CHAPTER 1

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

Nadia R. Cohen,[*,1] **Salil Garg,**[†,1] and **Michael B. Brenner**[‡]

Contents

* Harvard Division of Medical Sciences, Graduate Program in Immunology, Boston, Massachusetts, USA
† Harvard Division of Medical Sciences, Graduate Program in Immunology and Harvard-MIT MD PhD Program, Boston, Massachusetts, USA
‡ Division of Rheumatology, Immunology and Allergy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA
[1] These authors equally contributed to this work

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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

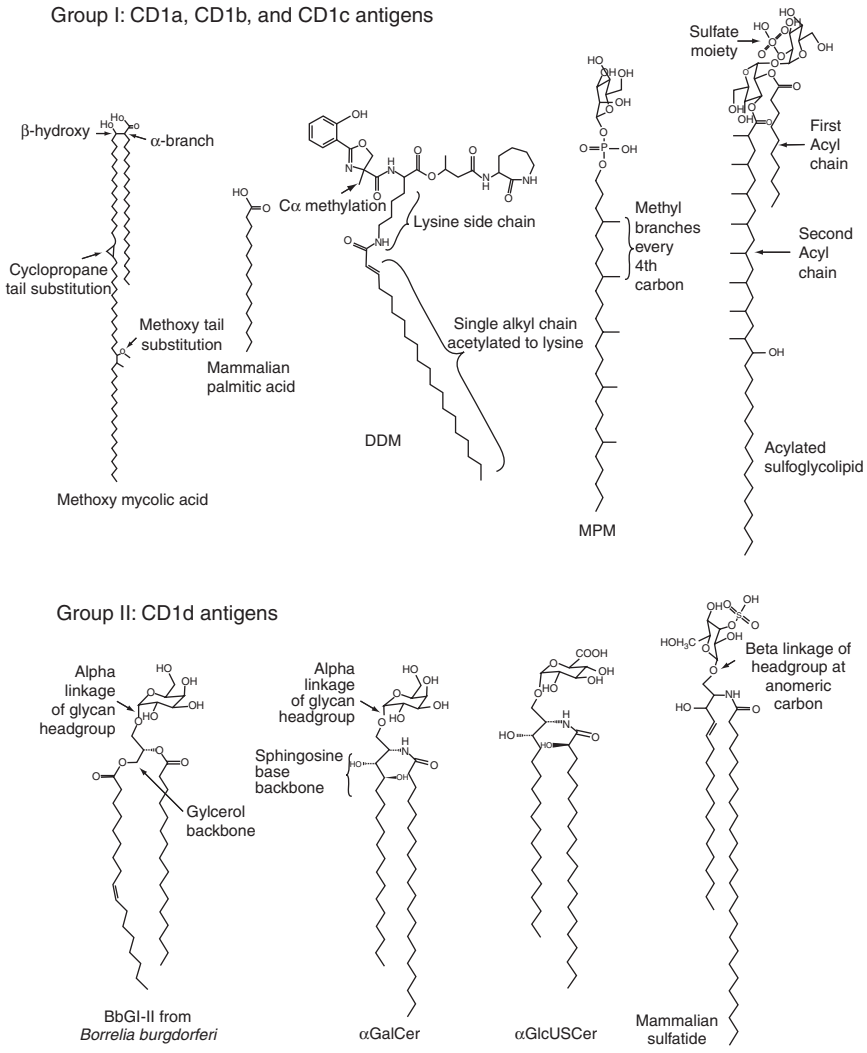

FIGURE 1.1 The structure of lipid antigens. Lipid antigens from a variety of chemical classes are presented for immune recognition by Group I and II CD1 molecules. Group I antigens (top panel) include the fatty acid-derived mycolic acid, the lipopeptide DDM, the isoprenoid-like structure mannosyl phosphomycoketide (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

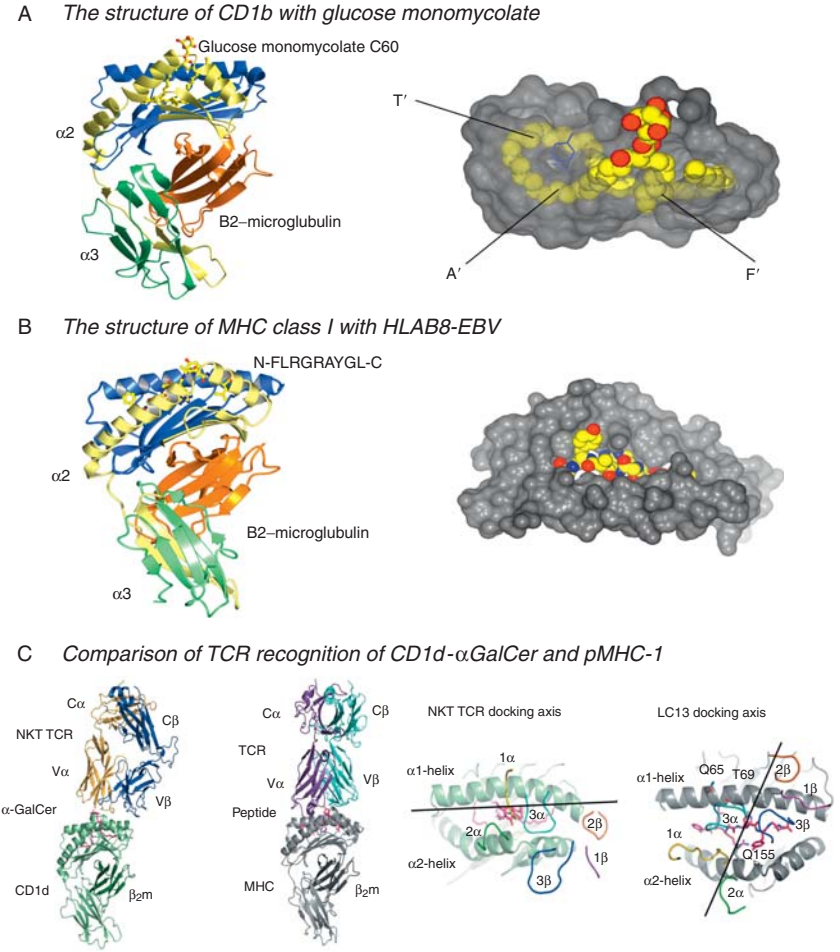

FIGURE 1.2 The structure of antigenic complexes. (A) A ribbon diagram of human CD1b crystal structure loaded with the microbial lipid GMM shown in stick representation. At right is a surface representation of the CD1b–lipid complex. The tails of the lipid antigen bury deep into the hydrophobic antigen-binding 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

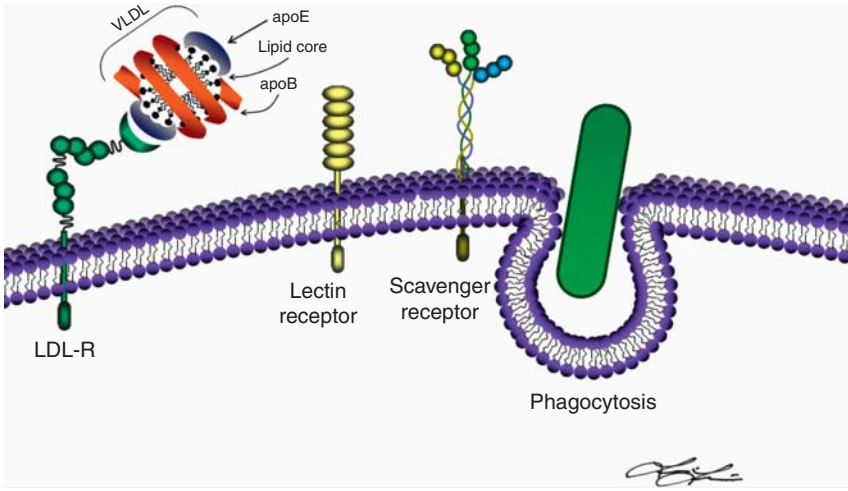

FIGURE 1.3 Pathways of lipid antigen acquisition. Lipids bound in complexes to lipoproteins may be taken up through receptor-mediated pathways utilizing cell surface lipoprotein receptors (e.g., LDL-R), Scavenger receptors and others. Lectin-like molecules (e.g., mannose receptor, DC-SIGN, Langerin) 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.

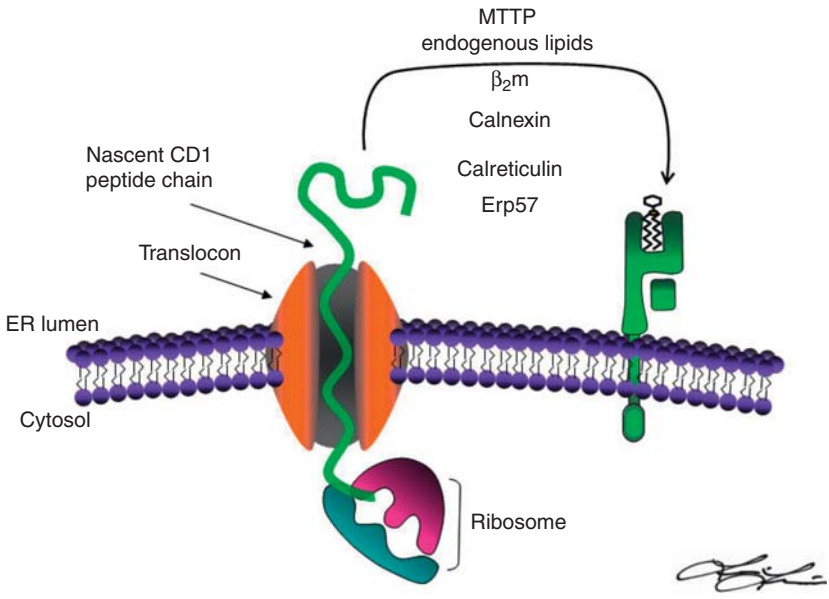

FIGURE 1.4 CD1 assembly in the ER. CD1 heavy chains (HC) are translocated from ribosomes in the cytosol into the ER lumen where N-glycan addition occurs. Mono-glucosylated N-glycan moieties of the CD1 HC bind calnexin and calreticulin. The oxidoreductase Erp57 also associates with HC during its folding 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;

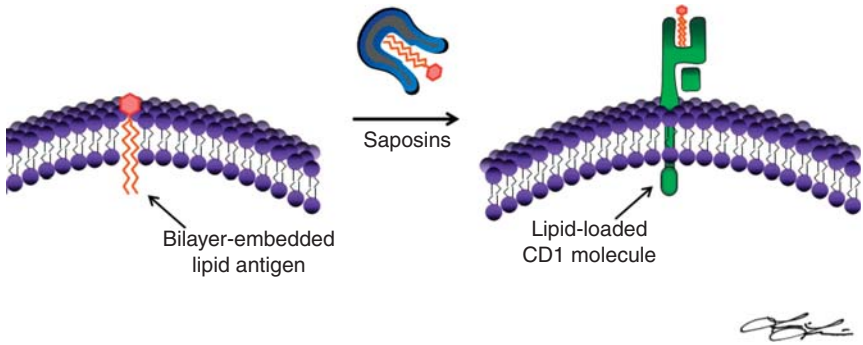

FIGURE 1.5 The role of saposins in lipid antigen loading. Lipid antigens typically localize in cellular membranes. In order to load into CD1, the lipid must be removed from a highly hydrophobic membrane and be transferred into the membrane distal antigen-binding domain of the CD1 HC. This process can 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

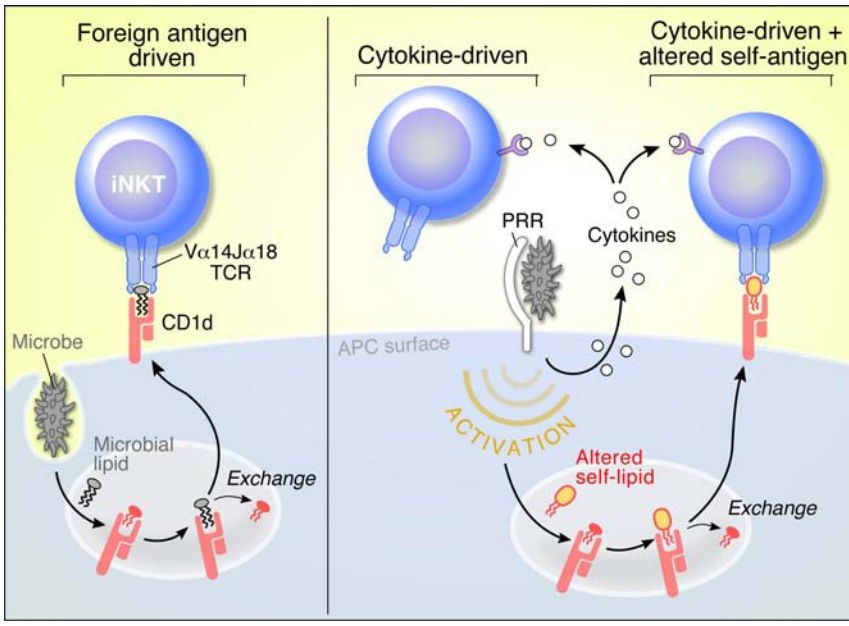

FIGURE 1.6 Mechanisms of iNKT cell activation by microbes. (Left panel) iNKT cells can become activated following cognate, TCR-mediated recognition of foreign microbial lipids presented in CD1d. (Right panel) iNKT cell activation can also occur independently of foreign lipid recognition. iNKT cells can 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

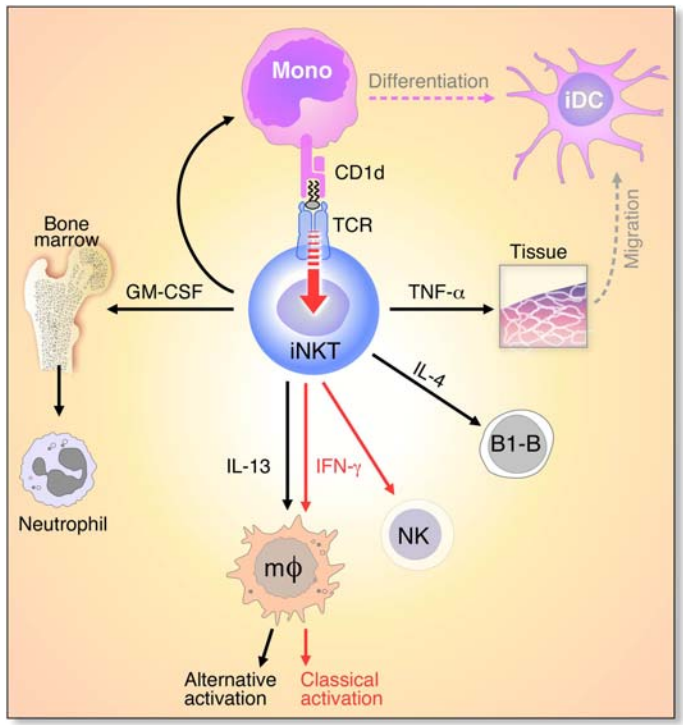

FIGURE 1.7 Activated iNKT cells elicit innate immune responses. Rapid secretion of cytokines by activated iNKT cells leads to mobilization of innate immune effectors such as macrophages (Mϕ), neutrophils, monocytes (Mono), natural killer (NK) cells and B1–B cells.

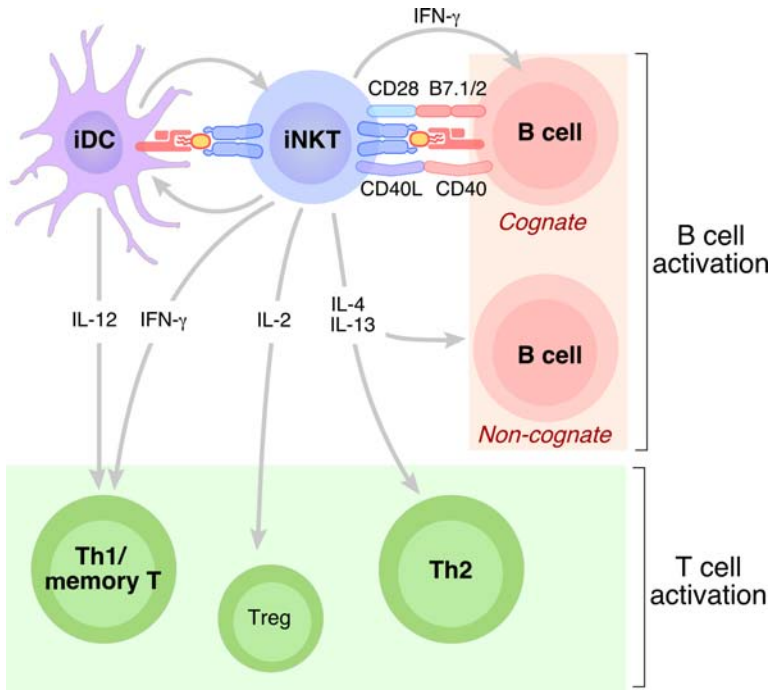

FIGURE 1.8 Activated iNKT cells elicit adaptive immune response. Through cytokine secretion and cell–cell interactions, activated iNKT cells contribute to shaping the adaptive immune response.

(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 $J\alpha18^{-/-}$ 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 $J\alpha18^{-/-}$ mice, suggesting that dNKT cells, which, unlike iNKT cells, are absent from $CD1d^{-/-}$ but not $J\alpha18^{-/-}$ 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.

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