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Carbohydrate specificity of the recognition of diverse glycolipids by natural killer T cells

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Summary: Most T lymphocytes recognize peptide antigens bound to or presented by molecules encoded in the major histocompatibility complex (MHC). The CD1 family of antigen-presenting molecules is related to the MHC-encoded molecules, but CD1 proteins present lipid antigens, mostly glycolipids. Here we review T-lymphocyte recognition of glycolipids, with particular emphasis on the subpopulation known as natural killer T (NKT) cells. NKT cells influence many immune responses, they have a T-cell antigen receptor (TCR) that is restricted in diversity, and they share properties with cells of the innate immune system. NKT cells recognize antigens presented by CD1d with hexose sugars in α-linkage to lipids, although other, related antigens are known. The hydrophobic alkyl chains are buried in the CD1d groove, with the carbohydrate exposed for TCR recognition, together with the surface of the CD1d molecule. Therefore, understanding the biochemical basis for antigen recognition by NKT cells requires an understanding of how the trimolecular complex of CD1d, glycolipid, and the TCR is formed, which is in part a problem of carbohydrate recognition by the TCR. Recent investigations from our laboratories as well as studies from other groups have provided important information on the structural basis for NKT-cell specificity.

Keywords: *natural killer T cells, T lymphocyte, antigen receptor, glycolipid, affinity*

Introduction

T lymphocytes are key cells of the adaptive immune system that help to initiate and regulate immune responses. Recognition of microbial, viral, or self-antigens by the αβ T-cell antigen receptor (TCR), the type of antigen receptor expressed by most T lymphocytes, has unique properties compared with antigen receptors expressed by B cells, which are the other main cell type of the adaptive immune system. B lymphocytes use immunoglobulins (Igs) to recognize free antigens. The TCR, by contrast, generally recognizes peptide antigens that are bound to or presented by major histocompatibility complex-encoded (MHC) class I or II molecules (1). Therefore, T-cell antigen recognition by αβ TCRs requires the formation of a trimolecular complex between the class I or class II antigen-presenting molecule, a peptide antigen bound to it, and the TCR which recognizes the complex of the peptide antigen with the antigen-presenting molecule.

The types of T-cell antigens have been expanded beyond the realm of peptides through the discovery and analysis of the CD1 family, a group of MHC class I-like antigen-presenting molecules (2). In contrast to the MHC-encoded antigen presenting molecules, which are highly polymorphic, CD1 proteins exhibit little polymorphism. CD1 proteins have been identified as lipid, lipopeptide, and most importantly, glycolipid antigen-presenting molecules.

In this article, we review some general features of the CD1 family and the properties of lipid-reactive T cells, with an emphasis on the glycolipid-reactive natural killer T (NKT) cells. We will describe the progress we have made in understanding the biochemical and structural bases of CD1 glycolipid binding, antigen presentation, and the specificity of NKT cells for particular types of glycolipid antigens.

Antigen presentation by CD1 molecules

CD1 genes and proteins

CD1 molecules are a third family of antigen presenting molecules that are conserved throughout vertebrate evolution (3). This gene family consists of five members in humans (*Cd1a–e*) (4), while mice have two *Cd1d* genes. The CD1a, CD1b, and CD1c proteins are referred to as group I CD1 molecules, based on their sequence similarity, while the more distantly related CD1d is classified into group II. CD1e does not present antigens on the cell surface, but instead is involved in lipid antigen loading in endolysosomal compartments (5). The CD1 heavy chains are associated with β_2-microglobulin (β_2m), which is also a component of the MHC class I molecules, and they are similar in structure to the MHC class I and class II antigen-presenting molecules (6). The hydrophobic antigens they present are almost always lipids, although presentation and recognition of non-lipidic small molecules also has been reported (7). The types of lipid antigens known to be recognized when presented by CD1 proteins are most often glycolipids, but the CD1-mediated presentation of phospholipids and lipopeptides is also well characterized (8–11).

Comparison with MHC antigen presentation

The MHC-encoded class I molecules present peptide antigens to $CD8^+$ T cells, while the MHC class II proteins present peptide antigens to CD4-expressing T lymphocytes. MHC-encoded antigen presenting molecules bind peptides in a shallow but relatively wide antigen-binding groove formed by two anti-parallel α-helices that sit atop a central β-sheet platform. Peptide binding specificity is achieved by polymorphism of residues that cluster in and around the peptide-binding groove, thus changing the shape and chemical properties of the entire groove (1). Individual amino acids of the peptide antigen point up away from the groove for TCR recognition, or down into the groove. The downward pointing or so-called anchor amino acid side chains bind in the major binding pockets of the class I or class II molecules, thereby providing a degree of specificity based on two to three amino acid side chains. Peptide MHC (pMHC)-reactive αβ TCRs bind to both the peptide antigen and MHC molecule through a total of six loops formed by the complementarity-determining regions (CDRs) of both the α- (CDR1-3α) and β-chains of the TCR (CDR1-3β) (12). Upon binding to MHC, the TCR adopts a diagonal orientation above the MHC molecule, with the α-chain situated toward the C-terminal half of the α2-helix, while the β-chain sits above the C-terminal half of the α1-helix of MHC.

Similar to the MHC class I molecules, CD1 molecules present antigens in a binding groove formed by two anti-parallel α-helices that sit atop an anti-parallel β-sheet platform (*Fig.* 1). In contrast to the MHC encoded class I or II proteins, the CD1 binding grooves are relatively narrow, deep, and hydrophobic, and therefore adapted for the binding of alkyl chains. In particular, the CD1d binding groove consists of two connected pockets, generally called A' and F'. While the A' pocket is larger, donut shaped, and more deeply buried, the F' pocket is more accessible to the solvent. Both pockets usually bind one alkyl chain of a dual alkyl chain glycolipid, while the carbohydrate portion is located at the CD1 surface for TCR interaction, and usually in a specific orientation stabilized by hydrogen-bond interactions with CD1 residues (*Fig.* 2). Therefore glycolipid binding involves anchoring within the hydrophobic groove by the lipid moiety, while the portion recognized by the TCR, referred to as the T-cell epitope, is mostly formed by the exposed carbohydrate as well as by the polar regions of the lipid backbone, which are mostly hydroxyl groups. Therefore, the majority of any CD1-presented antigen is buried in the groove of the antigen-presenting molecule, to an even greater extent than for peptide antigen recognition. The TCR therefore contacts a surface composed of amino acids on the α-helices of CD1 and the exposed and generally more hydrophilic portions of the CD1-bound antigen. As a consequence, the reactivity and specificity of CD1-reactive cells for glycolipids is in large part determined by the structure of the exposed carbohydrate.

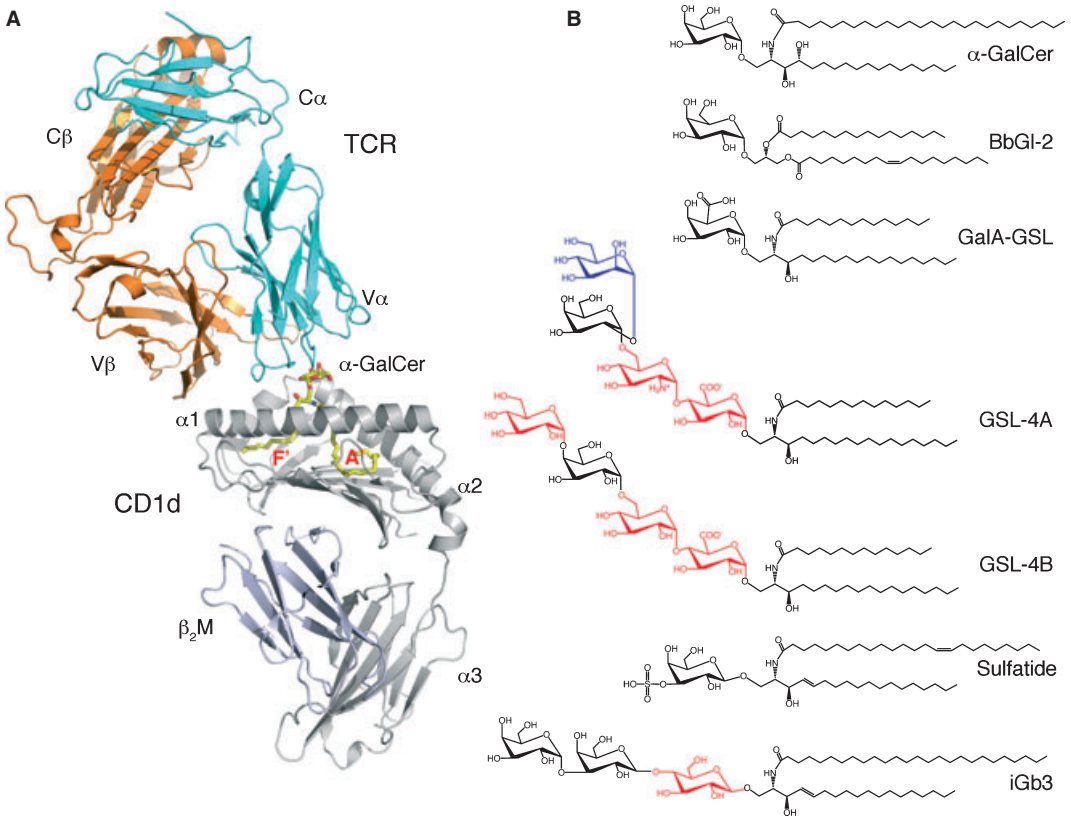

Fig. 1. Structures of the human CD1d–αGalCer–Vα24Vβ11 T-cell antigen receptor (TCR) ternary complex (PDB code 2po6) and glycolipid ligands. (A) Schematic representation of the protein backbone of a human Vα24-Vβ11 iNKT cell TCR (top) and human CD1d with bound αGalCer (bottom). The Vα24 chain (cyan) predominantly interacts with both the αGalCer ligand (yellow) and CD1d (grey), while the Vβ11 chain is offset to the C-terminal end of the α1-helix, without directly contacting the glycolipid ligand (107). (B) Chemical structures of several iNKT cell glycolipid ligands presented by CD1d. Monoglycosyl iNKT cell ligands presented by CD1d contain predominantly galactose or modified galactose (black) bound with an α-glycosidic linkage to either a ceramide or diacylglycerolipid backbone. More complex glycolipids also contain glucose or modified glucose (red) or mannose (blue) hexoses. Note that sulfatide is not an iNKT cell antigen, while the β-linked iGb3 is.

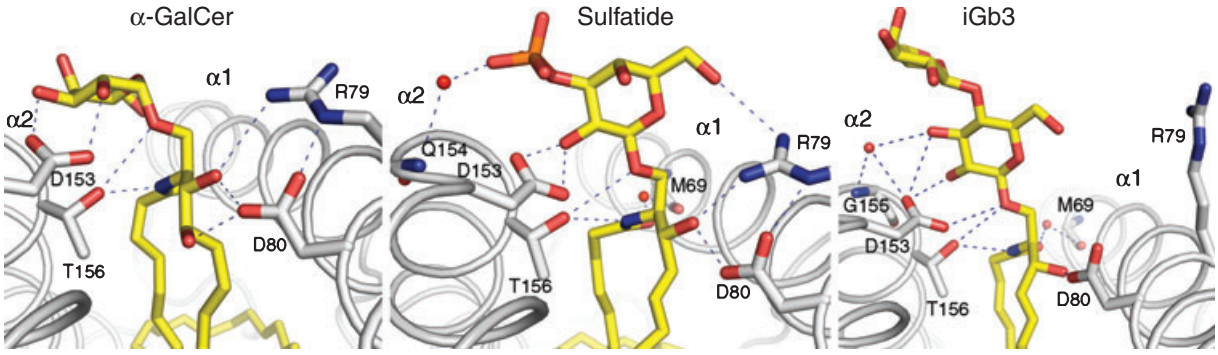

Fig. 2. CD1d hydrogen-bonding network. The CD1d ligands αGalCer, sulfatide, and iGb3 (yellow) are stabilized by hydrogen-bonds (dashed lines) between the α1 and α2-helices of mouse CD1d. Residues that participate in H-bond interactions are depicted. While αGalCer sits flat atop the CD1-binding groove, the β-linked ligands sulfatide and iGb3 are sitting perpendicular and across the α-helices. Note that the terminal α1–3 linked galactose of iGb3 is not depicted, as it was disordered in the crystal structure.

Types of CD1-reactive T cells

CD1-reactive cells with diverse TCRs

CD1-reactive T cells are only a minority compared with their MHC class I- and class II-reactive counterparts, but in a number of instances they have been reported to have a determining influence on the outcome of the immune response. The CD1-reactive T lymphocytes can be broadly characterized into three groups based on the type and diversity of their TCRs. Some of these cells express TCRs composed of γ and δ

chain, a minority TCR isotype in rodents and primates. For example, γδ T cells reactive to the group I CD1 molecule CD1c have been described, but the CD1c-presented antigen they recognize has not been characterized (13). It is likely, however, that the majority of CD1-reactive T cells in mice and humans express the αβ TCR, as this type of cell is generally more prevalent. A second category of CD1-reactive lymphocytes have diverse αβ TCRs, including those specific for the group I CD1 molecules, and some of the T lymphocytes reactive with the group II CD1 molecule CD1d (14–19). These cells with diverse TCRs are thought to be cells typical of the adaptive immune response, in that they are normally in a resting state and then, when they encounter antigen, they will proliferate and differentiate to acquire effector functions.

CD1-reactive cells with invariant TCRs

The members of the third category of CD1-reactive T lymphocytes also are reactive to CD1d, but they express αβ TCRs with a rather limited diversity, and they share features in common with innate immune cells. In mice, they have an invariant α chain encoded by a Vα14–Jα18 rearrangement with a conserved CDR3 region. They typically co-express either Vβ8.2, Vβ2, or Vβ7 β chain, but with highly diverse β chain CDR3 regions (20–22). These cells also express some receptors characteristic of natural killer (NK) cells, such as NK1.1 (NKRP1C), NKG2D, and members of the Ly-49 family (23). Because of the co-expression, they are typically known as natural killer T (NKT) cells. Humans have NKT cells that express an invariant Vα24–Jα18 rearranged α chain with Vβ11 (24, 25), although their CDR3 diversity is less pronounced (26). Human Vα24 and Vβ11 are orthologs of the mouse Vα14 and Vβ8 proteins, and the CDR3α regions are highly conserved comparing mouse and human. The evolutionary conservation of this specificity is striking, as mouse NKT cells recognize the same glycolipid antigen when presented by either mouse or human CD1d, and as is true for human NKT cells (27). This conservation of specificity, over a period greater than 60 million years, suggests these cells play a unique and important role in the immune system.

The simple classification of NKT cells as T lymphocytes that also express NK receptors is problematic (28), however, because many conventional T cells not reactive with CD1d can induce expression of NK receptors, including NK1.1/CD161, upon activation (29). Additionally, many CD1d-dependent NKT cells, including those with more diverse TCRs, and those in the third category with limited TCR diversity, do not express NK1.1. Because of the potential confusion arising from this complexity, there have been various attempts to classify NKT cells. We prefer a categorization based on TCR diversity. Therefore, the CD1d-reactive mouse cells with invariant TCRs are referred to here as Vα14 invariant (Vα14i) NKT cells, the corresponding human cells are known as Vα24i NKT cells, and the populations known generically as *i*NKT cells.

Properties of *i*NKT cells

Frequency and development

Vα14i NKT cells are found at the highest frequency in liver (>10% of liver lymphocytes), but are present at lower frequencies (typically <1%) in thymus, bone marrow, spleen, and blood, and at even lower frequencies in lymph nodes. They have a different developmental pathway with positive selection mediated by $CD1d^+$ double positive thymocytes rather than cortical epithelial cells (30, 31). Furthermore, there are a number of surface molecules, adapters, signaling molecules, and transcription factors that are uniquely required for Vα14i NKT cell differentiation and function (32). Their distinct developmental pathway in the thymus supports the hypothesis that Vα14i NKT cells constitute a separate T-cell sublineage. Human Vα24i NKT cells tend to be much less abundant than their Vα14i NKT cell counterparts, and they are not highly enriched in the liver, although their frequency in peripheral blood mononuclear cells can vary over two orders of magnitude in healthy individuals (33, 34), and they constitute only a small percentage of the cells that express an αβ TCR and NK receptors such as CD161. Like in the mouse, however, Vα24i NKT cells can rapidly produce effector cytokines after stimulation, and their development depends upon expression of the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), which is not required for conventional T lymphocytes in either species (35–37).

During thymic differentiation, Vα14i NKT cells proliferate and the chromatin in the loci encoding interferonγ (IFNγ) and interleukin-4 (IL-4) opens up (38). As a consequence, Vα14i NKT cells in the thymus and the periphery transcribe low amounts of these cytokine messanger RNAs (mRNAs) even before stimulation with exogenous antigen (38, 39). Following activation, they produce copious quantities of IL-4, IFNγ, and other cytokines within a few hours (40). Conventional naive T cells, by contrast, require days of proliferation and differentiation before they can produce effector-type cytokines. The rapid response by Vα14i NKT cells is similar to innate immune cells, and therefore Vα14i NKT cells may be thought of as innate immune lymphocytes.

Activation in the absence of foreign antigen

Perhaps as a result of their rapid production of effector cytokines, Vα14i NKT cells have been reported to exert a pivotal role in determining the outcome of a variety of immune responses and pathologic conditions in mice, including the development of autoimmunity, maintenance of self-tolerance, and the response to tumors and infectious agents (41). Surprisingly, several studies have highlighted the role of Vα14i NKT cells in inflammatory conditions, even when an exogenous antigen is not obviously present. These inflammatory conditions including the development of atherosclerotic lesions in mice on a high fat diet or deficient for apolipoprotein E (42, 43), a model of chronic obstructive pulmonary disease (COPD) in mice infected with Sendai virus (44), ischemia reperfusion injury in the liver (45), a cecal cauterization model of intestinal adhesion formation (46), and ozone induced airway hyperreactivity (47). Activation of Vα14i NKT cells in some of these models may be driven by exposure to self-antigens. Evidence strongly suggests that iNKT cells have a degree of self reactivity, because they have an antigen experienced phenotype in cord blood (26) and in germ-free mice (48), and CD1d autoreactivity can be measured *in vitro* under some conditions (49). Isoglobotrihexosylceramide (iGb3), a glycosphingolipid with the structure Galα(1,3)Galβ(1,4) Gluβ(1,1)ceramide, has been identified as a self-antigen that may be required for the positive selection Vα14i NKT cells (50). These data have been called into question by several studies, including the finding that iGb3 synthase gene-deficient mice have a normal number and function of Vα14iNKT cells (51), and therefore it is likely that there are additional, important self-antigens for these cells.

Response to infectious agents

Although Vα14i NKT cell activation, expansion, and recruitment have been shown following infection (52), this is not necessarily due to the recognition of microbial antigens by the iNKT cell TCR. For example, the recruitment of Vα14i NKT cells to granulomas does not require recognition of cognate antigen by the Vα14i NKT cell TCR, as it can occur even when these T cells are adoptively transferred to $Cd1d^{-/-}$ mice (53). Furthermore, Vα14i NKT cells can be activated *in vitro* and *in vivo* directly by cytokines, such as IL-12 plus IL-18 (54), or IL-12 alone, even in the apparent absence of a TCR signal (55). Therefore unlike the specific, TCR-mediated recognition of mycobacterial cell wall antigens presented by human CD1a, CD1b, and CD1c molecules, the recognition of microbial glycolipids presented by CD1d to the invariant Vα14i TCR may not be required for activation. Instead an indirect pathway of microbial recognition, mediated by cytokines such as IL-12, and perhaps in some cases in conjunction with the presentation of self-antigens by CD1d (56, 57), may be important. A CD1d-independent but IL-12-dependent pathway is required for the activation of Vα14i NKT cells after infection with mouse cytomegalovirus (58, 59). A similar pathway is likely to be involved in the stimulation of Vα14i NKT cells in some of the inflammatory models described above.

Vα14i NKT cells have been shown to participate in protection of mice from a variety of bacterial, viral, and protozoan parasites (41, 52, 60, 61). Much less is known about the role of human Vα24i NKT cells in infections, but because they express the antimicrobial peptide granulysin (62), they at least have the potential for anti-microbial activity. Patients deficient for the SAP have uncontrolled Epstein Barr virus infections, but they have other immune defects in addition to the absence of Vα24i NKT cells. Additionally, there is a report of an 11-year-old girl with disseminated varicella infection after vaccination who was found to have a deficiency of Vα24i NKT cells and no other immune defect (63).

Specificity of Vα14i NKT cells for synthetic antigens

The first exogenous antigen shown to be recognized by Vα14i NKT cells was α-galactosyl ceramide (αGalCer) (*Fig. 1*), which was isolated from a marine sponge in a screen for compounds that prevented tumor metastases. αGalCer has an unusual α anomeric linkage of the sugar to the lipid, unlike most of the glycosphingolipids in nature, which include many common compounds such as the gangliosides. Because of its unusual structure and isolation from a marine organism, it has been widely assumed that αGalCer cannot be the natural antigen recognized by iNKT cells. Subsequently αGalCer has been used in many different animal studies and a number of clinical studies including clinical trials for cancer (64, 65). iNKT cells in mice and humans can be specifically identified by binding of fluorochrome-conjugated, tetrameric complexes of CD1d loaded with αGalCer that we and others have developed (40, 66). Detection by these reagents is on the basis of specificity, and therefore is not dependent upon expression of a particular NK receptor or any other surrogate marker for these cells.

αGalCer binds to CD1d with the C26 acyl chain in the A′ pocket and the sphingosine base in the F' pocket (*Figs 1, 2*). This interaction is highly stable. The complex of glycolipid plus CD1d binds with a relatively high affinity to the iNKT cell TCR (67–70) (*Fig. 3*). The affinity is in the 100–300 nM range, with a half-life of the TCR interaction of approximately

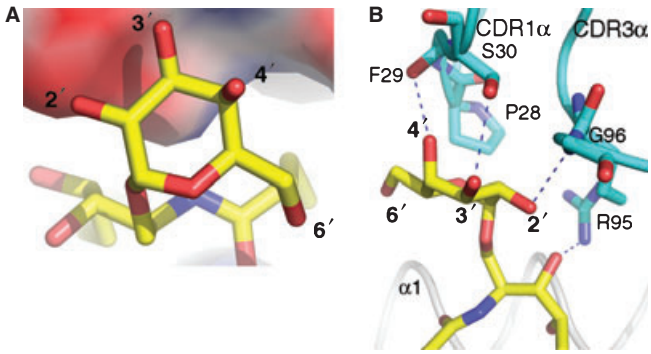

***Fig. 3.* Galactose presentation and recognition by the iNKT cell T-cell antigen receptor (TCR).** (A) Individual hydroxyl-groups of the galactose epitope of αGalCer and positions are indicated. (B) Overview of TCR contacts with αGalCer (PDB code 2po6). Hydroxyl positions and TCR contact residues are labeled with hydrogen bonds depicted as blue dashed lines.

3 min. This is a much longer half-life and much stronger equilibrium-binding constant than those observed for conventional, peptide reactive T cells.

We have carried out extensive structure–activity studies to determine how αGalCer/CD1d complexes are recognized by the TCR. The data indicate that the α linkage of the hexose sugar, which places the ring of the sugar parallel to the top of the CD1d groove, is required to form a stimulatory epitope for the invariant TCR (*Fig.* 3). There is also a very high degree of specificity for the galactose monosaccharide. For example, α-glucosyl ceramide (αGlcCer) is a weaker antigen than αGalCer. When measured in parallel with the same soluble TCR, the K_D was 3.8 μM for αGlcCer complexes with CD1d as opposed to 0.35 μM for αGalCer CD1d complexes, although these two sugars differ only by the orientation of the 4′ hydroxyl (70). Furthermore, α-mannosyl ceramide (αManCer) is almost completely inactive with no detectable binding of the TCR to αManCer complexes with CD1d, although compared with glucose, mannose has only a change in the 2′ hydroxyl to the axial orientation compared with equatorial in glucose and galactose. These data suggest that modifications of the orientation of the hydroxyls at the 2′ and 4′ positions of the sugar are critical, although modification of the 4′ position alone had a relatively modest effect on antigenic potency and TCR affinity. The supreme importance of the 2′ hydroxyl in the equatorial position was verified by testing 2′-deoxy, 2′-fluoro, 2′ N-acetyl- analogs of α-GalCer, and a galactose analog (talose) with the 2′ hydroxyl altered from equatorial to the axial position. All of these compounds were without any detectable antigenic activity (71). By contrast, modifications of the 3′ and 6′ positions were more permissive. Replacement of galactose with galacturonic acid, which has a carboxylic acid addition at the 6′ position compared with galactose, caused only a modest decrease in antigenic potency (72), as did the linkage of a second sugar to the 6′ position. Similarly, 3′-O-sulfo-αGalCer, with the addition of a sulfate group at the 3′ position, also had relatively little loss of activity (71). Our results are generally in agreement with those of others, although interestingly, recently it was shown that the hexose ring is not absolutely required, as a threitol containing ceramide also can stimulate iNKT cells (73).

Specificity of Vα14i NKT cells for bacterial antigens

Glycosphingolipids from *Sphingomonas spp.*

We found that *Sphingomonas* bacteria also contain glycosphingolipids with α-linked sugars that are antigenic for iNKT cells in mice and humans (74). *Sphingomonas* bacterial species are Gram-negative organisms that lack lipopolysaccharide (LPS) and that are highly abundant in the environment, including sea water (75, 76). Glycosphingolipids are not known to be present, however, in any other type of bacteria. It is likely, therefore, that *Sphingomonas spp.* are the only natural source of α-linked glycosphingolipids, and that the marine sponge that provided the original source of αGalCer contained these bacteria. *Sphingomonas* are well studied for their ability to degrade xenobiotic polymers, such as dioxins, but they are not known to be highly pathogenic, although they can cause infections (77, 78). We found that mice lacking Vα14i NKT cells are defective for clearance of *Sphingomonas yanoikuyae* at early times after infection, especially in the liver, although the bacteria were eventually cleared without signs of damage at the early times after infection. Others made similar observations (79, 80). Based on this, it seems possible that the recognition of α-linked glycosphingolipids provides a type of innate-like pattern recognition functionally similar to Toll-like receptor4 (TLR4)-mediated recognition of lipopolysaccharide (LPS).

Initially we tested purified and synthetic versions of *Sphingomonas* glycosphingolipids, with a galacturonic acid monosaccharide called GSL-1′ or GalAGSL (*Fig.* 1B). Glycosphingolipids purified from *Sphingomonas spp.* are heterogenous, however, with differences in both the ceramide lipid and the carbohydrate. Different bacterial strains may produce glycosphingolipids with predominantly GalA, glucuronic acid (GlcA), trisaccharides, or even tetrasaccharides. Two of these tetrasaccharide containing glycosphingolipids are known as GSL-4B, with the structure Glc(α1–4)Gal(α1–6)Glc(α1–4)GlcA(1)ceramide or GSL-4A with the structure: Man(α1–2)Gal(α1–6)GlcN(α1–4)GlcA(1)ceramide,

where GlcN = glucosamine (*Fig. 1B*). GSL-4B exhibited no antigenic activity, however, while GSL-4A had only very weak activity (81). The data indicated that in neither case could the tetrasaccharides be efficiently processed to form the much more antigenic monosaccharide GlcA-GSL. These findings are not in complete agreement with one earlier study that used purified material only; in that case, however, the oligosaccharide antigen could have had a small amount of contaminating monosaccharide (80). They are in good agreement with another study using synthetic antigens. These investigators attributed the inability to process the tetrasaccharide to the presence of the amino sugar, which can account for the poor antigenic activity of GSL-4A but not of GSL-4B (82). Regardless, it is certain that *Sphingomonas* bacteria synthesize a mixture of antigenic and non-antigenic glycosphingolipids. We speculate that although glycosphingolipids are very likely to be essential for survival, that *Sphingomonas* bacteria might alter the pattern of synthesis towards non-antigenic glycosphingolipids as an immune evasion mechanism.

Recognition of diacylglycerol antigens from *Borrelia burgdorferi*

Borrelia burgdorferi (*B. burgdorferi*) is a spirochete that is the causative agent of Lyme disease. It provides the first example of a pathogenic microbe that has glycolipid antigens that activate iNKT cells, and it also provides a striking demonstration that the glycolipid antigens do not have to contain ceramide as the lipid moiety (83). Infection with *B. burgdorferi* occurs by the bite of Ioxedes ticks, and if untreated, the resulting infection can lead to serious complications that involve the nervous system, joints, and the heart. *B. burgdorferi* has abundant glycosylated diacylglycerols (84, 85) with a single galactose sugar in α linkage to the *sn*3 position of the glycerol (*Fig. 1B*). Patients with chronic disease produce antibodies to these glycolipids. The response to *B. burgdorferi* has been analyzed in mice deficient for Vα14i NKT cells due either to genetic deficiency for CD1d, the required antigen presenting molecule for these cells, or deficiency for Jα18, required to form the invariant TCR. In both cases, mice lacking Vα14i NKT cells were less capable of clearing the spirochetes and they were more subjected to chronic joint inflammation (86–88). Moreover, Vα14i NKT cells were activated *in vivo* to produce cytokines such as IFNγ and IL-4 1 week after tick-mediated infection (86).

Purified and synthetic versions of the diacylglycerol antigen from *B. burgdorferi*, called BbGL-2c, proved to be stimulatory for both mouse and human iNKT cells. Although they are less potent than glycosphingolipid antigens containing ceramide lipids, this finding is important because unlike glycosphingolipids, glycosylated diacylglycerols are widely distributed in the microbial world. The *B. burgdorferi* antigens contain only a single α-linked galactose sugar, similar to αGalCer, but the length and degree of unsaturation of the fatty acids esterified to form the diacylglycerol are critical for antigenic activity. Mouse Vα14i NKT cells prefer compounds with fewer unsaturated bonds compared with their human Vα24i NKT cell counterparts. These preferences may reflect the role of the lipid in positioning the sugar properly for TCR recognition.

Transport and processing of glycolipid antigens

Lipids and glycolipids do not move freely in solution as monomers, and as a consequence there must be mechanisms for enhancing glycolipid antigen uptake into antigen-presenting cell (APC) and their loading into the grooves of CD1 molecules. Although potent antigens such as αGalCer can be loaded directly into CD1d molecules on the cell surface, antigen presentation generally is enhanced by the internalization of glycolipids into acidic endosomal compartments (89). For CD1d molecules, this usually involves antigen loading in late endosomes or lysosomes. Apolipoprotein E, which is secreted by a variety of cells including dendritic cells (DCs), enhances the presentation of glycolipid antigens to Vα14i NKT cells because it can interact with glycosphingolipid antigens and enhances their uptake (90). Once in late endosomes or lysosomes, the loading of glycosphingolipid antigens is facilitated by sphingolipid activator proteins, which include the saposins A, B, C, and D (91–95). These proteins are known to make glycosphingolipids accessible for catabolism by degradative enzymes (94), but they also interact with CD1 proteins and presumably they facilitate the antigen loading process by making the glycolipid antigens accessible by facilitating their removal from internal membranes.

For peptide antigen presentation, proteins must be processed to the nine-amino acid length that is favorable for MHC class I binding, or the slightly longer peptides favorable for MHC class II. The steps in processing needed to generate these peptides have been well characterized. Less is known, however, about the processing of the complex carbohydrates in glycolipids to generate the small carbohydrate epitopes that can be recognized by TCRs when presented by CD1 molecules. As noted above, the data indicate that the tetrasaccharide containing antigens of *Sphingomonas spp.* are poorly processed to the more antigenic glucuronic acid-containing monosaccharides (GalA-GSL). Using synthetic glycosphingolipids

antigens, however, we found that processing of carbohydrate antigens does occur in APCs (96). One example was provided by the compound Gal(α1,2)αGalCer, which has digalactose sugar linked to ceramide. Because of the critical nature of a free 2′ OH in the equatorial position of the galactose sugar in α linkage to ceramide, we reasoned that the second or outer sugar in α1,2 linkage to the inner galactose would have to be removed for antigenic activity. This turned out to be the case, and we demonstrated that the lysosomal enzyme α-galactosidase A is required to remove the terminal galactose, thereby generating the highly antigenic monosaccharide, αGalCer (96). Processing or removal of the outer sugar was also required for linkages to the 3′ position of the sugar, as in Gal(β1,3)αGalCer, but not when the outer sugar was linked to the 6′ position as in Gal(α1,6)αGalCer. There are still very few examples, however, of natural glycolipid antigens that efficiently undergo carbohydrate processing to generate a processed, antigenic species. One example is provided by hexamannosylated phosphatidyl-myo-inositols (PIM_6), which are derived from *Mycobacterium tuberculosis* and that are presented by CD1b. PIM_6 is processed to the dimannoside form PIM_2 by α-mannosidase, a process that is greatly enhanced by CD1e (5). Also, recognition of iGb3 is believed to require processing from iGb4 by an enzyme or enzymes having the β-subunit found in lysosomal hexosaminidases A and B.

Structural basis of glycolipid anchoring to CD1d

Several CD1d-glycolipid structures have been determined, and together they shed light on the structural details of glycolipid binding, especially how the carbohydrate moiety is bound and presented by CD1d (97). These structures include mouse CD1d in complex with either the glycosphingolipids PBS-25, which is a version of αGalCer with a shortened fatty acid (98), cis-tetracosenoyl-sulfatide (99), α-galacturonosylceramide or GalAGSL, a *Sphingomonas* antigen (100), the self-antigen isoglobotrihexosylceramide (iGb3) (101), as well as human CD1d with or without bound αGalCer (102). The structures of mouse CD1d bound to the diacylglycerolipids phosphatidylcholine (PC) (103) and phosphatidylinositol-dimannoside (PIM_2) (104) have also been determined. In addition, the structures of the mouse and human iNKT cell TCRs in unbound form (101, 105, 106) as well as the human CD1d-αGalCer-iNKT TCR trimolecular complex (107) have been solved (*Fig. 1*).

While much attention rightfully has been paid to the fine specificity of carbohydrate antigen recognition, the lipid backbone that anchors the glycolipid into the CD1-binding groove is responsible for overall binding. The two classes of glycolipids that have been structurally investigated are based on either a ceramide backbone (sphingolipids) or a diacyglycerol backbone (glycerolipids).

So far, all the determined CD1d-glycosphingolipid structures illustrate a similar mode of lipid binding. The N-amide linked fatty acid is always bound in the A' pocket, regardless of its size, while the sphingosine chain is accommodated in the F' pocket (97, 108). If the fatty acid chain is too short to fill the A' pocket completely, spacer lipids, such as a C16:0 fatty acids (palmitic acid) are recruited to occupy the remainder of the pocket, at least for proteins that were recombinantly expressed in insect cells (98, 100, 101). Whether that holds true in a physiological setting still remains unknown. With the lipid backbone anchored in the CD1d binding groove, the carbohydrate moiety is exposed at the CD1d surface for potential interactions with the TCR.

Changes in the lipid backbone, such as the elimination of the 4-OH group of the ceramide, as found in the *Sphingomonas* ligand GalA-GSL compared with the phytosphingosine αGalCer, lead to a slight tilt of the lipid backbone in the binding groove, due to an altered H-bond interaction of Asp80 with the 3-OH group of the ceramide. This tilt in turn laterally displaces the α-galacturonosyl headgroup by about 1Å and results in a significant reduction in antigenicity when compared with αGalCer. Apart from the observed tilt of the sugar, binding of the ceramide moieties of αGalCer, GalA-GSL, sulfatide and iGb3 to CD1d is highly similar (98–102). Therefore, the most notable difference in general is the reduced glycolipid-CD1d half-lives of the short chain ligands. For example, OCH, with its shortened sphingosine chain is not long enough to fully occupy the F' pocket. Two scenarios have been proposed to result from this incomplete occupancy. On the one hand, the F' pocket could partially collapse, thereby creating structural changes at the CD1d surface above this pocket (109). The altered CD1d landscape could then affect recognition by the TCR β chain. Such a conformational change might not take place, either because the partially filled pocket can maintain the conformation of the fully filled one, or because a short chain fatty acid (C_8–C_{10}) occupies the remainder of the pocket. It should be noted, however, that C_8–C_{10} short chain fatty acids are not abundant in eukaryotic cells (110).

Diacylglycerolipid antigens are characterized by fatty acids esterified to the *sn*-1 and *sn*-2 positions of the glycerol, while the more hydrophilic headgroup is bound to the *sn*-3 position. The glycerolipid backbone is less rigid than the ceramide backbone, due to the absence of the N-amide linkage.

Therefore, it is not surprising that two different binding orientations have been observed in the two available structures, one of CD1d-PC and the other of a CD1d-PIM_2 complex (103, 104). While PC binds with the *sn*-1 fatty acid in the F' pocket and the *sn*-2 fatty acid in the A' pocket, PIM_2 binds in the opposite orientation, with *sn*-1 pointing into the A' pocket and *sn*-2 into the F' pocket. As a result, the headgroups should be presented in slightly different position above the CD1d binding groove. Although PC is not a ligand for CD1d-restricted T cells, the different lipid binding orientations could possibly affect the antigenic potency of glycerolipid antigens.

Headgroup positioning by CD1d

The positioning of the carbohydrate epitopes above the CD1d groove drives antigen recognition by the TCR. The carbohydrate can either be attached via an α- or β-glycosidic bond to the lipid backbone. When glycosphingolipid antigens are bound, a core set of CD1d residues form extensive H-bond interactions with the various carbohydrate epitopes of the different ligands. Our studies have revealed how these interactions differ for α- and β-linked sugars.

Presentation of α-linked carbohydrates

Site-directed mutation studies established a crucial role for several CD1d amino acids in iNKT cell activation by αGalCer (111, 112). All of these residues have now been shown through structural analysis to either orient the galactose through a precise hydrogen-bond network for proper TCR engagement (Arg79, Asp80, Asp153, and Thr156, see *Fig.* 2), or they directly interact with the TCR (Glu83, Arg79) to facilitate formation of the CD1d-lipid antigen-TCR ternary complex (98, 100, 102, 107). Specifically, Asp80 in the α1-helix interacts with the 3-OH group of the sphingosine base, whereas Asp153 in mouse CD1d (151 in human CD1d) stabilizes the headgroup through interaction with either the 2′- and/or the 3′-OH groups of the galactose or galacturonic acid. Thr156 mouse CD1d (Thr154 in human CD1d) interacts with the oxygen of the 0-glycosidic linkage and with the sphingosine backbone nitrogen. Additional residues, such Arg79, appear to be more important in forming TCR contacts than in stabilizing the galactose.

Presentation of β-linked carbohydrates

Binding of sulfatide and iGb3 to mouse CD1d is very similar for the proximal sugar (3′-sulfated galactose for sulfatide and glucose for iGb3). In both cases the proximal hexose sugar forms hydrogen bonds with CD1d residues of the α2-helix, in particular with Asp153 and Thr156, and with Gln154 (sulfatide) or Gly155 (iGb3) through a water-mediated hydrogen bond. Residues from the α1-helix, Asp80 and Met69, primarily interact with and stabilize the lipid backbone, while Arg79, a key interacting residue with exogenous ligands with α-linked sugars, hydrogen bonds with the 6′-OH galactose of sulfatide, but does not interact with iGb3. In the iGb3 structure, the position of the third, terminal α1–3 galactose could not be determined because of its high degree of flexibility (101).

TCR engagement of CD1d-glycolipid complexes

The recent crystal structure of the human CD1d-αGalCer-Vα24Vβ11 TCR trimolecular complex (107) shed light onto the importance of the Vα24–Jα18 rearrangement in CD1d and glycolipid recognition. It also helped to illustrate the basis for the bias in Vβ usage in iNKT cells. The TCR docks in an unprecedented parallel binding orientation to the α1- and α2-helices of the antigen-presenting molecule. While the α-chain sits above the N-terminal half of the α1-helix, the β-chain is tipped toward the C-terminal end (*Fig* 1*A*). Interestingly, most of the contacts between the TCR, the glycolipid and CD1d are formed by CDR1α and CDR3α**,** while none of the CDR3β residues is in proximity to the glycolipid antigen. By contrast, CDR3α and CDR3β are equally important for antigen discrimination in pMHC-restricted TCR's.

Activation of iNKT cells is a characteristic feature of glycolipids with monosaccharides in α-linkage, such as αGalCer, GalA-GSL, and BbGl-2. However iGb3, a β-linked glycosphingolipid can also activate iNKT cells, while the structurally related sulfatide cannot. The common feature of all α-linked glycolipids is the flat presentation of the carbohydrate epitope, parallel to the top of the CD1d surface, which is in stark contrast to β-linked glycolipids (*Fig.* 2). In the α orientation, carbohydrates can only be engaged by the TCR from the top, while β-linked glycolipids could potentially also be engaged by certain TCRs in a 'tweezer' mode from both sides of the hexose ring. For αGalCer, the 2′, 3′, and 4′-OH groups of the galactose epitope hydrogen bond to the backbone oxygen of Phe29, Ser30 of CDR1α and the backbone nitrogen and oxygen of Gly96 of CDR3α of the TCR, respectively (*Fig.* 3*B*). Interestingly many other CDR3α residues, more precisely residues within the Jα18 region, bind to mouse CD1d amino acids, explaining the exquisite CD1d reactivity of iNKT cells. However, the current tri-molecular structure does not explain how iGb3, with a highly exposed β-linked glycolipid can be

recognized by the same, invariant TCR, especially because there are data suggesting that the TCR has a similar footprint when binding to CD1d-αGalCer or CD1d-iGb3 complexes (113). Our previous modeling studies on iGb3 indicated that the highly exposed tri-hexosyl headgroup could be squashed by the TCR to lie flat between the TCR and CD1d surface. Moreover, this model has recently been strengthened by the identification of synthetic synthetic iGb3 with an α-linked lipid proximal sugar as an iNKT cell ligand (114). In this case the α-linked headgroup is predicted to be already in a flat orientation suitable for TCR engagement, which could also explain the higher potentcy of α-iGb3 versus iGb3 (114). Therefore, we assume that the orientation of α-iGb3 and iGb3 upon TCR binding will be similar in the trimolecular CD1d-glycolipid-TCR complex, and that the iNKT cell TCR in general has the capacity to squash complex α-linked carbohydrate epitopes into an antigenic 'β-linked' conformation, as it has been observed for highly bulged peptides in the case of pMHC TCRs (115). The headgroups of β-linked monosaccharide glycolipids, such as β-GalCer or sulfatide are likely small enough to be accommodated between CD1d and the TCR without the need for 'flattening' and, therefore follow a different mode of recognition by other TCRs, as in the case for sulfatide-reactive T cells.

Conclusions and summary

We regard the combination of a hexose sugar α-linked to lipids, particularly galactose and closely related sugars, as a type of a microbe-specific structure or microbial pattern, analogous to LPS or double stranded RNA that is categorically different and therefore immediately recognized by an innate-like immune response. Research from our laboratories and others has contributed to the depth of knowledge concerning the precise nature of glycolipid antigen recognition by iNKT cells. As a result of this work, the means by which lipid antigens bind to CD1d is well understood. Furthermore, there is enormous specificity of the invariant TCR for certain carbohydrates protruding out of the CD1d groove, and this has been well analyzed in terms of the molecular structures required, such as the equatorial 2′OH, and the requirement for carbohydrate antigen processing to generate such structures. There is also information suggesting conformational changes in CD1d could be important for enhancing TCR affinity, at least in the case of the recognition of the potent antigen αGalCer presented by mouse CD1d.

While there are similarities between glycolipid recognition by iNKT cells and peptide antigen recognition by conventional T lymphocytes, there are also major differences that include the nature of the binding of antigens to the antigen presenting molecule, and the orientation of the invariant TCR to the glycolipid-CD1d complex. Many questions remain to be resolved. Of particular interest is the recognition of self-antigens, and how iGb3 is accommodated in the trimolecular complex that includes CD1d and the invariant TCR. Furthermore, because additional self-antigens are likely to exist, it remains to be determined if these also in some way mimic the type of antigens already characterized, or if the invariant TCR has the capability to use a different set of molecular interactions to bind to divergent structures. A second area requiring much further research is the nature of the interaction of glycolipid-CD1 complexes with other types of TCRs, including the more variable TCRs specific for CD1d, such as the sulfatide reactive cells, as well as TCRs reactive with group I CD1 molecules. Because iNKT cells have a unique biology, one that reflects the properties of innate immune cells as much as adaptive immune cells, it is possible that this will be reflected in a unique mode of TCR interaction for these cells that will not be found in other CD1-reactive T lymphocytes.

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