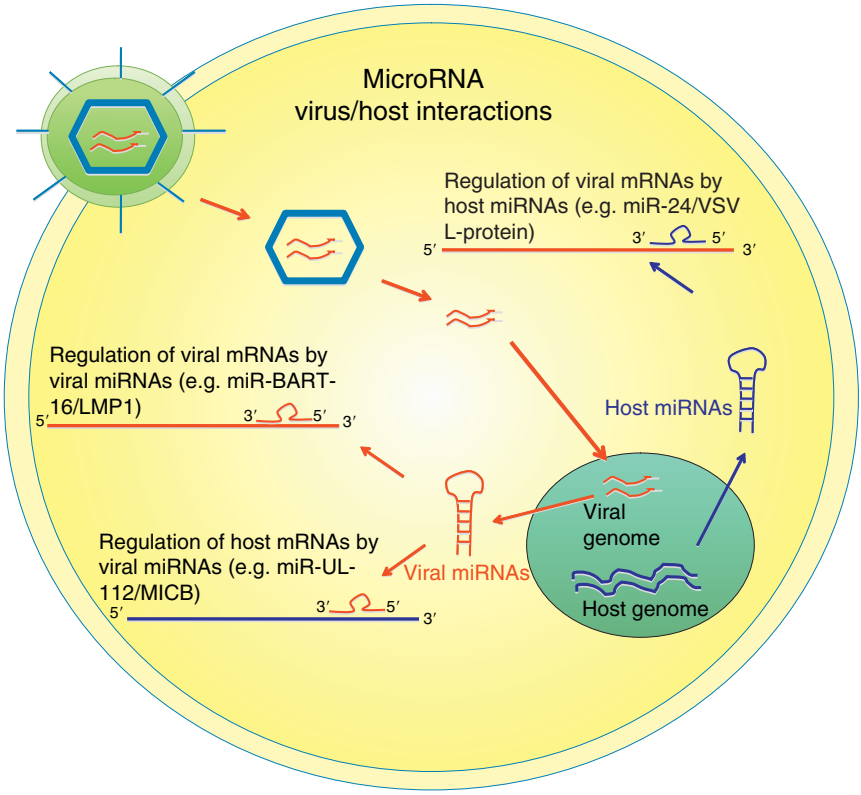

FIGURE 4.2 Both viral and host encoded miRNAs regulate gene expression to control viral infection.

or HTLV-1 (Pfeffer *et al.*, 2005). Both the Cullen laboratory (Lin and Cullen, 2007) and our unpublished data do not suggest that HIV encodes its own miRNAs.

Viral miRNAs have been most carefully studied for herpesviruses, especially those that cause human disease. Clustered miRNA polycistrons, expressed during Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein–Barr virus (EBV) latent phases and encoded in relatively conserved regions of the viral genome, regulate latency by suppressing opposite strand transcripts. One example is EBV miR-Bart2, which regulates the antisense BALF5 mRNA (Barth *et al.*, 2008). During latency, miRNAs may keep protein levels of viral genes to a minimum, facilitating evasion of immune surveillance (Huang *et al.*, 2007). Other herpesvirus miRNAs, identified in cytomegalovirus (CMV) and herpes simplex virus (HSV) lytic phase, which are not as conserved, appear to regulate viral and host transcripts during lytic infection (Buck *et al.*, 2007;

Dolken *et al.*, 2007; Dunn *et al.*, 2005; Grey *et al.*, 2005; Pfeffer *et al.*, 2005). Nonconserved viral miRNA/mRNA interactions also exist that significantly affect the viral cycle. For example, EBV miR-BART-16, miR-BART-17-5p, and miR-BART-1-5p modulate the expression of EBV latent membrane protein 1 (LMP1), preventing LMP1-induced apoptosis in infected cells (Lo *et al.*, 2007). Therefore, viral miRNAs might mediate both conserved and newly acquired biological functions to which viruses have recently adapted.

Some viral miRNAs regulate the expression of host genes influencing host cell behavior. KSHV miRNAs suppress thrombospondin expression to enhance angiogenesis and induce resistance to TGFβ signaling (Samols *et al.*, 2007). hCMV miR-UL-112 silences MHC1-related chain B (MICB) and A (MICA) to evade NK cell recognition (Stern-Ginossar *et al.*, 2007).

9. SOME VIRAL miRNAs MIMIC HOST miRNAs

Other viral miRNAs mimic host miRNAs. KSHV miR-K12-11 closely resembles cellular miR-155, sharing the first nine 5 nucleotides including the seed region. This viral miRNA functionally mimics miR-155, as determined by microarray and functional studies (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Since miR-155 is oncogenic and its overexpression leads to B cell lymphoma (Costinean *et al.*, 2006) and KSHV infection also causes B cell malignancies, it is likely that KSHV tumor induction may be linked, at least in part, to miR-K12-11 expression.

Other viral miRNAs with homology to known cellular miRNAs include EBV miR-BART5, rhesus lymphocryptovirus (rLCV) miR-rL1-8, and murine gammaherpesvirus 68 (MHV68) miR-M1-7-5p, all of which share perfect seed homology with cellular miR-18a, encoded by the miR-17–92 cluster, which is overexpressed in lymphomas and other cancers (Volinia *et al.*, 2006). Overexpression of this cluster in mice leads to lymphoma and autoimmunity (He *et al.*, 2005; Xiao *et al.*, 2008). Since miR-18a appears to silence CDKN1A (p21) (F. Petrocca, unpublished results), a key regulator of cell cycle progression and a central hub for a variety of tumor suppressor pathways, miR-BART5 may disrupt cell cycle control in EBV-infected cells and contribute to EBV-driven lymphomas and nasopharyngeal carcinomas.

10. HOST miRNAs ALSO REGULATE VIRAL REPLICATION

In primitive organisms that lack adaptive immunity, including plants, flies, and worms, RNAi is an important antiviral host defense mechanism (Li and Ding, 2006). Whether RNAi is important for mammalian antiviral

defense is still unclear. Several cellular miRNAs have been shown to target viral sequences, inhibiting viral replication. For example, impairment of the RNAi pathway by Drosha/Dicer knockdown enhances VSV replication, an effect that has been attributed to miR-24 and miR-93 downregulation (Otsuka *et al.*, 2007). Some host miRNAs may be essential for viral replication. For example, hepatocyte expression of miR-122, which binds to a seed-matching region in the hepatitis C virus (HCV) 5′-NCR, is needed for HCV replication by an unclear mechanism (Jopling *et al.*, 2005). Viruses can also reshape host miRNA expression to downregulate miRNAs that inhibit their own replication. For example, latent EBV infection of B cells leads to upregulation of both miR-155 and miR-146, the latter via NF-κB activation by LMP-1 (Lu *et al.*, 2008). HIV-1 infection downregulates expression of miR-17-5p and miR-20a in $CD4^+$ T cells. These miRNAs may inhibit HIV-1 replication by silencing PCAF, a transcriptional cofactor of Tat that is essential for viral replication (Triboulet *et al.*, 2007).

11. PROSPECTS FOR MANIPULATING miRNA FUNCTION FOR IMMUNOMODULATORY THERAPY

Since miRNAs have such a profound effect on immune function and response to pathogens, manipulating miRNA function in immune cells could potentially have therapeutic benefit. This approach might be able to alter expression of multiple genes at once to regulate key functional networks. miRNA function can in principle be augmented by transducing cells with double-stranded miRNA mimics that resemble the Dicer-processed endogenous miRNA or antagonized by ASO. The main obstacle to miRNA-based immune therapeutics is the challenge of transducing immune cells with nucleic acids *in vivo* (de Fougerolles *et al.*, 2007). Even *in vitro* transduction of lymphocytes is challenging. Manipulating miRNA function will likely be easier to accomplish for dendritic cells and macrophages to modulate the early steps of antigen recognition, innate immunity, and inflammation. Situations where local, as opposed to systemic, intervention could prove beneficial would also pose less of a delivery hurdle. The decade of drug development for antisense therapeutics has identified ways for *in vivo* transduction of some cells, including possibly myeloid antigen-presenting cells, with single-stranded oligonucleotides (as could be used to antagonize miRNAs).

Some progress has been made in developing methods for *in vivo* transduction of primary lymphocytes with siRNAs, which have the same chemical properties and pose the same delivery challenge as miRNAs. These involve targeted delivery of antibody fusion protein–RNA complexes (Song *et al.*, 2005) or antibody-coated siRNA-encapsulating

liposomes (Peer *et al.*, 2008). Fusion proteins have even been developed that induce silencing specifically only in activated lymphocytes by taking advantage of activation-associated changes in cell surface integrins (Peer *et al.*, 2007). Targeted intravenous delivery of antiviral siRNAs to primary lymphocytes was able to control HIV infection in humanized mice (Kumar *et al.*, 2008). Targeted delivery of cyclin D1 siRNAs encapsulated into β7-integrin-targeted liposomes administered intravenously was able to suppress experimental colitis by inhibiting T cell proliferation and cytokine production (Peer *et al.*, 2008). These encouraging examples suggest that the obstacles for therapeutic manipulation of miRNA expression in immune cells, although considerable, may be tractable.

ACKNOWLEDGMENT

This work was supported by NIH AI070302 (J.L.). We thank Klaus Rajewsky and members of the Lieberman laboratory for useful discussions.

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CHAPTER 5

Immune Pathways for Translating Viral Infection into Chronic Airway Disease[1]

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[1] The research of the authors reported herein was supported by the National Institutes of Health (National Heart, Lung, and Blood Institute and National Institute of Allergic and Infectious Diseases), Martin Schaeffer Fund, and Alan A. and Edith L. Wolff Charitable Trust, USA

Advances in Immunology, Volume 102
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)01205-X

Abstract To better understand the immune basis for chronic inflammatory lung disease, we analyzed a mouse model of lung disease that develops after respiratory viral infection. The disease that develops in this model is similar to asthma and chronic obstructive pulmonary disease (COPD) in humans and is manifested after the inciting virus has been cleared to trace levels. The model thereby mimics the relationship of paramyxoviral infection to the development of childhood asthma in humans. When the acute lung disease appears in this model (at 3 weeks after viral inoculation), it depends on an immune axis that is initiated by expression and activation of the high-affinity IgE receptor (FcεRI) on conventional lung dendritic cells (cDCs) to recruit interleukin (IL)-13-producing $CD4^+$ T cells to the lower airways. However, when the chronic lung disease develops fully (at 7 weeks after inoculation), it is driven instead by an innate immune axis that relies on invariant natural killer T (iNKT) cells that are programmed to activate macrophages to produce IL-13. The interaction between iNKT cells and macrophages depends on contact between the semi-invariant Vα14Jα18-TCR on lung iNKT cells and the oligomorphic MHC-like protein CD1d on macrophages as well as NKT cell production of IL-13 that binds to the IL-13 receptor (IL-13R) on the macrophage. This innate immune axis is also activated in the lungs of humans with severe asthma or COPD based on detection of increased numbers of iNKT cells and alternatively activated IL-13-producing macrophages in the lung. Together, the findings identify an adaptive immune response that mediates acute disease and an innate immune response that drives chronic inflammatory lung disease in experimental and clinical settings.

1. INTRODUCTION

One of the major tasks of current medical research is to define the pathogenesis of chronic inflammatory disease. In that regard, chronic inflammatory diseases of the lung such as asthma and chronic obstructive pulmonary disease (COPD) represent one of the most common types of chronic illness that affects humans. A critical step towards defining the molecular mechanisms underlying these illnesses came with formal recognition of the role of immunity and inflammation. Since then, evidence of excessive airway inflammation in concert with lung disease has led to a widening search for the types of inflammatory cells and mediators that might be responsible for abnormal airway function. Cell types implicated

in the development of airway inflammation include immune cells as well as parenchymal lung cells. Cell–cell interactions have been attributed to classes of mediators that include lipids, proteases, peptides, glycoproteins, glycolipids, and cytokines. The leading scheme for integrating this information has been based on the classification of the adaptive immune system, and especially the responses of T helper (Th) cells. In this scheme, $CD4^+$ T cell-dependent responses are classified into T helper type 1 (Th1) or type 2 (Th2). Th1 cells characteristically mediate delayed-type hypersensitivity reactions and selectively produce interleukin (IL)-2 and interferon (IFN)-γ, whereas Th2 cells promote B-cell dependent humoral immunity and selectively produce IL-4, IL-5, and IL-13. For the most part, Th2-based reactions constitute the fundamental response to allergen inhalation and thus account for the overproduction of Th2-derived cytokines that is characteristic of allergic asthma (Poston *et al.*, 1992; Ying *et al.*, 1995). By extension, the same reactions may underlie the inflammation and consequent airway hyperreactivity (AHR) and mucus overproduction that are also characteristic of the airway disease found in COPD. The Th1 versus Th2 paradigm has become more complicated by the recognition of Th17 (IL-17-producing) and Treg (IL-10- and TGF-β-producing) subsets of T cells, and these subsets may also contribute to inflammatory airway disease at least in part by increasing the Th2 response (Wang and Liu, 2008; Wilson *et al.*, 2008).

However, some research findings appear to challenge the Th2 hypothesis for asthma and related airway diseases. For example, studies in mice using adoptive transfer with Th1 and Th2 cells indicate that Th1 cells may also be necessary for initiating the allergic response (Castro *et al.*, 2000; Randolph *et al.*, 1999). Moreover, it is unclear how a Th2-polarized response accounts for airway disease that is also triggered by exposure to nonallergic stimuli, especially infection with respiratory viruses, that would ordinarily elicit a Th1 response. Indeed, the broader issue of the relationship between acute infection, especially due to viruses (including respiratory viruses), and the subsequent development of chronic inflammatory disease (including lung disease) remains uncertain. Perhaps by analogy to the allergic response, the link between infection and the subsequent development of chronic inflammatory disease has been attributed to alterations in the adaptive immune system (Fig. 5.1). However, the exact mechanism of how the immune system might direct a switch from an acute response to infection to a chronic inflammatory disease remains unknown. Because of these uncertainties, we have questioned whether other aspects of immunity and inflammation might also be critical for the pathogenesis of airway disease after viral infection. Therefore, we aimed to develop a model that better accounted for the development of allergy and asthma and was based on a more precise appraisal of the innate and adaptive systems in the airway. Accordingly, this review will summarize

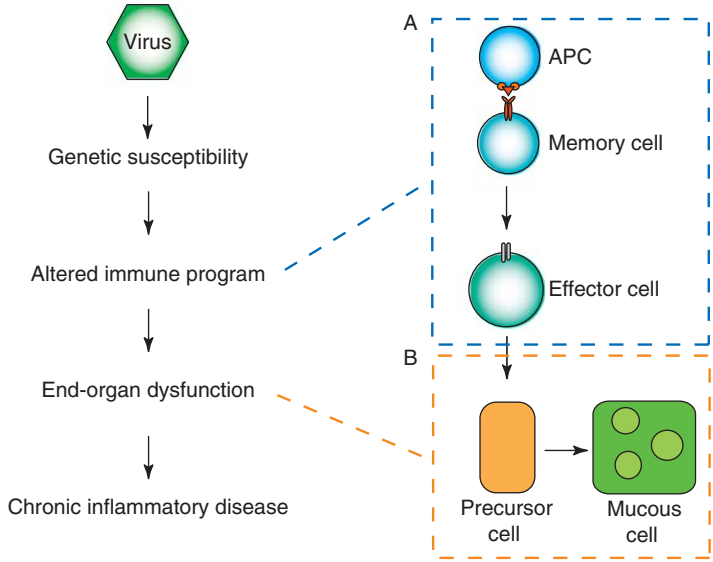

FIGURE 5.1 Proposed scheme for how viruses might trigger chronic inflammatory disease. In a susceptible genetic background, two broad issues must be defined: (A) an altered immune program with the development of a chronic immune response involving APCs, memory cells, and effector cells; and (B) end-organ dysfunction with a transition from epithelial precursor cells (such as Clara cells and ciliated cells) to mucous cells. The present review is focused on alterations in the immune program.

how our view of the immune response to respiratory viruses has evolved, based on the identification of specialized programming for host defense and pathogenic programming in chronic disease.

The review is divided into five major sections. Section 2 summarizes the background development of a high-fidelity mouse model for chronic inflammatory lung disease. This model relies on infection with a type of respiratory virus that has been linked to the development of chronic lung disease in humans. Section 3 summarizes work on this model that provides a scheme for the development of acute disease after viral infection. This work has uncovered an immune axis that links the antiviral response that is often Th1 in character to an allergic-type response that is generally Th2 in character. Section 4 summarizes studies of the same mouse model for the development of chronic lung disease and extends these findings to studies of human subjects with airway disease. This work has defined a novel innate immune axis that relies on T cell receptor semi-invariant natural killer T (iNKT) cells that are programmed to activate macrophages to produce IL-13 as well as other products that are characteristic of an alternative pathway for macrophage activation. Section 5 summarizes this review.

2. BACKGROUND FOR THE MODEL

To understand how a specific immune response leads to chronic lung disease, it was critical to generate a representative experimental model that demonstrated a close linkage between acute viral infection and the subsequent development of chronic lung disease. To be faithful to the clinical condition, the model should also include the element of genetic susceptibility as well as virology, immunology, and pathology components that are similar to what occurs in humans. In that regard, epidemiological studies of humans indicate that respiratory syncytial virus (RSV) is the most common cause of serious respiratory illness in infancy and that this particular paramyxovirus is frequently associated with the later development of persistent asthma (Castro *et al.*, 2008; Sigurs *et al.*, 2005). Some experimental evidence suggests that a related paramyxovirus known as human metapneumovirus (hMPV) can cause chronic airway disease in mice, but mucus production was increased for only a short period of time after infection (Hamelin *et al.*, 2006). There is also a reported association between rhinovirus infection and childhood asthma (Jackson *et al.*, 2008), though in this instance, rhinoviruses appear to be associated with acute exacerbation of existing disease rather than acting as a primary cause of chronic disease. Thus, RSV appears to contain distinct ingredients to drive the chronic inflammatory lung disease that develops after the resolution of acute infection.

Based on the epidemiological findings from clinical studies, investigators have often used infection with RSV to develop a model of virus-induced chronic lung disease. In general, however, RSV (like most human pathogens) replicates poorly in the mouse lung unless the virus is first adapted using serial passage or other experimental approaches. To circumvent these issues, RSV is delivered at a high inoculums, but the resulting all-or-none pattern of illness often includes a severe alveolitis rather than the primary bronchiolitis that is typical of human disease (Graham *et al.*, 1988). To better capture this critical feature of human airway disease, we selected an infectious agent known as Sendai virus (SeV). This virus is a mouse parainfluenza-type I virus that is similar to the other paramyxoviruses (e.g., RSV, hMPV, and human parainfluenza virus) that more commonly infect humans. In contrast to infection with human pathogens such as RSV and hMPV, SeV replicates at high efficiency in the mouse lung and causes an acute virus-mediated inflammation of the small airways that is essentially indistinguishable from the RSV-mediated disease observed in humans (Shornick *et al.*, 2008; Tyner *et al.*, 2005; Walter *et al.*, 2001). In particular, the pattern of illness after SeV inoculation resembles the so-called ''top-down'' infection found in humans. In this case, an intermediate inoculum causes infection limited to the airway mucosa and inflammation that is largely restricted to

peribronchial and bronchiolar tissues. Smaller inoculum will cause illness confined to the upper airways (i.e., sinusitis and bronchitis) whereas larger inoculums will cause disease that extends to the alveolar compartment (i.e., pneumonitis). Because chronic inflammatory disease is likely to be found in the small pulmonary airways, we suspect that a severe infection at this site is critical for the subsequent development of chronic lung disease (similar to the case in humans where severe RSV bronchiolitis is associated with chronic asthma).

Consistent with these principles, we found that the acute antiviral response to SeV infection is followed by a delayed but permanent switch to chronic airway disease in mice. This disease is characterized by overproduction of mucus [marked by mucous cell metaplasia (MCM)] and increased airway reactivity to inhaled methacholine (defined by AHR) (Patel *et al.*, 2006; Tyner *et al.*, 2006; Walter *et al.*, 2002). These disease traits of MCM and AHR are hallmark features of asthma and COPD in humans. These traits are also the primary causes of morbidity and mortality in these conditions, and therefore are primary targets for therapy. As developed in this review, the airway disease is first detected at 3 weeks after viral inoculation, reaches its maximal level at 7 weeks, and persists for at least a year later (Kim *et al.*, 2008; Patel *et al.*, 2006; Tyner *et al.*, 2006; Walter *et al.*, 2002). This time course is consistent with the one in humans, wherein chronic lung disease also lasts indefinitely after infection.

As noted above, another characteristic of chronic lung disease in humans that needs to be represented in the mouse model is genetic susceptibility. In that regard, we have observed that the development of chronic lung disease after SeV infection is manifested most vigorously in the C57BL/6J strain of inbred mice. By contrast, the BALBc/J strain of mice exhibits a very similar pattern of acute illness in the first week after viral inoculation, but fails to develop any significant acute disease at 3 weeks or chronic disease at 7 weeks after inoculation (Patel *et al.*, 2006). Other mouse strains (e.g., CV129, C3H/HeJ, or A/J) are so sensitive to SeV (and develop such severe alveolitis) that it is difficult to capture the top-down pattern of illness that is typical of severe RSV infection in humans. In both C57BL/6J and BALBc/J mice, the initial reverse transcriptase polymerase chain reaction (RT-PCR) analysis of virus in whole-lung homogenates indicated that SeV was completely cleared before the onset of airway disease on postinoculation week 3 (Patel *et al.*, 2006; Walter *et al.*, 2002). However, more sensitive PCR assays indicated that low levels of virus may persist for longer periods of time in each of these mouse strains (Kim *et al.*, 2008) and (E. Agapov and M. J. Holtzman, unpublished observations). The role for this remnant viral RNA in driving a chronic immune response still needs to be fully defined. This role as well as the one for host genetics will likely only be resolved after we define the type of immune response that causes the chronic disease

found in this model. In that regard, the C57BL/6J strain provides a suitable genetic background for transgenic and knockout mice that could be used to define the immune mechanism for chronic inflammatory disease after viral infection.

3. cDC T CELL IMMUNE AXIS

In this section, we review our analysis of the mouse model to define how the acute airway disease develops at 3 weeks after respiratory viral infection. This pattern of illness is also frequently found in humans, wherein illness is manifested by persistent cough, sputum production, or wheezing after respiratory viral infection. Based on the short-term course of illness, we assumed that the acute illness might be mediated by the innate immune system. We reasoned that inhibition of the acute inflammatory response could be achieved by targeted disruption of airway epithelial immune-response genes. These genes form a network that is directly induced by viral replication and is dominated by an array of IFN-responsive genes (Koga *et al.*, 1999; Look *et al.*, 1998; Walter *et al.*, 2001). Among candidate genes that might mediate immune cell traffic, the intercellular adhesion molecule (ICAM)-1 is the predominant determinant for epithelial-immune cell adhesion *in vitro* (Nakajima *et al.*, 1994, 1995; Taguchi *et al.*, 1998). Indeed, we found that ICAM-1 expression was induced primarily on host airway epithelial cells (AECs) by viral infection and was necessary for full development of acute inflammation and concomitant postviral AHR. Moreover, we demonstrated that the acute disease (but not the later chronic disease) depended on the upregulation of ICAM-1 (Walter *et al.*, 2002). Therefore, this study illustrated that the acute disease could be genetically segregated from the chronic disease (using ICAM-1-null mice), and thereby served to establish a separate pathogenic mechanism for acute versus chronic inflammatory disease. However, this study did not further define the immune basis of how the acute or chronic disease developed.

The next breakthrough in defining the immune basis for the persistent AHR and MCM after viral infection came when we discovered that these disease traits depended on the production of IL-13 in the lung (Kim *et al.*, 2008; Tyner *et al.*, 2006). Using high-speed flow cytometry in combination with real-time PCR analysis, we were able to identify a major cellular source of IL-13 production as $CD4^+$ T cells (Kim *et al.*, 2008). Although $CD4^+$ Th2 cells are a traditional source of IL-13 production during the allergic response, it is remarkable that this same cell population is also a source of chronic IL-13 production after viral infection. This finding raised the possibility that elements of an adaptive immune response characteristic of the one that develops after allergen exposure (a Th2

response) might also mediate the disease found after viral infection (a Th1 response). When we pursued the underlying mechanism for $CD4^+$ T cell production of IL-13 in this model, we discovered a novel pathway that links acute viral infection to chronic lung disease (Fig. 5.2). This pathway is initiated when viral induction of type-I IFN production drives expression of the high-affinity IgE receptor (FcεRI) on cDCs. Subsequent activation of FcεRI causes production of the chemokine CCL28 and consequent recruitment of $CD4^+$ T cells that produce IL-13 (Grayson *et al.*, 2007a). As described below, the scheme is based on the findings that (1) viral infection induces type-I IFN receptor (IFNAR)-dependent expression of FcεRI on cDCs that are resident in mouse lung tissue; (2) expression of FcεRI on cDCs is followed by generation of antiviral IgE; (3) IgE-dependent activation of FcεRI on cDCs causes release of the T cell chemoattractant CCL28; (4) blockade of CCL28 inhibits MCM after viral infection; (5) loss of FcεRI (in *FceRIa*$^{-/-}$ mice) leads to a decrease in CCL28 production, decreased recruitment of IL-13-producing $CD4^+$ T cells to the lung, and inhibition of MCM after viral infection, while adoptive transfer of wild-type cDCs to *FceRIa*$^{-/-}$ mice restores this immune cascade to wild-type conditions. The findings thereby establish a pathway that mediates chronic IL-13 production by $CD4^+$ T cells after viral infection, and serves to explain how an antiviral response, that is generally Th1 in character, can drive an allergic/asthmatic response that is generally Th2 in character.

3.1. Viral infection causes FcεRIα expression on lung DCs

Respiratory viral infection is a recognized stimulus of IgE production, and IgE levels are known to regulate FcεRI expression (Rager *et al.*, 1998; Skoner *et al.*, 1995; Welliver *et al.*, 1986). We therefore asked which cell type(s) in the lung might express FcεRI during the course after viral infection. In rodents, the high-affinity IgE receptor had been identified only on mast cells, basophils, and possibly eosinophils (Dombrowicz *et al.*, 2000). However, in humans, the receptor may also be found on skin and peripheral blood cDCs, as well as bronchoalveolar lavage fluid plasmacytoid dendritic cell (pDCs), monocytes, and Langerhan cells, albeit as a trimeric form (FcεRIαγγ) that lacks the FcεRIβ chain (Bieber *et al.*, 1992; Foster *et al.*, 2003; Schroeder *et al.*, 2005). We therefore questioned whether this form of the receptor might also be found on resident lung cDCs after viral infection. Using forward/side scatter characteristics and a high level of CD11c expression to identify lung cDCs, we observed that SeV infection leads to a rapid and sustained decrease in the number of lung cDCs, but at least a portion of this cell population remains in the lung and becomes more mature and differentiated (Grayson *et al.*, 2007b). Because the function of this resident population of cDCs was uncertain, we examined it in more detail.

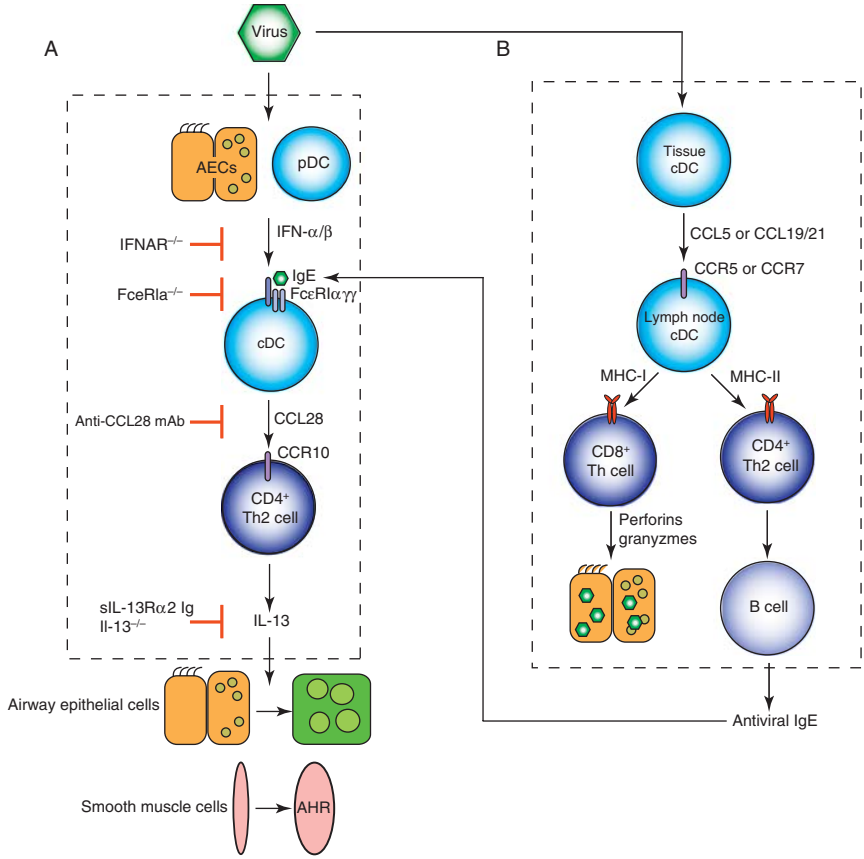

FIGURE 5.2 Cellular and molecular scheme for an adaptive immune axis leading to acute lung disease after viral infection. Viral infection activates two major immune pathways. (A) In one of these pathways, viruses cause AEC and pDC production of type-I IFN. Subsequent IFNAR signaling leads to upregulation of FcεRIα expression on resident lung cDCs. In turn, FcεRI activation by viral antigen and antiviral IgE leads to production of CCL28 and recruitment of CCR10-expressing IL-13-producing Th2 cells to the lung. Persistent IL-13 production drives differentiation of AEC precursors towards mucous cells (MCM) and airway smooth muscle cells to become more reactive to contractile agonists (AHR). (B) In another pathway, viral infection also leads to maturation of lung cDCs that use CCL5-CCR5 and CCL19/21-CCR7 interactions to migrate to regional lymph nodes. In the nodes, these cDCs regulate MHC Class I-dependent generation of $CD8^+$ cytotoxic T cells as well as MHC Class II-dependent production of $CD4^+$ Th cells and consequent B cell production of virus-specific IgE. This IgE is thereby available to participate in FcεRI signaling in the pathway described in (A). Modified from Grayson *et al.* (2007a).

We found that lung cDCs began to express the α-chain of the high-affinity receptor for IgE (FcεRIα) at SeV postinoculation Day 3, and this expression remained detectable for 2–3 weeks after inoculation (Grayson *et al.*, 2007a). Viral induction of FcεRIα expression was specific to cDCs resident in the lung, since there was no detectable expression of FcεRIα on cDCs isolated from draining lymph nodes or spleens of mice after SeV infection. No difference was noted in other surface molecule expression (MHC-II, B220, CD11b, CD80, CD86, or CD23) on lung cDCs from wild-type or *FcεRIα*$^{-/-}$ mice. The level of expression of FcεRIα on lung cDCs at postinoculation Day 7 was similar to levels found on c-kit^{+} lung mast cells. However, mast cells also expressed FcεRIα at baseline before inoculation. We did find a similar level of induction of FcεRIα on pDCs in the lung, but we did not detect expression of FcεRIα on lung CD4^{+} or CD8^{+} T cells, B220^{+} B cells, Mac-3^{+} macrophages, or GR-1^{+} neutrophils.

Based on work with isolated cells (particularly mast cells), mice have been reported to be obligate expressers of the classical tetrameric form (FcεRIαβγγ) of FcεRI (Blank *et al.*, 1989). To determine whether mouse lung cDCs were indeed expressing the tetrameric or trimeric (FcεRIαγγ) form of the receptor, we analyzed mouse lung cDCs for expression of each FcεRI component chain. Based on immunoprecipitation, immunoblotting, and real-time PCR assays for individual FcεRI components, we showed that mouse lung cDCs appear to express only the trimeric (FcεRIαγγ) form of FcεRI (Grayson *et al.*, 2007a). This is the same form of FcεRI as expressed on human antigen-presenting cells (APCs), although expression in mouse cDCs requires induction by a productive viral infection. This requirement may explain the failure to detect FcεRI expression on mouse DCs in previous work. The loss of *FceRIa* gene expression was not associated with an altered immune response against SeV, as evidenced by clearance of SeV. Further, development of an adaptive immune response was not impaired given the appropriate expansion of SeV-specific CD8^{+} T cells in the lung.

3.2. Type-I IFN regulates FcεRIα expression

We next explored what component of the antiviral response is responsible for induction of FcεRIα expression on lung cDCs during viral infection. Serum IgE level tightly regulates expression of the high-affinity IgE receptor in humans, so we reasoned that total IgE and, more likely, SeV-specific IgE might drive FcεRI receptor expression after viral infection in mice. However, we found that serum total IgE and SeV-specific IgE do not increase until 1 week after inoculation (Grayson *et al.*, 2007a). This time course lags behind the onset of expression of FcεRIα on lung cDCs, suggesting that the level of IgE is not driving the expression of FcεRIα under these conditions. In fact, we found that IgE-deficient (*IgE*$^{-/-}$) mice

continued to develop the same increase in FcεRIα expression on lung cDCs as wild-type littermates ($IgE^{+/+}$) after viral infection. Therefore, IgE levels did not appear to regulate the appearance of FcεRIα on lung cDCs after viral infection in mice.

Given that initial expression of FcεRIα developed at the same time as IFN-dependent responses in this viral model, we examined whether IFN signaling was necessary for FcεRIα expression. We found that IFNAR deficient ($IFNAR^{-/-}$) mice no longer exhibited an increase in FcεRIα expression on lung cDCs during viral infection (Grayson *et al.*, 2007a). By contrast, mice that were deficient for *CD1d*, *CD4*, *CD8*, *perforin*, or *MyD88* gene expression showed no defect in *FceRIa* expression after viral infection. Thus, while IFNAR is critical for expression of FcεRIα on lung cDCs after viral infection, there is no significant role for NKT cells, $CD4^+$ T cells, or $CD8^+$ T cells, or for the innate antiviral perforin- or MyD88-dependent Toll-like receptor pathways in this process. We also determined that IFNAR expression on cDCs was not necessary for FcεRIα expression after viral infection. For these experiments, we purified lung cDCs from $IFNAR^{-/-}$ and wild-type control mice and used carboxyfluorescein diacetate succinimidyl ester (CFSE) to discriminate from endogenous cDCs after transfer into wild-type or $IFNAR^{-/-}$ recipients. We found that either $IFNAR^{-/-}$ or wild-type cDC transfer into wild-type mice allowed for expression of FcεRIα on cDCs. By contrast, transfer of either genotype of cDCs into $IFNAR^{-/-}$ mice did not permit expression of FcεRIα on cDCs after viral infection.

These findings imply that IFN acts through another intermediate cell to activate expression of FcεRI on lung cDCs. Using similar cell transfer experiments, we later demonstrated that neutrophils are required for mediating the IFNAR signal to cDCs after viral infection (Grayson *et al.*, 2008). Moreover, the absence of dipeptidyl peptidase I (DPPI), a lysosomal cysteine protease found in neutrophils, dampens the acute inflammatory response and the subsequent MCM that develops after viral infection in mice (Akk *et al.*, 2008). This attenuated phenotype is accompanied by a significant decrease in the accumulation of neutrophils and the local production of CXCL2, TNF, IL-1β, and IL-6 in the lung of infected $DPPI^{-/-}$ mice. Adoptive transfer of DPPI-sufficient neutrophils into $DPPI^{-/-}$ mice restored the levels of CXCL2 and enhanced cytokine production on Day 4 after inoculation and the subsequent MCM at 3 weeks after inoculation. Together, these results indicate that DPPI-dependent neutrophil recruitment also contributes to the acute disease after viral infection.

3.3. FcεRI activation drives CCL28 production

Because IL-13 drives postviral MCM, and Th2 cells are a source of IL-13 in this process (Kim *et al.*, 2008; Tyner *et al.*, 2006), we reasoned that generation of Th2 cells might be linked to FcεRI engagement on cDCs. Using a

cell culture system that contains $CD4^+$ T cells and cDCs, we showed that antigen-specific $CD4^+$ T cells produced IL-13 when cultured with antigen-pulsed cDCs regardless of whether cDCs were isolated from wild-type or *FceRIa*$^{-/-}$ mice or from infected or uninfected mice (Grayson *et al.*, 2007a). Furthermore, cross-linking FcεRI had no effect on T cell production of IL-13 or proliferation. Thus, lung cDCs are capable of driving the development of IL-13 producing $CD4^+$ Th2 cells, but this process does not require expression or activation of FcεRI.

Since we did not detect a requirement for cross-linking FcεRIα in the development of the T cell cytokine response, we next assessed whether FcεRI might be involved in the generation of T cell chemoattractants. Lung cDCs were purified from wild-type mice and were then cultured with a cross-linking antibody against FcεRIα or a control hamster IgG. Supernatants from these cultures were then used in a modified Boyden chamber assay to assess whether a functional T cell chemoattractant had been produced. Supernatants from FcεRIα cross-linked cDCs induced significantly more $CD4^+$ T cell migration than did IgG control supernatants, indicating that engagement of the receptor led to production of a $CD4^+$ T cell chemoattractant (Grayson *et al.*, 2007a). To identify the chemokine receptor for this T cell chemoattractant, we added individual T cell chemokines (CCL5, CCL22, and CCL27) to the upper chamber and evaluated the effect on chemotaxis. Only CCL27 (C-TACK) inhibited this T cell migration, indicating that the $CD4^+$ T cells were moving in response to a CCR10 agonist. Only two known CCR10 agonists have been identified, CCL27 and CCL28 (MEC). Using blocking mAbs, we found that the chemotactic activity was entirely due to CCL28. Relevant to this finding, we noted that CCL28 was associated with both human asthma and mouse models of asthma (English *et al.*, 2006; John *et al.*, 2005; Wang *et al.*, 2000).

To establish that CCL28 expression was inducible in cDCs after a more physiological form of FcεRI activation, we loaded lung cDCs from post-inoculation Day 7 with ovalbumin (Ova)-specific IgE. We found that addition of Ova (by cross-linking IgE bound to FcεRI on the cDC) caused a mark increase in *CCL28* mRNA levels (Grayson *et al.*, 2007a). Similar results were obtained when using the anti-FcεRIα antibody to directly cross-link the receptor. To determine whether FcεRI activation also caused CCL28 expression *in vivo*, we returned to experiments with the mouse model of virus-induced lung disease. As the description of development below, we found that lung levels of CCL28 mRNA were increased after SeV infection (Grayson *et al.*, 2007a). In addition, we found a significant decrease in MCM in mice treated with an anti-CCL28 blocking mAb versus control IgG2b. This finding suggested that CCL28 is a chemotactic factor for $CD4^+$ T cells producing IL-13 and that this particular chemokine is necessary for the full development of MCM after viral infection.

3.4. FcεRI on cDCs is required for acute lung disease

To verify our proposed FcεRI-driven pathway *in vivo*, we next monitored each of the downstream steps in mice that were deficient in *FceRIa* (*FceRIa*$^{-/-}$). Both *FceRIa*$^{-/-}$ and wild-type control mice exhibited similar morbidity (as monitored by weight loss), development of an adaptive immune response (as evidenced by the development of SeV-specific CD8$^+$ T cells), and clearance of virus from the lung (based on SeV copy number) during the acute phase of viral infection. Despite a similar acute response to viral infection, we found that *FceRIa*$^{-/-}$ mice exhibited decreased expression of CCL28, decreased frequency of lung CD4$^+$ T cells, a decreased levels of *IL-13* and *GATA-3* mRNA in lung CD4$^+$ T cells, a decrease in CCR10$^+$ CD4$^+$ T cells, a decreased level of *GATA-3* mRNA (typically found in Th2 cells) compared to *T-bet* mRNA (typically found in Th1 cells), and a marked decrease in the number of Muc5ac-expressing epithelial cells and total lung *Muc5ac* mRNA after viral infection (Grayson *et al.*, 2007a). These findings further supported the proposal that inhibition of MCM in *FceRIa*$^{-/-}$ mice was based on decreased accumulation of IL-13-producing CD4$^+$ T cells due to a lack of production of CCL28 by the resident FcεRIα$^+$ lung cDCs. Together, these findings provide further support for the proposal that activation of FcεRIα on cDCs leads to preferential accumulation of CD4$^+$ Th2 cells in the lung after SeV infection. (CD4$^+$ GATA-3$^+$ IL-13-producing T cells are referred to here as Th2 cells, although they do not appear to produce IL-4 under these conditions.)

To further prove that FcεRI expression on cDCs was necessary for recruitment of CD4$^+$ Th2 cells to the lung and MCM after viral infection, we performed adoptive cell transfer experiments with cDCs from wild-type or *FceRIa*$^{-/-}$ mice transferred into *FceRIa*$^{-/-}$ recipients followed by SeV inoculation. We found that reconstitution with wild-type cDCs restored postviral MCM in *FceRIa*$^{-/-}$ recipient mice. By contrast, transfer of *FceRIa*$^{-/-}$ cDCs was unable to restore the development of MCM after viral infection. Furthermore, we found that CD4$^+$ T cells isolated from lungs of *FceRIa*$^{-/-}$ mice that had received wild-type cDCs expressed *IL-13* and *GATA-3* mRNA at significantly higher levels than mice that received cDCs from *FceRIa*$^{-/-}$ mice. Together, these results indicate that FcεRI on lung cDCs is critical for Th2 cell recruitment and MCM that develops at 3 weeks after viral infection. We note that FcεRI expression returns to baseline levels by 3 weeks after viral inoculation (Grayson *et al.*, 2007a). Consistent with this finding, it appears that *FceRIa*$^{-/-}$ mice are able to manifest chronic lung disease by 7 weeks after viral inoculation (L. A. Benoit, D. E. Byers, M. H. Grayson, and M. J. Holtzman unpublished observations). Thus, another immune pathway must mediate the chronic lung disease that develops after viral infection in the mouse model.

4. NKT CELL–MACROPHAGE IMMUNE AXIS

As summarized in the previous section, our discovery of the cDC-T cell pathway presents a relevant paradigm of how the adaptive immune response can drive acute inflammatory disease after viral infection. This finding was unexpected since the adaptive immune response is most often proposed to drive chronic inflammatory disease and accordingly to have a central role in the development of a variety of inflammatory diseases (Anderson and Bluestone, 2005; Busse and Lemanske, 2001; Herrick and Bottomly, 2003; Jones *et al.*, 2006; Seino and Taniguchi, 2006). By contrast, the innate immune system is believed to mediate the acute response to an infectious agent and is not thought to be solely responsible for a chronic inflammatory state (Mattner *et al.*, 2005). Thus, as we approached the issue of defining an underlying mechanism for the chronic inflammatory lung disease in this model, we might have predicted that chronic inflammation was most likely mediated by the adaptive rather than the innate immune response. Unexpectedly, we next learned that the innate immune response can be solely responsible for the development of chronic inflammatory disease.

In this section, we summarize our work on defining the innate immune pathway for the chronic inflammatory lung disease that develops fully at 7 weeks after respiratory viral infection. The initial insight into the immune basis for chronic lung disease in this model was the identification of lung macrophages as a significant source of IL-13 in the long-term response that developed after viral infection. When we pursued the underlying mechanism for this observation, we uncovered a second immune pathway (summarized in Fig. 5.3) that developed independently of an adaptive immune response and depended on iNKT cells to drive macrophage production of IL-13 (Kim *et al.*, 2008). Analysis of lung tissue obtained from patients with asthma and COPD indicated that this innate immune axis was also activated in humans with chronic obstructive lung disease. Together, the findings from studies of mice and humans provided a mechanism for the transition of an acute viral infection into a chronic inflammatory disease and new mechanistic insight into the pathogenesis of chronic inflammatory disease. Here, we present an outline of these findings and point out how this novel immune pathway differs from other reports of NKT cell and macrophage activation in asthma and asthma models (Akbari *et al.*, 2003, 2006; Lisbonne *et al.*, 2003; Sen *et al.*, 2005; Vijayanand *et al.*, 2007).

4.1. IL-13 requirement for chronic lung disease

After C57BL/6J mice are infected with SeV, airway disease is first detected at 3 weeks after viral infection (as developed above in the previous section), but disease does not become maximal until 7 weeks

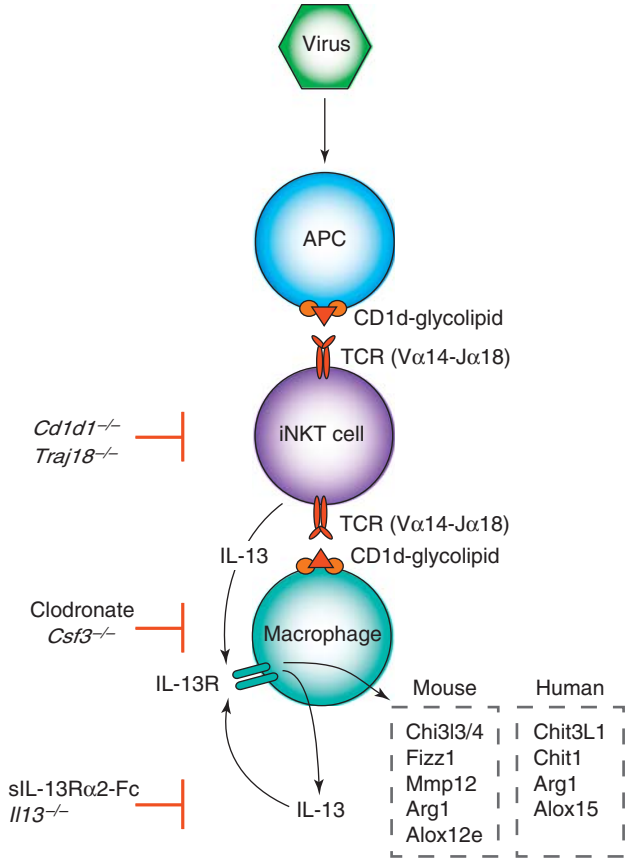

FIGURE 5.3 Cellular and molecular scheme for an innate immune axis leading to chronic lung disease after viral infection. Virus may directly or indirectly activate an APC and thereby facilitate CD1d-dependent antigen presentation and consequent activation of invariant $CD4^-$ NKT cells. NKT cells then interact directly with lung macrophages via IL-13 production and binding to the IL-13 receptor (IL-13R) as well as contact between invariant Vα14 TCR and glycolipid-loaded CD1d. This interaction leads to increased expression of IL-13R and production of IL-13 that drives a positive feedback loop to amplify IL-13 production and alternative activation of macrophages, including *Chi3l3/4*, *Fizz1*, *Mmp12*, *Arg1*, and *Alox12e* gene expression in mice and *Chit3L1*, *Chit1*, *Arg1*, and *Alox15* in humans. Modified from Kim *et al.* (2008).

after infection (Kim *et al.*, 2008; Tyner *et al.*, 2006; Walter *et al.*, 2002). Disease is manifested by leukocyte infiltration as well as AHR (signified by increased pulmonary resistance in response to inhaled methacholine) and MCM (marked by increased lung levels of *Muc5ac* mRNA and Muc5ac immunostaining). Under these conditions, MCM is manifested by overexpression of the predominant airway mucin Muc5ac and

depends on differentiation of mucous cells from airway epithelial precursor cells, that is, ciliated cells and Clara cells that are normally present in the airway epithelium (Tyner *et al.*, 2006). Despite the high levels of IL-13 production in the lung, we did not observe pulmonary emphysema or fibrosis at any time after viral infection (Kim *et al.*, 2008). These additional abnormalities can be found after overexpression of the *Il-13* gene in transgenic mouse models (Chapoval *et al.*, 2007), but the cellular pattern of IL-13 expression in these models may not reflect endogenous cytokine production that is found naturally after viral infection or perhaps other conditions.

Since IL-13 has been widely identified as a critical mediator of lung disease in experimental animal models (including the acute disease that developed after viral infection) and in human asthma, we investigated the role of IL-13 in the development of chronic disease in this model. Similar to the time course for appearance of airway disease, IL-13 production was first detected at 3 weeks and reached maximal levels at 7 weeks after viral inoculation (Kim *et al.*, 2008; Tyner *et al.*, 2006). Furthermore, mice treated with an IL-13 decoy receptor (sIL-13-Rα2-Fc) or targeted to disrupt *Il-13* gene expression ($Il\text{-}13^{-/-}$), no longer developed either MCM or AHR at any time after viral infection (Kim *et al.*, 2008). The inhibition of AHR was independent of any changes in baseline airway caliber. By viral plaque-forming assay, infectious virus is no longer detectable by 2 weeks after infection (Patel *et al.*, 2006; Walter *et al.*, 2002), and, by real-time PCR assay, SeV-specific RNA is decreased to trace levels before airway disease appears at 3 weeks after infection (Kim *et al.*, 2008). Lung levels of viral RNA were similar in $Il\text{-}13^{-/-}$ and wild-type mice (Kim *et al.*, 2008). Therefore, the inhibition of chronic lung disease in $Il\text{-}13^{-/-}$ mice does not appear to depend on any difference in viral replication or clearance. Nonetheless, virus-specific RNA may persist for long periods of time, and this remnant viral RNA may serve to drive a chronic immune response leading to disease. This issue will likely only be resolved after we define the type of immune response that causes the chronic disease traits found in this model.

4.2. IL-13 production by macrophages

We next investigated the cellular sources of IL-13 in the lung after viral infection. As noted above, $CD4^+$ T cells ($CD3^+CD4^+$) contributed the highest amount of *Il-13* mRNA among immune cells in the lung at 3 weeks, but macrophages ($Mac1^+CD68^+$) became the predominant cellular source of *Il-13* mRNA by 7 weeks after viral inoculation (Kim *et al.*, 2008). For $CD4^+$ T cells and macrophages, the contribution to lung *Il-13* mRNA levels was based on an increased number of cells recruited to the lung as well as increased production of *Il-13* mRNA per cell. Other minor

sources of *Il-13* mRNA included NK cells and NKT cells. However, there was no detectable *Il-13* mRNA produced by mast cells, basophils, neutrophils, DCs, B cells, or $CD8^+$ T cells. We also found that lung macrophages cultured from SeV (but not SeV-UV) at 7 weeks after infection expressed *Il-13* mRNA and produced IL-13 protein. IL-13 protein release was further increased by treatment with PMA-ionomycin. Regardless of PMA-ionomycin treatment, IL-13 protein production was increased in lung macrophages compared to $CD4^+$ T cells and in cells isolated from SeV-inoculated mice compared to SeV-UV-inoculated control mice. This pattern for IL-13 protein production was consistent with the analysis of *Il-13* mRNA levels. Furthermore, cells with typical macrophage morphology and CD68 expression were positive for IL-13 immunostaining in alveolar, interstitial, and epithelial locations in wild type but not in *Il-13*$^{-/-}$ mice. In some sections, we found a marked accumulation of macrophages in the airway epithelium that was not observed in uninfected control. The high production of IL-13 per macrophage compared to other cell types (e.g., $CD4^+$ T cells) may be responsible for the detection of $CD68^+IL\text{-}13^+$ but not $CD4^+IL\text{-}13^+$ cells in tissue. Together, these findings provided evidence that lung macrophages are a significant cell source of chronic IL-13 production after viral infection. Ongoing work is aimed at defining the precise subset of macrophages that manifest IL-13 production.

To determine whether macrophages are necessary for chronic lung disease, we next examined macrophage-deficient mice. We first studied *op/op* mice that are macrophage-deficient due to a loss of function mutation in the *Csf1* (also known as *M-csf*) gene. Since macrophages are required for antiviral defense (Tyner *et al.*, 2005), these mice were infected with a reduced viral inoculum. Compared to wild-type mice inoculated with the same amount of SeV, the *op/op* mice exhibit markedly decreased levels of *Il-13* and *Muc5ac* gene expression (Kim *et al.*, 2008). To preserve the ability to develop an acute immune response to viral infection, we next studied wild-type mice that were treated with clodronate-containing liposomes using a protocol that selectively depletes lung macrophages (Tyner *et al.*, 2005). Liposome treatment was begun after clearance of infectious virus to avoid any possible effect on the acute infection. Thus, there was no need to decrease the viral inoculum to preserve survival, and the usual inoculum was administered in these experiments. We found no difference between control and clodronate-liposome treated mice in the levels of *Il-13* or *Muc5ac* mRNA at 3 weeks after viral inoculation, which was consistent with a lack of IL-13 production by macrophages at this early time point (Kim *et al.*, 2008). However, macrophage-depleted mice exhibited significantly decreased levels of *Il-13* and *Muc5ac* gene expression at 7 weeks after viral inoculation compared to mice that were treated with empty liposomes. Immunostaining of lung sections indicated that

clodronate-treated mice were depleted of Il-13-producing macrophages. The loss of IL-13 producing macrophages caused a decrease in *Il-13* mRNA and MCM that was in proportion to the macrophage contribution to total *Il-13* mRNA production in the lung at 7 weeks after viral inoculation.

4.3. NKT cell activation of macrophages

We reasoned that persistent pressure from the immune system was necessary to activate macrophages for prolonged periods after viral clearance. As noted above, this type of chronic pressure is generally attributed to activation of the adaptive immune response. However, we found that MHC Class II-deficient *H2-Ab1*$^{-/-}$ mice that lack most CD4$^+$ T cells continued to develop MCM in concert with increased *Il-13* and *Muc5ac* mRNA levels after viral infection (Kim *et al.*, 2008). We found the same susceptibility to develop chronic airway disease in *CD4*$^+$ and *CD8a*$^+$ T cell-deficient mice. Similar to wild-type mice, these immunocompromised mice contained no detectable levels of viral RNA at baseline and only trace levels of viral RNA in the lung by 3 weeks after viral inoculation. To fully avoid interfering with the acute antiviral response, we also achieved T cell depletion using antibody treatment that (like sIL-13Rα2-Fc and clodronate treatment) was not initiated until after clearance of infectious virus. Similar to mice with a genetic T cell deficiency, mice that were antibody-depleted of CD4$^+$ T cells, CD8$^+$ T cells, or both CD4$^+$ and CD8$^+$ T cells also showed the expected increase in the number of IL-13-producing macrophages, induction of *Il-13* or *Muc5ac* gene expression, and MCM after viral infection. These findings do not exclude a role for T cells in the development of chronic lung disease after viral infection. Indeed, we find that CD4$^+$ T cells are a significant source of IL-13 production at 3 weeks and 7 weeks after viral inoculation. The findings with T cell depletion do, however, suggest that other types of immune cells may also influence the chronic disease traits that develop after viral infection.

The unexpected findings in the context of T cell deficiency led us to consider whether a new type of cellular mechanism could mediate a chronic immune response. In that context, we noted that, in addition to T cells and macrophages, the NKT cell population was recruited to the lung at 7 weeks after viral inoculation. This increase was observed irrespective of whether NKT cells were detected using a α-galactosylceramide (α-GalCer) analog-loaded CD1d-tetramer or by costaining for the NK1.1 and CD3 antigens. Further analysis of the lung NKT cell population indicated that the CD4$^-$ as well as CD4$^+$ NKT cells were recruited into the lung. In fact, CD4$^-$ NKT cells appeared in greater numbers in the lung, especially at 7 weeks after viral inoculation. The involvement of CD4$^-$ NKT cells in driving chronic inflammation was consistent with our

finding that treatment with a CD4-depleting mAb did not decrease *Il-13* or *Muc5ac* mRNA production.

Chronic NKT cell activation after viral infection was associated with a relatively selective increase in *Il-13* mRNA production. Thus, we found relatively little induction of *Ifn*-β, *Ifn*-γ, or *Il-4* gene expression by NKT cells (particularly the predominant $CD4^-$ NKT cell population) in the setting of chronic lung disease after viral infection. This pattern of low *Il-4* mRNA production was also found for $CD4^+$ T cells at 7 weeks after viral inoculation. Consistent with these findings, treatment with an anti-IL-4 mAb from postinoculation Day 12 to Week 3 (or Week 7) caused no significant change in lung *Il-13* or *Muc5ac* mRNA levels or corresponding airway reactivity or mucous cell levels. Therefore, the profile of Th2-type cytokine production after viral infection is distinct from the one after allergen challenge, where IL-4 production is more prominent and often contributes significantly to the development of disease. NKT cell contribution to overall levels of *Il-13* mRNA in the lung is relatively small compared to macrophages at 7 weeks after viral inoculation. Nonetheless, the persistent production of *Il-13* mRNA and the accumulation of NKT cells in the lung suggested that this cell population might act in a regulatory role to drive the chronic production of IL-13 by macrophages in the lung.

The majority of mouse NKT cells express the semi-invariant V_a14-J_a18 TCR chain that recognizes glycolipid antigen presented by the oligomorphic CD1d, an MHC Class-I-like protein (Bendelac *et al.*, 1995; Kowano *et al.*, 1997). To investigate the role of NKT cells in our virus-induced model of chronic lung disease, we studied mice that were deficient in NKT cells due to loss of *Cd1d* or *Ja18* gene expression (Cui *et al.*, 1997; Mattner *et al.*, 2005). We found that $Cd1d^{-/-}$ mice have markedly decreased levels of IL-13-expressing macrophages in lung tissue as well as decreased levels of lung *Il-13* and *Muc5ac* mRNA after viral infection. Moreover, the decrease in IL-13-producing macrophages as well as *Il-13* and *Muc5ac* mRNA levels in the lung were observed at 7 weeks but were not observed at 3 weeks after viral inoculation. This finding was consistent with the time course for increased macrophage production of IL-13 and with the effects of macrophage depletion in the experiments described above. Furthermore, $Cd1d^{-/-}$ mice at 7 weeks after viral inoculation also had decreased AHR relative to wild-type control mice, and this decrease was independent of any changes in baseline airway caliber. We also observed similar inhibition of virus-induced *Il-13* and *Muc5ac* gene expression in the lungs of $Ja18^{-/-}$ mice. In both strains, lung levels of viral RNA were similar to wild-type mice. Together, these findings indicated that NKT cells (independent of CD1d-dependent actions on NKT cells or APCs) were necessary for chronic lung disease after viral infection.

4.4. Basis of NKT cell–macrophage interaction

Based on the findings in NKT cell-deficient mice, we postulated that NKT cells might directly influence the population of IL-13-producing macrophages after viral infection. We reasoned that NKT cells could act by recruiting macrophages to the lung and activating this cell population to produce IL-13. A role for NKT cell-dependent recruitment of macrophages was substantiated when we found that the usual increase in lung macrophages found in wild-type mice was blocked in NKT cell-deficient mice at 7 weeks after viral inoculation (Kim *et al.*, 2008). In support of a mechanism for NKT cell-dependent recruitment of macrophages, we observed that purified lung $CD4^-$ NKT cells released biologically relevant amounts of the monocyte/macrophage chemokines (predominantly CCL3) after stimulation with PMA-ionomycin. Furthermore, lung NKT cells isolated from lungs at 7 weeks after viral inoculation produced increased levels of macrophage chemokine mRNA compared to NKT cells from age-matched control mice without virus-induced lung disease. The predominant chemokine mRNA produced by NKT cells after viral infection was *Ccl3* (consistent with the profile for chemokine production at the protein level), and increased *Ccl3* (as well as *Ccl2* and *Ccl4*) mRNA levels were found exclusively in $CD4^-$ rather than $CD4^+$ NKT cells (consistent with the increased activity of $CD4^-$ NKT cells in chronic lung disease after viral infection). These findings suggest that NKT cells may recruit macrophages to the lung after viral infection, but additional work is required to better define the functional role of NKT cell-derived chemokines *in vivo*.

To determine whether NKT cells might directly stimulate macrophage production of IL-13, we next established a system for coculture of purified NKT cells and macrophages (Kim *et al.*, 2008). In this system, we used NKT cells and macrophages from mice without SeV inoculation to achieve low background levels of IL-13 production. We used α-GalCer-analog-loaded CD1d-tetramer to isolate NKT cells and thereby obtained the same iNKT cell population that was targeted in $Cd1d^{-/-}$ and $Ja18^{-/-}$ mice. The system was also constructed so that NKT cells could be removed after cell–cell interaction to allow for monitoring IL-13 production derived only from adherent macrophages.

A role for lung NKT cell-mediated activation of macrophage IL-13 production was established when we found that lung NKT cells, when cocultured with lung macrophages, caused macrophage production of *Il-13* mRNA and release of IL-13 protein (Kim *et al.*, 2008). The NKT cell effect on macrophage production of IL-13 was relatively specific for lung NKT cells since it was not found for naïve $CD4^+$ T cells. NKT cell stimulation of macrophage IL-13 production was significantly inhibited by treatment with anti-CD1d mAb, indicating that this NKT

cell–macrophage immune axis required direct contact of Vα14-Jα18-TCR on the NKT cell with CD1d present on the macrophage. Furthermore, NKT cell-dependent activation of lung macrophages was increased by 10-fold in the presence of TCR-CD1d ligand α-GalCer, achieving IL-13 production levels similar to PMA-ionomycin stimulation of macrophages. NKT cell-derived IL-13 was also necessary for NKT cell-dependent activation of macrophage IL-13 production, since activation was lost if coculture was performed with NKT cells from *Il-13*$^{-/-}$ mice. CD4^{-} NKT cells are more abundant than CD4^{+} NKT cells in the lung, both at baseline and after SeV infection. To determine whether CD4 expression also influenced NKT cell–macrophage interaction, we studied liver NKT cells to provide a more abundant source of CD4^{+} and CD4^{-} NKT cells. We found that liver NKT cells were less effective than lung NKT cells in driving macrophage activation, in that liver NKT cells required α-GalCer ligand to stimulate macrophage production of *Il-13* mRNA. Nonetheless, pure preparations of either CD4^{+} or CD4^{-} liver NKT cells were both capable of activating lung macrophages when CD1d glycolipid ligand was added to the coculture system. Thus, while CD4^{+} NKT cells have been the focus for studies of the response to allergen in the lung (Akbari *et al.*, 2003), it appears that CD4 expression is not necessary for NKT cell capacity to drive macrophage production of IL-13. Instead, NKT cell-dependent macrophage activation appears to depend primarily on invariant TCR-CD1d and IL-13–IL-13 receptor (IL-13R) interactions.

4.5. IL-13 production and alternative pathway activation

We next further characterized the downstream events in the NKT cell–macrophage–IL-13 immune axis that we had identified. It was possible that persistent activation of this immune axis could be driven by upregulation of a cytokine or cytokine receptor. We therefore applied oligonucleotide microarrays to analyze mRNA isolated from the lungs of mice at 3 and 7 weeks after viral inoculation and examined the microarray gene expression data to identify any chronic changes in cytokine or cytokine receptor expression after viral infection. The only significant change among cytokines or cytokine receptors was induction of Il-13 receptor alpha chain (*Il-13ra1*) gene expression (Kim *et al.*, 2008). Real-time PCR analysis of whole lung samples indicated that *Il-13ra1* mRNA was upregulated in wild-type and *Il-13*$^{-/-}$ mice but not in *Cd1d*$^{-/-}$ mice. This finding indicated that NKT cells stimulated an increase in *Il-13ra1* mRNA.

The relatively small increase in *Il-13ra1* mRNA after viral infection in whole lung samples suggested that increased expression might be restricted to a subpopulation of lung cells such as macrophages. We therefore repeated the real-time PCR analysis of *Il-13ra1* mRNA using macrophages that were FACS-purified from whole lung samples.

Relative to whole lung samples, we found that there was a much greater increase of *Il-13ra1* gene expression in lung macrophages isolated after viral infection, and this increase was also blocked in $Cd1d^{-/-}$ mice. Similarly, immunohistology revealed that *Il-13rα1* was colocalized with the macrophage marker CD68 as well as with IL-13 in lung tissue sections obtained from mice at 7 weeks after inoculation. We therefore hypothesized that the interaction of IL-13 with IL-13 receptor (IL-13R) could amplify macrophage production of IL-13. Indeed, we found that blockade of IL-13 action by sIl-13Rα2-Fc caused a marked decrease in the levels of IL-13-producing macrophages, which, in turn, caused a decrease in *Il-13* mRNA levels in the lung. Together, these findings suggest a positive feedback loop in which IL-13 signaling causes increased production of IL-13 by macrophages. This feedback loop may be amplified by an increase in *Il-13ra1* mRNA expression that is driven by NKT cells. Together with the data from NKT-macrophage coculture, it appears that the IL-13 receptor on macrophages could respond to IL-13 from NKT cells or macrophages to further drive IL-13 production.

We also further characterized the nature of macrophage activation in the immune axis that we had identified. The microarray gene expression data was therefore reexamined to identify any additional chronic changes in gene expression after viral infection. Relative to mice inoculated with UV-inactivated virus, we observed a pattern of gene expression that is characteristic of an alternative pathway for activation of macrophages (Gordon, 2003; Pouladi *et al.*, 2004). The mRNAs encoding chitinase-like proteins (*Chi3l3/4* and *Fizz1*) arginase (*Arg1*), matrix metalloproteinase (*Mmp12*), and arachidonate 12-lipoxygenase (*Alox12e*) were significantly upregulated at 7 weeks, and these same mRNAs were also upregulated but to a lesser degree at 3 weeks after viral inoculation (Kim *et al.*, 2008). Real-time PCR assays for these gene products confirmed the microarray data. Furthermore, these changes were completely blocked in $Il\text{-}13^{-/-}$ mice, and they were partially inhibited in $Cd1d^{-/-}$ mice after viral infection. Immunostaining for Chi3l3/4 protein indicated that the increase in the amount of this protein was localized predominantly to lung macrophages and occurred at the same time as the increases in *Chi3l3/4* mRNA levels. Moreover, FACS-purified lung macrophages exhibited upregulation of the same markers of alternative activation as whole lung samples at 7 weeks after inoculation in wild-type mice as well as downregulation of expression in NKT cell-deficient mice. The findings indicate that viruses can trigger an NKT cell–macrophage–IL-13 immune axis that, when properly amplified, can effectively drive the alternative pathway for activation of macrophages in a chronic response.

Previously, immune pathways promoting IL-13 production were viewed primarily as protection against parasitic infection (Gordon, 2003; Skold and Behar, 2003). Infection by extracellular parasites is known to

stimulate a Th2 cell response with production of IL-4 and IL-13 that can in turn activate macrophages by an alternative pathway (Gordon, 2003). A similar type of response is found after allergen challenge in mice and in allergic asthma in humans (Webb *et al.*, 2001; Zhu *et al.*, 2004). By contrast, intracellular bacteria and viruses characteristically activate a Th1 response with production of IFN-γ and consequent activation of macrophages via the classical pathway. Development of this IFN-dependent pathway is proposed to downregulate the allergic response and protect against allergic airway disease (von Mutius, 2007). However, these general patterns were largely defined within the context of an acute immune response (Holtzman *et al.*, 1996). The present observations indicate that viruses can also trigger long-term activation of NKT cells and achieve chronic production of IL-13 by macrophages themselves. In combination with IL-13 receptor signaling, this innate mechanism can establish a state of persistent macrophage activation that is typical of the alternative pathway. Presumably, this mechanism evolved to mount a long-term innate immune response independently of $CD4^+$ or $CD8^+$ T cells and thereby enable a response to low-level endemic pathogens that are poorly presented by MHC but adequately presented by CD1d. However, in at least some genetic backgrounds, it appears that this type of innate immune activation can also lead to the development of chronic inflammatory airway disease in an experimental model. The next question was whether similar immune activation occurs in humans with chronic inflammatory airway disease.

4.6. NKT cell–macrophage pathway in humans

In that context, we extended our analysis of the NKT cell–macrophage immune axis to humans with chronic airway disease. Based on the established association of acute respiratory viral infection and the subsequent development of chronic asthma, we first investigated whether the NKT cell–macrophage–IL-13 pathway was activated in patients with asthma. We detected an increased number of macrophages that immunostained positive for IL-13 in bronchoalveolar lavage samples obtained from patients with severe asthma relative to the number in samples from healthy control subjects (Kim *et al.*, 2008). Ongoing work aims to address whether the development of IL-13-producing macrophages is specific for severe asthma relative to mild and moderate forms of the disease.

To obtain suitable samples of lung tissue for more detailed analysis, we analyzed lung tissue obtained from lung transplant recipients with severe COPD and lung donors that did not have COPD. The lung tissue obtained from COPD patients exhibited significant MCM as evidenced by an increased number of $MUC5AC^+$ mucous cells and higher levels of *Muc5ac* mRNA in the lung (Kim *et al.*, 2008). Similar to our findings in the

mouse model of chronic lung disease, we also detected an increased level of *IL-13* mRNA in COPD lungs with chronic MCM. The increase in *IL-13* mRNA levels was associated with an increase in the number of cells that immunostain for IL-13 protein in COPD lungs. These IL-13$^+$ cells were identified as lung macrophages based on typical morphology and positive immunostaining for CD68. Furthermore, these IL-13$^+$CD68$^+$ macrophages were found in increased numbers in lungs from COPD patients compared to non-COPD controls. The results from immunostaining indicate that only a subset of macrophages develop the capability for IL-13 production, so additional work is needed to better define the characteristics of this subset.

In that regard, it appears that markers of alternatively activated macrophages (AAMacs) may also be found in concert with increased production of IL-13 in humans with severe asthma or COPD. These markers include arachidonate 15-lipoxygenase, arginase 1, and chitinase and chitinase-like proteins (e.g., Chitinase 3L1 and Chitinase 1 (Chupp *et al.*, 2007; Shannon *et al.*, 1993; Siebold *et al.*, 2008; Zimmerman *et al.*, 2003) and (E. Agapov, J. Battaile, and M. J. Holtzman, unpublished observations). An initial report suggested that AMCase levels are also increased in asthma (Zhu *et al.*, 2004), but this finding was not confirmed in subsequent studies (Siebold *et al.*, 2008). These findings underscore the likelihood that genes from the mouse and human may be homologous for coding sequence without sharing regulatory elements. In addition, small samples of tissues or populations may lead to inaccurate assessment of biomarkers. This issue must be carefully addressed, since a specific immune axis (e.g., the NKT cell–macrophage axis) is likely activated in only a subset of patients with airway disease, and larger populations will need to be studied to obtain accurate molecular phenotypes for complex diseases.

Nonetheless, we have obtained additional evidence for activation of the NKT cell–macrophage system in chronic inflammatory airway disease in humans. Thus, in addition to evidence of macrophage activation, we have also begun to analyze the behavior of lung NKT cells under normal and disease conditions. In an initial analysis, we were able to detect NKT cells in lung tissue based on immunostaining for the invariant Vα24 T cell receptor chain. In concert with increased levels of mucous cells, IL-13 production, and IL-13$^+$ macrophages, we also found that Vα24$^+$ NKT cells were present in increased numbers in the lungs of patients with COPD compared to non-COPD controls (Kim *et al.*, 2008). Recent data indicates that increased levels of *Va24* mRNA are also detectable in lung samples from COPD patients (J. Battaile and M. J. Holtzman, unpublished observations). Together, the findings suggest that an innate NKT cell–macrophage–IL-13 immune axis may be activated in human disease conditions that are similar to the mouse model of virus-induced chronic airway disease.

Our findings for NKT cell–macrophage behavior in mice and humans are distinct from previous reports of immune abnormalities in chronic inflammatory disease in general, and lung disease in particular. For example, iNKT cells were necessary for AHR in a mouse model of allergen-induced asthma and were found in increased numbers in allergic asthma patients (Akbari *et al.*, 2003, 2006; Lisbonne *et al.*, 2003; Sen *et al.*, 2005). However, subsequent reports indicated that NKT cells were not necessary for airway inflammation in the mouse model and that the numbers of iNKT cells in BAL fluid or endobronchial biopsies were no different from normal in either asthma or COPD patients (Das *et al.*, 2006; Vijayanand *et al.*, 2007). Our findings thereby highlight the utility of a more complete assessment of the innate immune response in a mouse-to-man translational approach. When this type of analysis was done, we found that respiratory viral infection provided a more effective stimulus than acute allergen challenge for chronic activation of the immune system in an experimental mouse model. In addition, we achieved a more accurate assessment of a small subset of lymphocytes by using the relatively larger amounts of sample tissue that can be obtained in lung resection or transplantation in patients with airway disease. Together, these resources provided for a synchronized analysis of the mouse model in combination with human patients, and this combination was essential to establish a previously unrecognized role for $CD4^-$ NKT cells and IL-13-producing macrophages in the inflammatory process that drives chronic disease. The results thereby provide the first evidence that the innate immune system, which was formerly thought to act only acutely and transiently, can instead manifest a persistent response that leads to chronic inflammatory disease.

5. SUMMARY

In summary, there is a critical clinical need for better understanding and treatment of chronic inflammatory lung diseases such as asthma and COPD. To address this issue, we analyzed new mouse and cell-based models of chronic inflammatory lung disease and extended our findings to studies of human subjects to better understand the pathogenesis of severe asthma and COPD. In the experimental mouse model, we showed that chronic airway disease could develop after infection with a common type of respiratory virus is cleared to trace levels. The postviral lung disease is manifested by persistent MCM and AHR that is also characteristic of chronic airway disease in humans. When the acute disease appears, it depends on an immune axis that is initiated by IFN-dependent expression of the high-affinity IgE receptor (FcεRI) on cDCs. Crosslinking of this receptor causes cDCs to produce the chemokine CCL28 that

subsequently recruits IL-13-producing $CD4^+$ T cells to the airway. This pathway thereby links the antiviral response that is often Th1 in character to an allergic-type response that is generally Th2 in character. When the chronic lung disease develops, it is unexpectedly independent of an adaptive immune response. Instead, the chronic inflammation is driven by an innate immune axis that relies on T cell receptor iNKT cells that are programmed to activate macrophages to produce IL-13. Studies of mice as well as isolated cells in coculture show that the interaction between iNKT cells and macrophages depends on contact between the invariant Vα14Jα18-TCR on lung iNKT cells and the nonpolymorphic MHC Class I-like protein CD1d on macrophages as well as NKT cell production of IL-13 that binds to the IL-13R on the macrophage. The activated lung macrophages produce IL-13 and overexpress the IL-13R, and this combination of events establishes a positive feedback loop that promotes the persistent expression of IL-13 as well as other IL-13-induced gene products (e.g., chitinase-like proteins and arachidonate 12/15-lipoxygenase) that are characteristic of an alternative pathway for macrophage activation. This innate immune axis is also activated in the lungs of humans with chronic airway disease due to severe asthma or COPD based on detection of increased numbers of iNKT cells and AAMacs in the lung. These findings provide new insight into the pathogenesis of chronic inflammatory disease with the discovery that the transition from respiratory viral infection into chronic lung disease requires persistent activation of a novel NKT cell–macrophage innate immune axis.

Together, our studies identify an adaptive immune response that mediates acute disease and an innate immune response that drives chronic inflammatory lung disease in experimental and clinical settings (as summarized in Fig. 5.4). However, major questions still need to be addressed to better define the basis for chronic inflammatory airway disease. For example, the presence of a persistent innate immune response suggests that there must be ongoing immune stimulation. In that regard, we did not detect any evidence of infectious virus by 2 weeks after inoculation. However, using highly sensitive PCR probes and virus-clean isolation rooms, we were able to detect very low levels of virus-specific RNA in lung tissue until at least 1 year after viral inoculation (E. Agapov and M. J. Holtzman, unpublished observations). Whether viral persistence is necessary for a chronic immune response is still being defined. For example, cDCs are sites of virus uptake, are activated acutely and perhaps chronically after SeV infection, and are capable of activating NKT cells at low levels of antigen (Cheng *et al.*, 2007; Grayson *et al.*, 2007a,b). Our results (like that of others) indicate that iNKT cells may also react to CD1d-expressing macrophages by a mechanism that does not require but can be enhanced by high-affinity agonists such as αGalCer (Hegde *et al.*, 2007). Additional work must therefore be directed

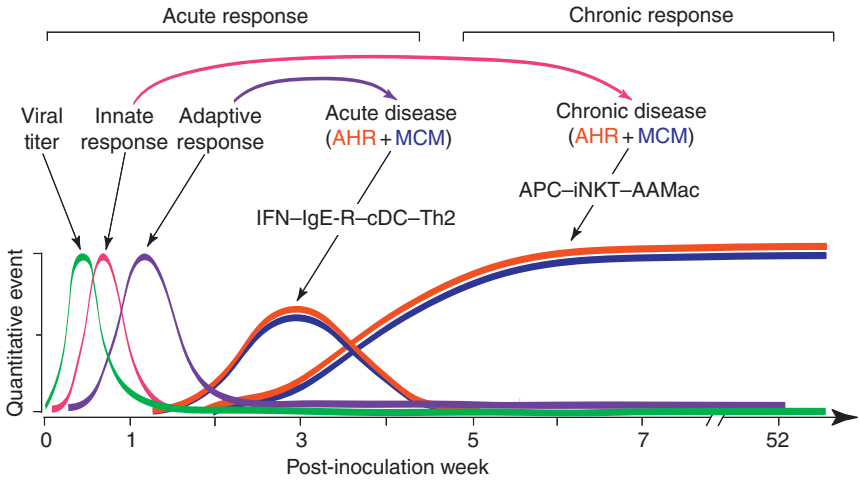

FIGURE 5.4 Time course for immune events in the development of acute and chronic lung disease after respiratory viral infection. Viral replication is maximal by postinoculation Days 3–5 in concert with immune-response gene expression and subsequent immune cell infiltration into the site of infection. The development of acute disease is characterized by acute AHR and MCM and is manifested at 3 weeks after viral inoculation; this disease is driven by a combined innate and adaptive immune response that includes type-I IFN production, high-affinity IgE receptor expression and CCL28 production by cDCs, and recruitment of IL-13-producing Th2 cells. The development of chronic disease is fully manifested at 7 weeks after viral inoculation and is regulated by APC-dependent activation of iNKT cells that drive the generation of alternatively activated macrophages (AAMacs). Modified from Holtzman *et al.* (2002).

at the cause of chronic NKT cell–macrophage activation after viral infection, and the possibility that viral remnants contained in cDCs, macrophages, or other cell types might drive this process.

In addition to questions over the role of the viral trigger for the development of chronic disease, there are additional uncertainties over the full nature of the immune response that leads to IL-13-dependent disease after viral infection. For example, we unexpectedly found that conventional T cell-deficient mice continued to develop the same level of IL-13-producing macrophages and IL-13-associated disease after viral infection. We interpreted these findings with T cell deficiency to suggest that other types of immune cells can also influence the chronic disease traits that develop after viral infection. This interpretation prompted further study of the innate immune response and the discovery of the NKT cell–macrophage immune axis. However, we also recognize that there may be additional immune cell populations that contribute to IL-13 production after viral infection. For example, a distinct non-B non-T (NBNT) cell lineage with no expression of T cell, B cell, or NK cell

markers is also a potent source of IL-13 (Fallon *et al.*, 2006), and preliminary observations suggest that a similar cell population may also be activated after viral infection (Byers *et al.*, 2009). It is also possible that T cell depletion additionally removes an immune cell population that downregulates production of IL-13. For example, a population of $CD4^+$ regulatory T cells (Tregs) is induced by IL-13 and is capable of inhibiting effector T cell responses (Skapenko *et al.*, 2005). We can detect a persistent increase in the number of Tregs in the lung after viral infection (Y. You and M. J. Holtzman, unpublished observations), but the functional role of this cell population still needs to be determined. Moreover, we have provided initial evidence that conventional $CD4^+$ T cells contribute to IL-13 production after viral infection, but even the full role of this cell population in the development of postviral lung disease is still uncertain. We must eventually define how these additional immune pathways respond to viral infection and furthermore, how they interact with the new NKT cell–macrophage pathway to provide a comprehensive scheme for chronic inflammatory disease after infection. Future work must also define more precisely how to correct these abnormalities in susceptible individuals.

ACKNOWLEDGMENTS

The authors gratefully acknowledge their laboratory colleagues for valuable assistance and advice in the course of this work.

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