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Review

Polyisoprenyl glycolipids as targets of CD1-mediated T cell responses

D. B. Moody

Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Smith Building Room 514, 1 Jimmy Fund Way, Boston, Massachusetts 02115 (USA), Fax +1 617 525 1010, e-mail: bmoody@rics.bwh.harvard.edu

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Abstract. T cells are well known to recognize peptide antigens presented by major histocompatibility (MHC) class I or class II molecules. More recently, the CD1 family of antigen-presenting molecules has been shown to present both mammalian and microbial glycolipid antigens for specific recognition by T cells. Human CD1c proteins mediate T cell recognition of polyisoprenyl glycolipids, evolutionarily conserved phosphoglycolipids, which function in glycan synthesis pathways. This family of antigenic molecules is particularly attractive for the study of the molecular features that control T cell recognition of self and foreign glycolipids because natural polyisoprenols from mammals, fungi, protozoa, mycobacteria and eubacteria differ in structure. Moreover, these naturally occurring structural differences can influence their recognition by CD1c-restricted T cells. This review of the structural diversity and evolutionary relationships of polyisoprenoid glycolipids emphasizes those features of polyisoprenyl glycolipid biosynthesis that are relevant to their functions as targets of CD1-mediated T cell responses.

Key words. Polyisoprenol; dolichol; antigen presentation; CD1; T cell; mycobacteria; N-linked glycosylation.

Peptide and glycolipid antigens for T cells

The central task of the immune system is to recognize and destroy pathogens, and also to recognize host cells as belonging to self so that they are not damaged during immune responses. Lymphocytes accomplish this by specific interactions with foreign antigens, small molecules that bind immunological receptors and differ in structure between the host and microbial pathogens. The ability of certain microbes such as viruses, protozoa and intracellular bacteria to reside within host cells presents a special challenge for the cellular immune system because it must recognize microbial antigens generated within host cells that are intermixed with abundant self antigens.

Cellular immune responses have long been known to be carried out by T cells that possess clonally variable T cell antigen receptors (TCRs), which interact with foreign peptide antigens bound in the grooves of major histocompatibility class I (MHC I) or class II (MHC II) molecules. In the proper environment, high-affinity TCR interactions with peptide-MHC complexes lead to activation of T cells, which lyse infected target cells and recruit other effector cells to aid in the clearance of pathogens. Thus, cellular mechanisms of host defense rely on receptor-mediated discrimination of the amino acid sequence of microbial peptides [1–3]. More recently, the discovery of antigen presentation pathways mediated by the CD1 family of proteins has demonstrated that T cells also recognize glycolipid components of cellular membranes [4–10]. Since glycolipids differ fundamentally from proteins in the nature of their biosynthetic pathways, biophysical properties and cellular functions, the study of

glycolipid antigen recognition requires application of lipid biochemistry to classical paradigms of immune function.

The human family of CD1 proteins presents glycolipid antigens to T cells

The human CD1 locus is outside the MHC on chromosome 1 and consists of five genes, all of which are now known to encode funtional proteins, CD1a, CD1b, CD1c CD1d and CD1e [11–15]. CD1 antigen-presenting molecules are immunoglobulin-like transmembrane proteins that noncovalently associate with β-2 microglobulin and have demonstrable amino acid sequence homology with MHC I and MHC II [12]. Calabi and Milstein originally proposed that human CD1 genes be divided into group 1 (CD1A, CD1B, CD1C) and group 2 (CD1D), with CD1E having features of both groups [16]. This classification was based on comparison of their predicted amino acid sequences.

More recent insights into the functions of each of the CD1 isoforms have generally supported this separation of CD1 proteins into two groups. Human and murine group 2 CD1 proteins (CD1d) mediate activation of a large population of NK T cells that express certain natural killer (NK) surface markers and a limited repertoire of TCR genes, Vα24 in humans and Vα14 in mice [17–19]. Because CD1d-restricted NK T cells are present in certain organs at a high precursor frequency and have limited TCR and ligand heterogeneity, they have been proposed to be effectors of innate immunity, which regulate other immune cells [20]. In contrast, T cells that recognize antigens presented by group 1 CD1 proteins are not known to have conserved TCR gene usage, and there is evidence for heterogeneity of antigen structure [21]. In addition, only the group 1 CD1 proteins have been shown to recognize mycobacterial glycolipids and been implicated in the physiological immune response to these intracellular pathogens.

CD1 proteins are generally expressed on cells with specialized functions in antigen presentation, including monocyte-derived dendritic cells, Langerhans cells, B cells and thymocytes [22]. The structural similarity of CD1 proteins to known antigen-presenting molecules, as well as their expression on cells with a specialized role in antigen presentation, initially prompted the hypothesis that CD1 could mediate antigen recognition by T cells. Porcelli, Brenner and colleagues provided the first evidence for a functional role of CD1 in glycolipid antigen presentation in their description of human T cells that were activated in a CD1b-dependent manner by a long chain, α-branched, β-hydroxy mycolic acid from mycobacteria [4, 5]. It is now known that four of the human CD1 isoforms present glycolipid antigens for specific recognition by T cells [4, 8, 10, 17, 23, 24]. Chemical analysis of the antigens presented by CD1 has demonstrated that they are lipids or glycolipids composed of mycolic acids, diacylglycerols, ceramides or polyisoprenols [5, 7–10, 23, 25]. In addition, certain hydrophobic peptides are presented by CD1d [26].

Analysis of murine CD1 protein crystals by Wilson and colleagues has demonstrated that the distal face of CD1 is organized into a groove structure that is at least superficially similar in its overall architecture to the grooves found in MHC I and MHC II [27]. However, the CD1 groove is much larger and deeper than those found in peptide antigen-presenting molecules, and the interior of the CD1 groove is lined almost exclusively by amino acids with hydrophobic side chains. Human CD1 proteins have not yet been crystallized, but they show homology with mCD1 and also have hydrophobic amino acids at the positions that are predicted to line the interior surface of the groove [22, 27]. The hydrophobic surface of the mCD1 groove is thought to interact with the aliphatic hydrocarbon chains of amphipathic glycolipids, binding them within the groove so that the hydrophilic elements of the antigen protrude for direct contact with antigen-specific TCRs [7, 21, 27, 28]. Thus, the molecular targets of CD1-restricted T cells are now widely believed to be CD1-glycolipid complexes (fig. 1).

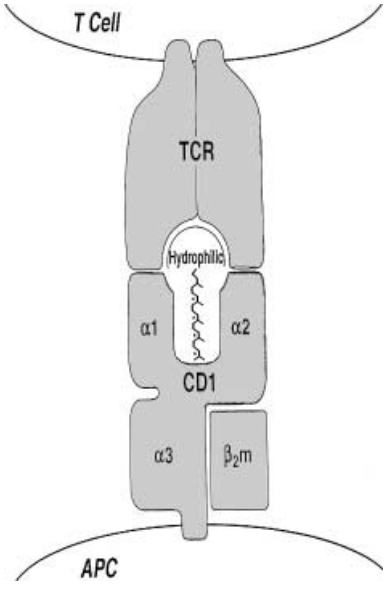

Figure 1. The trimolecular model of glycolipid antigen presentation by CD1 proteins to T cells. Amphipathic glycolipids are thought to insert their lipid moieties into a groove formed by the α1 and α2 domains of CD1 on the membrane distal surface of the protein [7, 27, 39, 40]. The rigid and hydrophilic elements of the antigen, to protrude from the groove to directly contact variable regions of the T cell antigen receptor [21, 28, 54]. In the case of polyisoprenyl glycolipids, the prenyl units are predicted to lie within the hydrophobic cavity, positioning the phosphoglycan moiety on the membrane distal face of CD1c.

In addition to CD1-restricted cells that recognize exogenous antigens of known structure, both $\alpha\beta$ and $\gamma\delta$ T cells can directly recognize CD1-expressing antigen-presenting cells [17, 29–31]. In particular, the discovery of CD1c-dependent activation of $\gamma\delta$ T cells suggests that CD1 proteins could be the long sought after ligands for $\gamma\delta$ T cells [30]. The direct activation of CD1 autoreactive T cells by CD1-expressing antigen-presenting cells is specific for the CD1 isoform structure, but does not require an exogenous glycolipid antigen. Although it remains formally possible that the molecular targets of such autoreactive T cell responses are unliganded CD1 proteins, it is more likely that CD1 autoreactive T cells recognize CD1 bound to endogenous lipids. This is supported by observations that cellular CD1 proteins bind and present self glycolipid antigens and is discussed in more detail in later sections [25, 32, 33].

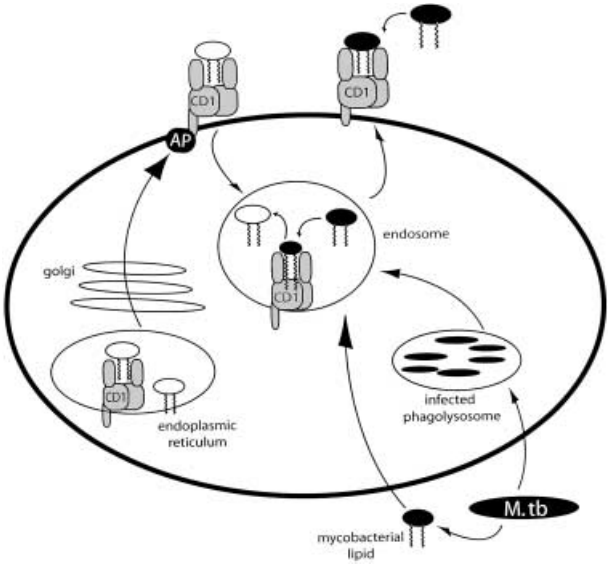

Figure 2. Cellular pathways leading to the presentation of glycolipids to T cells. CD1 proteins are translated in the endoplasmic reticulum, and to egress to the surface via the secretory pathway. CD1b, CD1c and CD1d proteins possess tyrosine-containing motifs in the cytoplasmic tail, which interact with adaptor protein complexes. This interaction promotes the reinternalization of these CD1 isoforms into endocytic compartments where they may be degraded in terminal lysosomes or be recycled to the cell surface for interactions with T cells. Mycobacterial lipids may enter into presentation pathways by binding CD1 at the cell surface, being internalized and binding to CD1 in intracellular compartments or by persistent infection of phagolysosomes that generates CD1-glycolipid complexes within the cell.

Cellular pathways of mycobacterial glycolipid antigen presentation

To date there is no evidence for CD1d-mediated recognition of mycobacterial glycolipids, but a number of studies suggest that CD1a- CD1b- and CD1c-mediated T cell responses could play a physiological role in the human immune response to mycobacterial infection [5–7, 10, 23, 24, 34, 35]. Group 1 CD1 proteins are expressed on activated macrophages and dendritic cells, which phagocytose mycobacteria and are the likely reservoir of mycobacteria during prolonged latent infections [22, 36]. CD1a, CD1b and CD1c mediate human T cell activation by glycolipid components of the mycobacterial cell wall in vitro [5–7, 23, 24]. These T cells generally secrete large mounts of γ-interferon, which promotes the clearance of both natural and experimental mycobacterial infections [24, 37, 38]. In addition, T cells that recognize mycobacterial lipids lyse infected cells and produce granulysin, two additional effector mechanisms that have at least a presumptive role in resolving mycobacterial infections [24, 35]. Recently, polyclonal, glycolipid-specific T cell responses that are mediated by CD1c have been detected in humans after natural *Mycobacterium tuberculosis* infection, providing direct evidence that T cell recognition of glycolipids occurs during the physiologic immune response to this pathogen [10]. Thus, CD1 proteins have been proposed to bind and present glycolipid components of the mycobacterial cell wall for recognition by T cells that could mediate killing of these intracellular pathogens.

The cellular pathway of glycolipid antigen presentation involves the passage of CD1 proteins through multiple cellular subcompartments, where they sample cellular lipids by loading them into the hydrophobic CD1 groove and transporting them to the cell surface for direct contact with T cell receptors (fig. 2). A heterogeneous mixture of phosphatidylinositol-containing compounds has been directly eluted from CD1d proteins, and recombinant CD1 proteins have been shown to bind and present glycolipids added in aqueous solution [32, 39, 40]. However, most CD1 presented glycolipids are not soluble at the concentrations that are required to activate T cells. This suggests that CD1 proteins acquire cellular glycolipids from lipid-binding proteins, membranes or other lipid aggregates, although the precise molecular mechanism of antigen loading onto CD1 proteins in cells is not understood. After glycolipids are inserted into the groove to form CD1-antigen complexes, they are transported to the surface for direct interactions with T cell receptors (fig. 2) [41].

Increasingly, there is evidence that CD1a, CD1b and CD1c differ in their trafficking patterns within cells and their molecular requirements for binding glycolipids. For example, CD1b proteins traffic to the cell surface by the secretory pathway and are thought to undergo reinternalization to late endosomal or lysosomal compartments. This recycling from the cell surface through endosomes is mediated by a tyrosine-containing amino acid motif in the CD1b tail that interacts with adaptor protein com-

plexes, promoting the entry of CD1b complexes into clathrin-coated vesicles for delivery to late endosomes [37, 42, 43]. The low pH of late endosomes is thought to facilitate the binding of mycobacterial glycolipids to CD1b [39, 44].

In contrast, CD1a proteins lack the tyrosine-containing motif that diverts transmembrane proteins to endosomes, so they accumulate at the cell surface at steady state and are thought to bind antigen at the cell surface [45]. CD1c has an intermediate phenotype, as it is present at the cell surface and in endosomes, and may acquire antigens in both compartments [43, 46]. These observations suggest that each of the group 1 CD1 proteins is specialized to survey different intracellular compartments, but together can comprehensively survey cells for antigenic glycolipids [41].

Mycobacterial glycolipid antigen can be introduced to antigen-presenting cells via intracellular and extracellular pathways during tissue infections. During the acute phase, phagocytosis of dead or dying mycobacteria or cell wall debris may provide extracellular antigen that can be phagocytosed and delivered to the endosomal network, where it is likely to intersect with CD1b and CD1c antigen presentation pathways. During the latent phase, mycobacterial infection proceeds primarily as an intracellular process in which mycobacteria persist within infected phagolysosomes. Thus, mycobacterial glycolipids, including those glycolipids that are uniquely produced by live mycobacteria growing within infected cells, also enter presentation pathways from within cells by escape from the phagolysosomes to other intracellular compartments (fig. 2) [47–51]. Delineating the precise compartments where CD1 and glycolipid antigen-trafficking pathways intersect remains an active area of research, as these pathways control the availability of antigens to CD1 proteins and therefore will likely also control their recognition by T cells.

The role of glycolipid antigen structure in the control of T cell responses

CD1 proteins mediate T cell recognition of glycolipid components of mammalian cells and bacterial cell walls. Certain antigens such as mycolyl glycolipids are found only in mycobacteria and related species and may be considered foreign to the mammalian immune system because homologues do not exist in mammalian cells [4, 5, 7, 44]. On the other hand, human T cells can also be activated by glycolipids derived from human or other mammalian sources. Known self antigens include GM_1 gangliosides and two glycolipids which are quite ubiquitously present in mammalian cellular membranes, phosphatidylinositol and phosphatidylethanolamine [25, 33, 52]. Since activation of T cells by self glycolipids in vivo would result in autoimmune damage to tissues, cells likely have mechanisms that prevent T cell activation by these lipids. These observations raise a central immunological question that has been studied in the context of peptide antigens for decades. What is the basis for T cell discrimination of self from foreign glycolipid antigens?

The precise chemical structures of glycolipids, particularly the hydrophilic elements of the antigens that are thought to contact the TCR, determine whether encounter with a glycolipid leads to T cell activation. T cell antigen receptors are well established to precisely interact with the structures of peptides bound onto MHC-encoded antigen-presenting molecules [1, 3]. Alteration of a single amino acid side chain in a peptide antigen can determine whether a TCR binds with high affinity, and subsequently whether a T cell becomes activated or undergoes programmed cell death [53]. It has now been formally demonstrated by experiments involving transfection of TCR α and β chains that the variable regions of TCR chains also mediate the CD1-restricted recognition of glycolipid antigens [10, 21, 48]. These TCR-mediated responses are highly specific for the structure of the distal face of CD1-glycolipid complexes, including both the CD1 protein itself and glycolipid antigens that are bound within the groove.

A role for isoform-specific elements of the CD1 protein in the specificity of the T cell response is supported by the observation that both autoreactive and antigen-dependent T cells are activated by only one, rather than multiple CD1 isoforms [4, 10]. In addition, a series of single point mutations of the α helices on the distal face of CD1b were able to block CD1b-mediated T cell recognition. The particular amino acids that affect recognition conform to a pattern that is similar to the known contact region of TCRs with MHC-encoded antigen-presenting molecules [54]. This suggests that α helices on the distal face of the CD1 protein contribute to the specificity of the T cell response by directly contacting the T cell antigen receptor (fig. 1).

There are now many studies that document the fine specificity of CD1-restricted T cells for the carbohydrate components of glycolipid antigens. For example, CD1b-restricted T cells that respond to glucose monomycolate do not cross-react with mannose monomycolate or galactose monomycolate, glycolipids that differ from GMM only in the orientation of a single hydroxyl group on the hexose sugar [7, 48]. Similarly, CD1d-restricted T cells that recognize α-galactosyl-ceramides do not respond to β-galactosyl ceramides or α-mannosyl-ceramides [8, 55]. These patterns of T cell fine specificity for antigen document the marked precision with which CD1-restricted T cells can discriminate among structurally related glycolipids which is comparable to that of MHC-restricted T cells for peptide antigens. Thus, it is likely that the fine

structure of natural glycolipid antigens will control CD1-restricted T cell responses during natural infections in vivo.

The general model of recognition suggests that the volume of the CD1 groove and the requirement for the glycolipid to be positioned between CD1 and the TCR place certain limits on the size of the antigen (fig. 1). However within these broad limits, the known antigens may have either one or two alkyl chains and range in overall size of the lipid moiety from C_{30} (mycobacterial phosphoisoprenoids, MPI) to C_{80} (mycobacterial glucose monomycolate) or even C_{95} (mannosyl phosphodolichol) [7, 10]. In addition, the core carbohydrate structure can range from zero (mycolic acid) to 1 (glucose monomycolate, α-hexosyl ceramides, MPI), 5 (GM_1 gangliosides) or more than 20 saccharide units (lipoarabinomannan) [5–8, 10, 52]. Thus, size range of known glycolipid antigens would encompass almost any cellular glycolipid, and the general rules that govern which particular classes of glycolipids are loaded onto CD1 proteins and function to activate T cells remain poorly defined.

Receptors on immune cells preferentially bind structures that are produced by microbes

Since the main function of immune cells is to alert the organism to microbial infection, the receptors that mediate both innate and acquired immune responses are generally activated by biochemical structures that are abun-

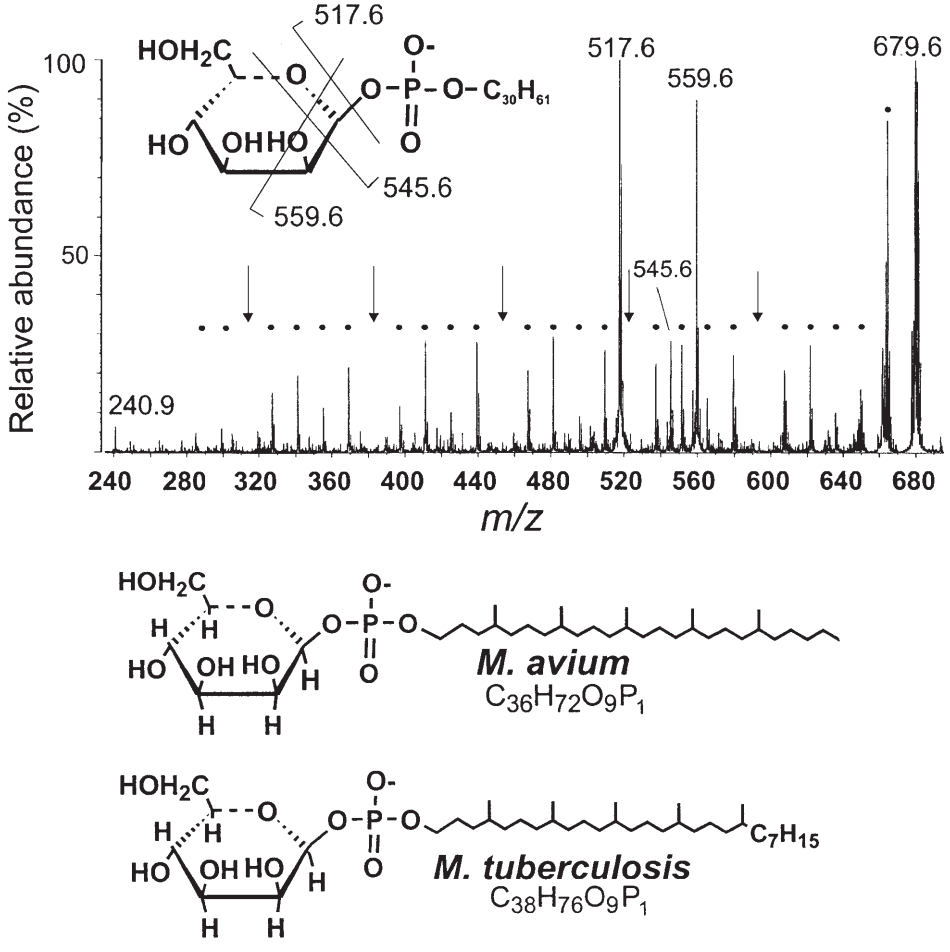

Figure 3. The natural *M. avium* mannosyl phosphoisoprenoid (MPI) presented by CD1c to human T cells. The structure of the MPI was determined by high-energy collision-induced dissociation tandem mass spectrometry using a linked scan of electric and magnetic sectors. A product ion at 517 identified the loss of a hexosyl residue, and ions *m/z* 559 and 545 corresponded to products generated when the phosphate is anomerically linked. These latter ions are reproducibly identified in hexose-1-phosphates with a cis hydroxyl at the 2 position, suggesting the presence of a β-anomeric linkage of the mannose residue [94]. A series of ions separated by 14 *m/z* correspond to alkane series (dots), and the absence of an ion at every fifth position (arrow) in the series indicates the presence of a methyl branch at every fourth carbon on the alkane backbone. These studies identify a previously unknown MPI that is structurally related to a known family of mannosyl-β-1 phosphopolyprenols (MPPs) including human mannosyl phosphodolichol (MPD) [10]. Reprinted by permission from Nature (404: 885) copyright (2000) Macmillan Magazines Ltd.

dant in microbes and rare in the host. For example, products that are unique to microbial pathogens such as lipopolysaccharide (LPS), bacterial deoxyyribonucleic acid motifs, N-formyl methionine peptides and terminal mannosyl residues on glycans potently activate receptors of the innate immune system [56]. MHC-restricted T cells that escape negative selection in the thymus are more likely to target amino acid sequences that comprise microbial rather than mammalian proteins. If CD1-restricted T cells function in host defense against microbial infection, then their activation may be controlled by elements of glycolipid structure that are common in pathogens but lacking in mammalian cells.

This hypothesis can now be considered in detail because human CD1c-restricted T cells have been found to recognize mycobacterial polyisoprenyl glycolipids that are members of a larger family of evolutionarily conserved lipids, which are present in microbes and mammalian cells [10]. Although polyisoprenyl glycolipids are present in all cellular organisms, their precise structures differ systematically among organisms with distant evolutionary relationships [57–60]. In particular, there are consistent differences in the structures of mammalian and microbial polyisoprenyl glycolipids that could control the selective activation of T cells during infection. The following discussion of the structural diversity and evolutionary relationships of long-chain acyclic polyisoprenols emphasizes the structural features of these lipids that could provide the molecular basis for their recognition as self or foreign antigens.

Discovery of MPI antigens

The first known glycolipids presented by the human CD1c protein were recently established to be two previously unknown mycobacterial mannosyl-β-1-phosphopolyisoprenols [10, 23]. These antigens were discovered by analyzing the responses of human T cell lines that were activated only in the presence of CD1c-expressing antigen presenting cells and a complex mixture of mycobacterial components from *M. tuberculosis* and *Mycobacterium avium* cell walls. Mass spectrometric analysis of purified stimulatory compounds demonstrated that they were structurally related phospholipids, mannosyl- and glucosyl-β-1 linked phospholipids with C_{30} or C_{32} linear alkyl chain with methyl branch at the δ-carbon and every fourth carbon thereafter (fig. 3). These two terpenoid phosphoglycolipid antigens were referred to as mannosyl phosphoisoprenoids (MPI) because they contained a methyl branching pattern that is similar to conventional polyisoprenols (fig. 3). Since these mycobacterial antigens were similar in structure to eukaryotic polyisoprenyl glycolipids, particularly mannosyl-β-1 phosphodolichol (MPD), this raised the possibility that human T cells could recognize both mammalian and microbial versions of these antigens.

Biosynthesis of structurally diverse polyisoprenyl glycolipids

Evaluating the hypothesis that foreign isoprenoid glycolipids represent physiologic targets of immune responses during microbial infection requires consideration of species-specific variations in lipid structure among self and foreign organisms. The pathways leading to production of polyisoprenoid lipids via the sequential condensation of C_5 isopentenyl pyrophosphate (IPP) units have been extensively reviewed [58, 61–63]. This discussion focuses on the biosynthetic mechanisms that lead to structural variation among long chain acyclic polyisoprenyl glycolipids that resemble the mycobacterial antigens presented by CD1c. The term polyisoprenol applies to all long-chain, acyclic polyisoprenoid lipids produced from IPP. Dolichols are polyisoprenols that are saturated in the most proximal prenyl unit (α unit), and polyprenols are α-unsaturated polyisoprenols.

Eukaryotes and prokaryotes synthesize polyisoprenols through the classical (mevalonate-dependent) pathway, whereas certain bacteria utilize nonclassical (mevalonate-independent) pathways. In the classical pathway, acetate is converted to mevalonate, which is in turn pyrophosphorylated and decarboxylated to yield IPP, the basic structural unit for all polyisoprenols. More recently, it has been demonstrated that certain bacteria possess a mevalonate-independent pathway of IPP synthesis. This pathway does not generate mevalonate, but instead utilizes glyceraldehyde 3-phosphate and pyruvate to produce isopentanyl units that are chemically indistinguishable from those generated by the classical pathway [61]. In addition to the pathways that use IPP to make conventional polyisoprenols, bacterial polyketide synthesis pathways can generate branched alkane lipids with an enormously diverse array of structures, including those that are similar to polyisoprenols, by varying both the substrates and the enzymatic subunits [64].

By varying the number, orientation, ring formation, saturation, phosphorylation, glycosylation and oxidation of the prenyl units, a tremendous variety of polyisoprenyl glycolipids are produced naturally. Isopentanyl pyrophosphate units are repeatedly transferred by *cis*-isoprenyl transferases onto the prenyl chain to generate progressively longer di-*trans*, poly-*cis* acyclic polyisoprenols. The length of the resulting polyisoprenol is determined by the number of times that isoprenyl transferases act on the substrate, and the final product has an overall length with an integer number of C_5 units. Natural polyiso-

prenols can vary substantially in length among different species; however, polyisoprenols isolated from a given organism do not typically vary in length by more. Moreover, members of implying the existence of species-specific mechanisms for the termination of prenyl elongation [57, 65–68].

Once the full-length polyisoprenol pyrophophosphate has been generated, eukaryotic species produce polyprenol reductases that selectively reduce the prenyl unit most proximal to the alcohol, converting polyprenols to α-saturated polyisoprenols known as dolichols [69, 70]. The primary alcohol of dolichols and polyprenols can be acted upon by enzymes to generate a variety of polyisoprenyl compounds including phosphates, pentose phosphates, hexose phosphates, mycolyl hexose phosphates, pyrophosphates, pyrophosphoglycans and fatty acyl chains [60, 71, 72]. Thus, the hydrophilic moiety of natural polyisoprenyl glycolipids can be quite varied in structure.

Cellular function of long-chain acyclic polyprenyl glycolipids

Long-chain acyclic polyisoprenols are semirigid due to the saturation present in each of the isoprene units. The unusual biophysical properties of these lipids are thought to promote local areas of disorder in packing of membrane diacylglycerols, facilitating the energetically unfavorable process of translocating phosphoglycans to the contralateral surface of membranes [73]. This action may be mediated by flippases that have yet to be identified [74, 75]. After translocation, enzymatic hydrolysis of the phosphate ester allows transfer of glycosyl units for glycan synthesis in a variety of pathways in both eukaryotic and prokaryotic cells such as N-linked glycosylation, O-mannosylation, C-mannosylation, glycosyl phosphatidylinositol anchor (GPI) synthesis and bacterial cell wall assembly [60, 76–79].

Perhaps the most extensively studied of these pathways is the eukaryotic dolichol cycle, which involves the translocation of the hydrophilic head group of MPD from the cytoplasmic face to the lumenal face of the endoplasmic reticulum membrane. Mannosyl phosphodolichol functions as the substrate for the enzymatic transfer of mannose to dolichyl pyrophosphorylglycans, which are subsequently transferred to asparagine units during N-linked glycosylation. The resulting pyrophosphoryldolichol is recycled for subsequent rounds of glucose or mannose transfer. Deletion or mutation of MPD synthase genes in yeast, human and hamster cells leads to the deficient mannosylation of N-linked glycans, demonstrating that no other cellular glycolipid can substitute for this function of MPD [80–83].

Analogous pathways exist in prokaryotes whereby polyprenol mono- and pyrophosporylglycans translocate carbohydrates across the cytoplasmic membrane and donate them to bacterial cell wall structures [60]. The function of the CD1c-presented MPI antigens from mycobacteria has not been established. However, given their structural similarity to polyisoprenyl glycolipids of known function, it is likely that MPIs also function in translocation of mannosyl residues for glycan synthesis within the mycobacterial cell wall [10, 71].

Evolutionary relationships of polyisoprenyl glycolipids

Since long-chain acyclic polyprenols are required for the basic function of transmembrane glycan transport, it is thought that all cellular organisms produce at least one member of this class of lipids. Unlike certain lipids that have conserved structures among even evolutionarily unrelated organisms, polyisoprenols vary systematically among distantly related groups of species in their length and saturation. However, within groups of evolutionarily related species, polyisoprenol structure is conserved, so that it is possible to summarize the phylogenetic relationships of polyisoprenols in a systematic way as seen in figure 4. These variations may be understood in terms of the evolutionary history of the genes that determine the fine structure of these lipids.

An important example of this is seen in the structure of dolichols produced by eukaryotic species and certain primitive bacteria that are saturated in the α unit, as contrasted with α-unsaturated polyprenols from eubacteria [67, 70]. Dolichol production results from enzymatic modification of the α-prenyl unit by polyprenol reductases that are found in eukaryotes and in certain primitive bacteria, but are lacking in most eubacteria, including the major pathogens of mammals [70, 84]. Although dolichyl phosphates function primarily in glycan synthesis pathways in the endoplasmic reticulum, their potential roles as antigens may require that they traffic through acidic intracellular compartments (fig. 2) [46]. Saturation of the α unit stabilizes the phosphate ester of dolichyl phosphates to hydrolysis by high temperatures or low pH, which may allow dolichol phosphates to remain intact within the relatively low pH endosomal compartments present in eukaryotic cells [85]. Although the function of the α-saturated unit in phosphodolichol antigen presentation within acidic endosomes remains unproven, the presence of the α-saturation reliably distinguishes eukaryotic polyprenols from most polyprenols found in most eubacterial pathogens of mammalian species.

Among different eukaryotic organisms, dolichols vary substantially in length from C_{50} to C_{100}. However, among phylogenetically related groups of organisms, dolichol

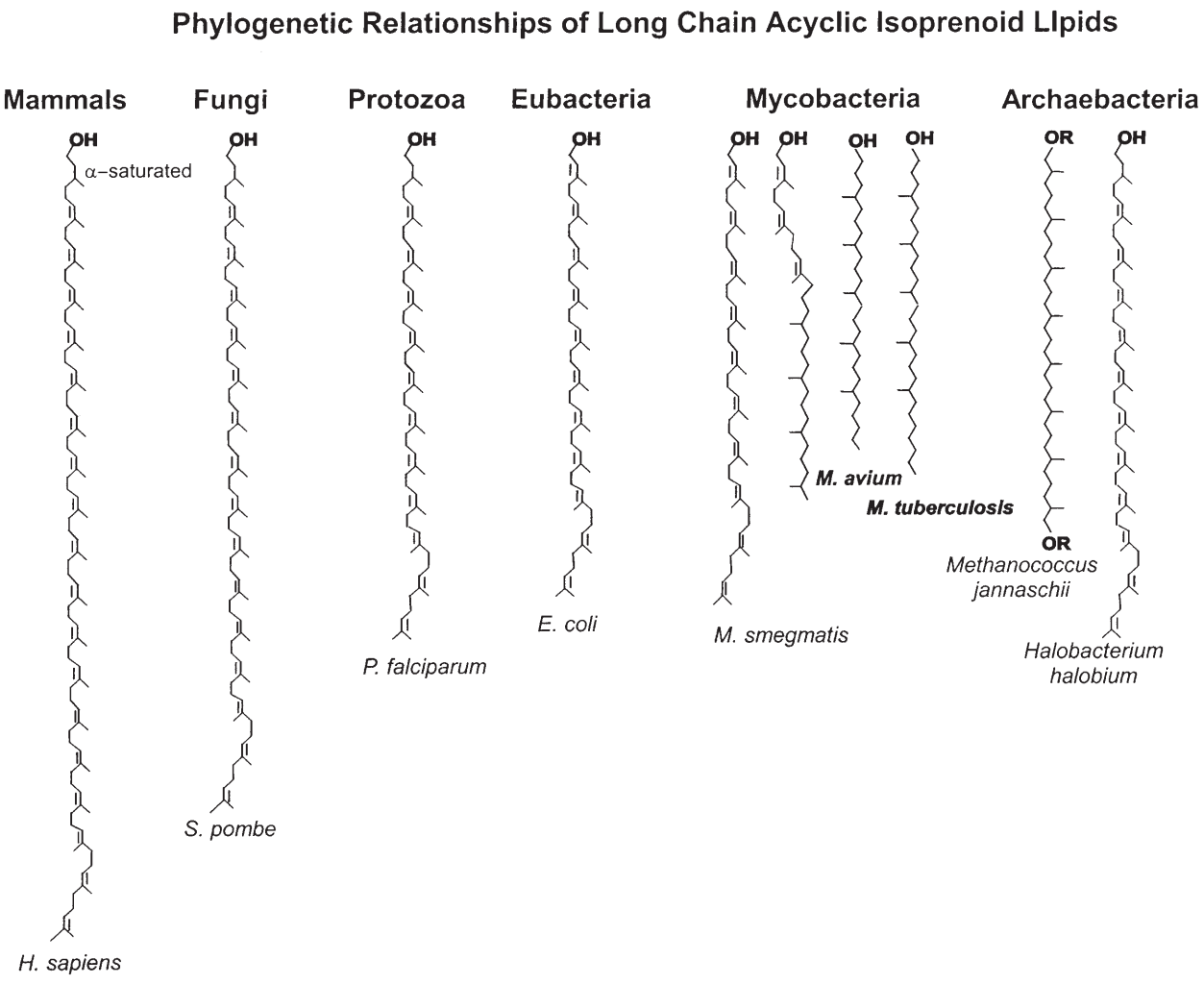

Figure 4. Phylogenic relationships of longchain acyclic isoprenoid lipids. Polyprenols differ systematically in structure among different groups: mammals [57], fungi [70], protozoa [88], eubacteria [96], mycobacteria [10, 65, 71] and archeabacteria [64, 92]. However, members of various species within each group generally synthesize polyprenols that are similar or identical in structure to the representative polyprenol depicted here. Mycobacteria are an exception because they produce polyprenols that vary substantially in length and saturation as indicated. The biosynthetic mechanism leading to production of fully saturated components of mycobacterial and archaebacterial lipids remains to be defined. The polyprenols produced by pathogens are shorter in overall length than those produced by mammals.

length is conserved. For example, multicellular eukaryotes, including humans, produce dolichols that range in length from C_{90} to C_{100} [57]. Fungi produce dolichols that are somewhat shorter in length, typically C_{75-85} [86, 87]. Protozoa, including the human pathogens such as leishmania and trypanosomes, produce yet shorter C_{50-70} dolichols [88, 89]. Polyprenols produced by most eubacteria are generally C_{50-60} in length and are commonly known by their trivial name, undecaprenols [60]. Thus, mammals generally produce polyisoprenols that are longer than those made by organisms that colonize or infect mammals.

In addition to the variations in the structure of the polyprenol unit itself, the hydrophilic groups esterified to polyisoprenol are quite varied [65]. For example, eukaryotic cells utilize dolichyl pyrophosphates as the membrane anchors for synthesis of glycans that are subsequently transferred to asparagine residues in N-linked glycosylation. Thus, dolichyl pyrophosphoglycan intermediates found in the eukaryotic N-linked glycosylation pathway represent a whole range of differentially glycosylated cellular polyisoprenyl glycolipids [60, 90]. Certain of hydrophilic modifications of polyisoprenols are characteristic of evolutionarily related groups of organisms. For example, eukaryotic cells produce glucosyl- and mannosyl-β-1-phosphodolichol, whereas mycobacteria synthesize at least two pentosyl phosphopolyprenols, ribosyl- and arabinosyl-phosphopolyprenol [91]. Thus, at least some of the hydrophilic modifications of polyisoprenyl glycolipids are thought to occur only in microbes.

The structural differences in polyisoprenols isolated from eukaryotes and most eubacteria can be systematically categorized because all of these lipid structures

are derived from the classical IPP unit and therefore do not display substantial microheterogeneity of fine structure. However, certain primitive bacteria produce unusual isoprenoid glycolipids that may or may not be derived form IPP and do not readily fall into the categories depicted in figure 4. For example, certain archaebacteria produce terpenoid lipids that are composed of an integer number of C_5 units, but unlike typical isoprenoids of eubacteria and eukaryotes, the lipids are fully saturated (fig. 4) [92].

Mycobacteria also produce polyisoprenols that vary from classical polyisoprenol structural motifs. Takayama and colleagues have isolated MPDs containing either C_{35} or C_{50} polyprenols with a single unsaturation in each of the prenyl units, similar to the structure of eubacterial polyprenols (fig. 4) [65]. Besra, Brennan and colleagues subsequently identified a mycolated phospholipid (Myc-PL) with mycolic acid esterified to a mannosyl-1-phosphopolyprenol in which the distal units are saturated [71]. The discovery of fully saturated mycobacterial MPI antigens completes the spectrum of the extent of saturation found in mycobacterial polyprenols (fig. 4) [10]. In addition, the lipid portion of MPIs from *M. avium* and *M. tuberculosis* are much shorter than the prenyl units seen in MPDs from other mycobacterial species. This variability of mycobacterial polyisoprenol structure among closely related species is distinctly atypical, as most cellular organisms produce polyisoprenols that are invariant in saturation and only slightly variable in length. These naturally occurring differences in fine structure are candidates for being the molecular determinants by which T cells distinguish self from foreign polyisoprenyl glycolipids.

Glycolipid structural variability over evolutionary time

Although the preceding discussion emphasizes the naturally occurring variations in the structures of polyisoprenyl glycolipids, these rather small differences came about only on the broadest evolutionary time scale (fig. 4). In fact, the natural variations in the structure of homologous polyisoprenyl glycolipids among unrelated individuals of the same species or members of closely related species, if they exist at all, are much less extensive than peptide targets of T cell responses. This is true because the production of polyisoprenyl glycolipids, like that of all complex glycolipids, results from the action of enzymes on substrates that yield intermediates, which are in turn acted upon by additional enzymes in a stepwise fashion. Enzymes specifically act on certain substrates and are often incapable of acting on molecules that differ even subtly in structure from those which are normally found in a given cell type. For example, Waechter and colleagues have shown that yeast MPD mannosyl transferase preferentially acts on synthetic MPD analogs that recapitulate the structure of natural mannosyl-β-1-dolichols from yeast and could not efficiently utilize structurally related glycolipids in mannose transfer [93]. Thus, genetic mutations that lead to altered glycolipid intermediates would be selected against, unless the new intermediates could be utilized in downstream reactions, or the loss of the entire pathway were beneficial to the organism.

This basic point is highly relevant to consideration of glycolipid, in contrast to peptide, antigens for T cells. T cell recognition of a peptide sequence that leads to destruction of a microbe provides strong selective pressure against those organisms that express proteins containing that particular amino acid sequence. DNA sequences are highly mutable, and relatively conservative mutations that alter the epitope recognized by T cells, but which do not alter the function of the protein bearing this epitope, may provide a substantial selective advantage in survival during infection. In this way, T cell responses directed at protein products of mutable DNA sequences may rapidly lead to selection of resistant organisms. Such pressures are thought to underlie the evolution of high levels of polymorphism among MHC antigen-presenting molecules, allowing them to present a broad array of peptide sequences.

It has been possible to define general motifs that determine which peptides bind to MHC-encoded antigen-presenting molecules [1, 3]. However, MHC I and MHC II polymorphism, variability of peptide structure and effects of antigen exposure on the T cell repertoire, make it difficult to formulate general rules that predict which particular amino acid sequences in a pathogen's genome will serve as the immunodominant targets of in vivo T cell responses. In contrast, CD1 proteins do not demonstrate substantial polymorphism, and the functional interdependence of enzymes in the biosynthesis of glycolipids renders organisms much less able to rapidly alter glycolipid structure in response to selective pressures. This is particularly true for pathways leading to glycolipid products with essential functions for which no alternate pathway exists, as is the case for polyisoprenols.

Therefore, it may be possible to define certain features of glycolipid structure that generally control in vivo polyclonal T cell responses among unrelated humans. Furthermore, as discussed in previous sections, certain aspects of polyisoprenyl glycolipid structure are conserved among multicellular eukaryotes including mammals, but differ systematically between mammals and the pathogens that infect mammals (fig. 4). Thus, the fine structure of polyisoprenyl glycolipids contains information that could allow a T cell to detect the presence of a foreign organism. Again, this situation contrasts with that of peptides, which are derived from essentially

the same pool of amino acids in mammals and microbes and differ only in the order of the amino acid sequence. These observations suggest that naturally occurring differences in prenyl length, saturation, and glycosylation that systematically distinguish mammalian polyisoprenoid glycolipids from those of microbes are particularly good candidates controlling human T cell responses.

T cell discrimination of polyisoprenyl glycolipid fine structure

If mycobacterial polyisoprenyl glycolipids function as natural targets for human immune recognition in host defense, then cellular mechanisms likely exist that allow T cells to distinguish microbial polyisoprenyl glycolipids from their mammalian counterparts. This has been investigated by testing the ability of long-term T cell lines to discriminate among purified isoprenoid glycolipids that differ in prenyl length, saturation and glycosylation (fig. 5). T cells that recognize semisynthetic mannosyl-β-1-phosphodolichol (C_{35} MPD) failed to recognize glucosyl-β-1-phosphodolichol (C_{35} GPD) or phosphodolichol (C_{35} PD) analogs (fig. 5) [10]. Thus, CD1c-restricted T cells are capable of precise discrimination of the hydrophilic structure of the carbohydrate portion of the antigen, similar to results obtained in studies of CD1b- and CD1d-presented glycolipids [7, 8, 28, 55]. Though existing CD1c-restricted T cell lines are known to recognize only

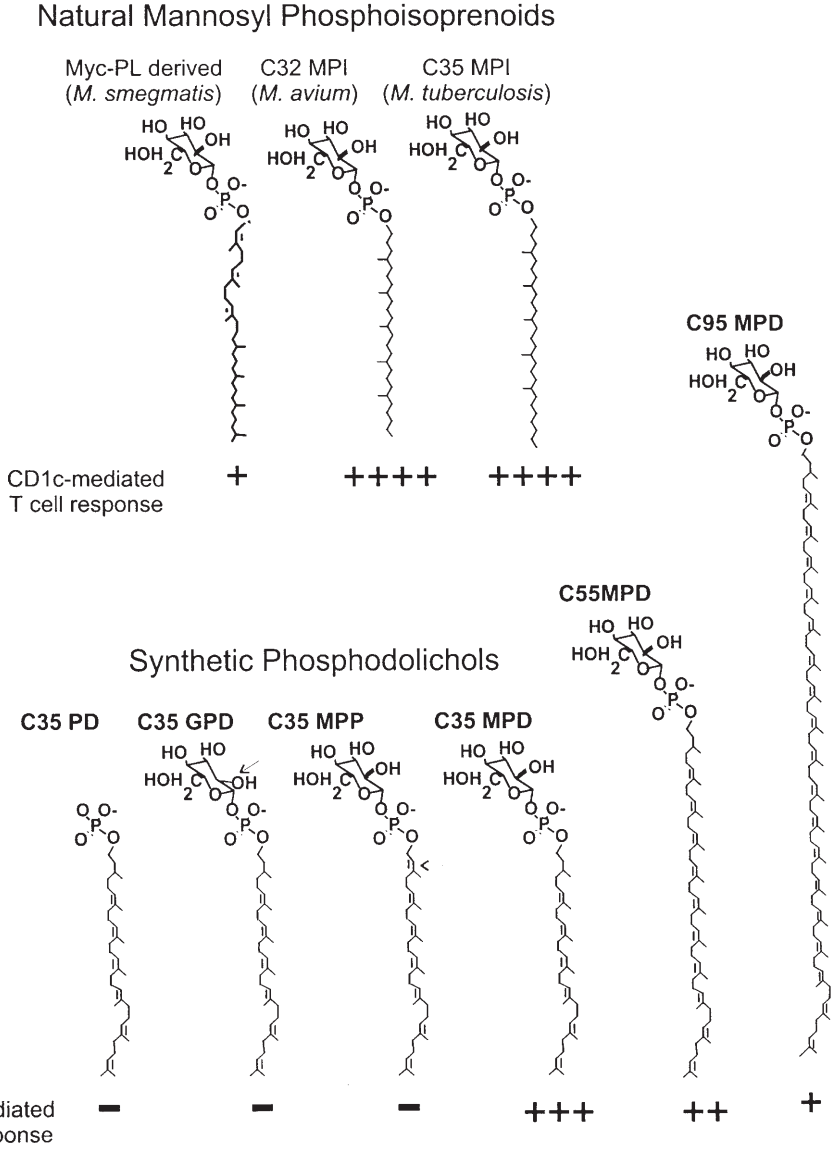

Figure 5. Human T cells discriminate differences in length, saturation and glycosylation of isoprenoid glycolipids. The human T cell line CD8-1 and other CD1c-restricted T cells respond most strongly to natural and synthetic antigens with a mannosyl residue, α-saturated polyprenol and a short prenyl unit [10].

mannosylated glycolipids [10, 27], it is possible that T cells bearing TCR α and β chains other than those described here may differ in their specificity for interacting with the hydrophilic substitutions of isoprenoid glycolipids [21, 28].

In contrast, the prenyl units are predicted to interact with nonpolymorphic regions of the CD1c groove (fig. 1). Thus, the influences of the structure of the distal prenyl units on binding to CD1 would presumably affect all responding T cells, regardless of which TCR is expressed. Analysis of the role of the polyisoprenyl unit in T cell activation demonstrated that T cells which responded to the fully saturated mycobacterial MPI also responded to synthetic MPD analogs with di-*trans,* poly-*cis* dolichyl units (C_{35} MPD, C_{55} MPD, fig. 5) [10]. Thus, the unusual, fully saturated structure of mycobacterial MPI antigens was not required for activation of long-term T cell lines or polyclonal T cell populations from infected patients, and instead could be substituted by polyunsaturated di-*trans*, poly-*cis* dolichols, including those with structures corresponding to natural MPDs from eukaryotic organisms [10].

MPD analogs that differ in prenyl chain length vary in their potency for activating one CD1c-restricted T cell line. T cells that were highly activated by the synthetic C_{35} MPD were less activated by C_{55} MPD and gave only trace responses to a semi-synthetic C_{95} MPD (fig. 5) [10]. The synthetic C_{55} MPD mimics the structures of natural isoprenoid glycolipids from important classes of human pathogens such as leishmania, plasmodia and other protozoa. The synthetic C_{95} MPD was produced from human hepatic dolichol by phosphorylation and glycosylation in vitro, so it represents a semisynthetic analog of a natural self antigen produced by all human cells (fig. 5) [10]. Thus, it is of interest that this CD1c-restricted T cell line is preferentially activated by glycolipids with shorter prenyl chains, which are typical of microbial rather than mammalian cells.

It is not yet clear whether these analyses of several human T cell lines can be generalized to many patients, so more comprehensive analysis of polyclonal T cell responses during natural infection remain an important focus of ongoing study. However, these data do establish that T cells, which recognize polyisoprenyl glycolipids are activated during natural mycobacterial infection. Furthermore, T cells use the TCR to discriminate among antigens that differ in their fine structure, including natural modifications that characterize polyisoprenyl glycolipids from particular groups of foreign organisms.

Since the polyisoprenyl unit is predicted to interact with nonpolymorphic regions of the CD1c protein, prenyl length is an attractive candidate for a general determinant of CD1c-restricted T cell responses to glycolipids of this class, particularly since natural polyisoprenols produced by pathogens are generally shorter than mammalian dolichols. The surface area of the mCD1d protein is known from crystallographic studies to be approximately 1360 Å^2 [27]. Mycobacterial MPIs have a prenyl unit that is of the appropriate size to fit within a groove of this size, and human C_{95} dolichols exceed the predicted volume of the CD1c groove. Thus, selective loading of short-chain polyisoprenyl glycolipids onto CD1c proteins could lead to preferential activation of T cells by antigens from microbial pathogens, while avoiding autoimmune responses to the homologous class of self glycolipid.

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