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Interplay between carbohydrate and lipid in recognition of glycolipid antigens by natural killer T cells

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Abstract

Natural killer T (NKT) cells are a T cell subpopulation that were named originally based on co-expression of receptors found on natural killer (NK) cells, cells of the innate immune system, and by T lymphocytes. The maturation and activation of NKT cells requires presentation of glycolipid antigens by CD1d, a cell surface protein distantly related to the major histocompatibility complex (MHC)-encoded antigen presenting molecules. This specificity distinguishes NKT cells from most CD4⁺ and CD8⁺ T cells, which recognize peptides presented by MHC class I and class II molecules. The rapid secretion of a large amount of both Th1 and Th2 cytokines by activated NKT cells endows them with the ability to play a vital role in the host immune defense against various microbial infections. In this review, we summarize progress on identifying the sources of microbe-derived glycolipid antigens recognized by NKT cells and the biochemical basis for their recognition.

Keywords

microbial infection; immune responses; glycolipid antigens; CD1; NKT cells

Introduction

NKT cells have fascinated immunologists because they have a number of unique properties. First, mouse and human NKT cells carry out immediate, innate-like response that, in some circumstances, leads to the production of diverse cytokines, including IFN- γ , IL-4, and TNF [1, 2]. Because of this vigorous cytokine production NKT cells have been reported to influence many types of immune responses and disease processes, particularly in mouse models [3–5]. Second, most NKT cells in mice express an invariant T cell antigen receptor (TCR) α chain and a homologous invariant α chain is found in many other mammalian species, including humans [1, 2, 6]. Because the TCR β chain is more variable, this TCR is sometimes described as being semi-invariant. Third, as noted, rather than recognizing peptides, NKT cells recognize glycolipids presented by a CD1d, a nonpolymorphic antigen-presenting molecule [5, 7, 8]. The trimolecular structures of the NKT T cell TCR bound to glycolipid-CD1d complexes have been solved, and these illustrate the structure of the CD1d-bound antigen, with the hydrophobic lipid buried in the CD1d antigen-binding groove. Despite this, the buried lipid still contributes to antigen recognition by positioning the sugar above the CD1d surface for the TCR.

Mouse and human NKT cells recognize essentially the same structures, but why has this specificity been conserved over 80 million years of evolution? Glycolipid antigens have been found in several pathogens, and NKT cell responses to these infectious agents are highly protective [5, 9, 10]. In agreement with others [11], we propose that the canonical or invariant TCR acts as a kind of pattern recognition receptor that recognizes widely expressed microbial glycolipids with α linked hexose sugars. Therefore, highly pathogenic bacteria with such glycolipid antigens may have driven the evolutionary conserved of the specificity of human and mouse NKT cells.

The CD1 family of antigen presenting molecules

In the battle of the mammalian immune system against invading pathogens, antigen presenting cells (APCs) must capture, process and present antigens to T lymphocytes. The classical antigen-presenting molecules, the highly polymorphic MHC class I and class II polypeptides, bind self or foreign peptides for presentation to CD8⁺ or CD4⁺ T cells, respectively [12–14]. A third family of antigen-presenting molecules, the CD1 family was first identified as a thymocyte antigen in 1979 and designated with the name CD1 in 1984 [15, 16]. These proteins are not highly polymorphic, and they present antigenic glycolipids to diverse T cell subpopulations, including CD4⁺, CD8⁺ and double-negative $\alpha\beta$ T lymphocytes as well as $\delta\gamma$ T cells [6, 17]. The CD1 family consists of four members that present glycolipids on the cell surface, CD1a–CD1d, which are divided into two groups according to the sequence identities of their antigen-binding $\alpha 1$ and $\alpha 2$ domains. CD1a, CD1b, and CD1c constitute group 1; CD1d is the only group 2 member. Humans and most other mammals have all of these CD1 proteins, while muridae rodents, including laboratory mice and rats, only have an ortholog of group 2 CD1d [5, 7, 8, 18]. CD1a, CD1b, CD1c, and CD1d are expressed predominantly by professional APCs, especially dendritic cells (DCs), macrophages and marginal zone B cells, as well as by thymocytes [15, 19–24], although expression of CD1d by other cell types, including hepatocytes and intestinal epithelial cells, has been reported. All CD1 proteins are type I integral membrane proteins, with a heavy chain consisting of extracellular $\alpha 1$ and $\alpha 2$ domains, which bind hydrophobic antigens, and a more membrane proximal $\alpha 3$ domains, which are immunoglobulin (Ig) super family homology units. The CD1 proteins are highly glycosylated polypeptides with the heavy chain non-covalently coupled with a light chain, $\beta 2$ -microglobulin, also a member of the Ig super family [25–28]. CD1e is fifth family member that is localized in lysosomes. CD1e facilitates the processing and consequent loading of complex glycolipids onto other CD1 proteins [29].

In acidic endosomal and lysosomal compartments, CD1 molecules bind to glycolipid antigens. In some cases, the carbohydrate portions of these antigens are hydrolyzed to their antigenic form by specific glycosidases [30], and this form of antigen processing and CD1 loading is facilitated by lipid transfer proteins, such as CD1e and the saposins [31–34]. After arriving at the cell surface via exocytosis, CD1 molecules present antigens [30, 35]. During more than a decade of research, subpopulations of CD1-reactive T lymphocytes have been intensively investigated. T lymphocytes that recognize CD1a, CD1b and CD1c express TCRs with diverse combinations of α chain and β chains, and recent evidence suggests that some of these cells, such as those reactive to CD1a, are abundant in the peripheral blood of humans [36]. In addition to these lymphocytes, CD1 reactive T lymphocytes that express $\gamma\delta$ TCRs also have been reported, particularly for recognition of CD1c [37].

NKT cells are reactive to CD1d

CD1d reactive T cells were defined as NKT cells because most cells of this population co-express NK cell makers, such as NK1.1, with their TCR [38]. However, it was found later

that expression of NK cell markers is not the exclusive characteristic of this T subset, and not all cells in this subset uniformly express NK receptors [1, 2, 4, 39, 40]. The discovery of α -galactosylceramide (α GalCer) (Fig. 1), a synthetic, glycolipid antigen that can specifically stimulate the CD1d reactive T cells with the semi-invariant TCR, and the development of α GalCer loaded CD1d tetramers that bind to the semi-invariant TCR, greatly contributed to the unambiguous definition and identification of this T subset [40, 41]. These cells now are most often referred to as invariant NKT cells ($\mathcal{I}$ NKT cells), or type I NKT cells, to distinguish them from CD1d reactive T lymphocytes expressing diverse TCRs, called type II NKT cells [1, 2, 6, 42].

$\mathcal{I}$ NKT cells exhibit an antigen experienced or memory phenotype. They are further activated when their TCR recognizes glycolipid antigens presented by CD1d, by the synergistic stimulation by glycolipid antigens and cytokines, particularly IL-12, or even by cytokines alone [43–46]. $\mathcal{I}$ NKT cells are striking because a single cell is able to secrete a large amount of Th1 and Th2 cytokines rapidly after stimulation, consequently activating and influencing various immune cells [47–51]. Therefore, although $\mathcal{I}$ NKT cells account for a relatively small portion of total T lymphocytes, <1%, they play a critical role in amplifying innate immune responses against infectious pathogens and they provide a bridge linking innate immunity to acquired immunity [3, 4]. In mouse models, $\mathcal{I}$ NKT cells are involved in the surveillance for tumors, the prevention of autoimmune diseases, the clearance of viral and bacterial pathogens [10, 52–55], and they participate in various inflammatory reactions such as ischemia reperfusion injury, ozone-induced asthma, and the formation of atherosclerotic plaques [56–59]. Furthermore, various human diseases have been correlated with reduced $\mathcal{I}$ NKT cell number or altered function [60, 61]. Herein, we provide an overview focusing on the sources of bacterial glycolipid antigens, their structure and presentation by CD1d, and the biochemical basis for their induction of $\mathcal{I}$ NKT cell activation.

Synthetic antigens for $\mathcal{I}$ NKT cells

In 1994, in a screen of marine sponge glycolipids for their anti-tumor activity, Natori et al. identified agelasphins derived from the marine sponge *Agelas mauritanus* as novel antitumor and immunostimulatory cerebroside [62]. Later, some minor structural modifications on the agelasphins were performed that produced α -galactosylceramide (α GalCer), commercially designated as KR7000 (Fig. 1). α GalCer possesses a ceramide backbone comprised of a C18 phytosphingosine with hydroxyl groups at the 3' and 4' carbon positions, and a C26 fatty acid chain. The ceramide lipid was conjugated to a single galactose carbohydrate head group via an α -anomeric linkage (Fig. 1). In 1997, Kawano *et al.* identified α GalCer as an exceptionally potent ligand for $\mathcal{I}$ NKT cells. Surface plasmon resonance studies have documented the unusually strong affinity of the semi-invariant TCR for α GalCer/CD1d complexes, measured in the 11–350 nM range [63–68]. A striking characteristic of α GalCer is the α -anomeric linkage between the ceramide moiety and the carbohydrate head group, given that mammalian cells only synthesize β -linked glycolipids. The α -linkage is critical for the potent antigenic activity of α GalCer because the β -anomeric isomer (β GalCer) was not able to stimulate $\mathcal{I}$ NKT cells or stimulated them only weakly [69].

A number of studies have probed structural variants of α GalCer in order to understand the biochemical basis for the extraordinarily high affinity of the semi-invariant TCR. Not surprisingly, the sugar protruding from the CD1d groove plays a major role in determining TCR affinity. Substitution of glucose for galactose reduced $\mathcal{I}$ NKT cell TCR affinity by approximately 10-fold [65, 68], and substitution to mannose completely abrogated the stimulation of $\mathcal{I}$ NKT cells [41, 65]. On the galactose sugar, all modifications of the 2" hydroxyl group of the sugar (Fig. 1) that were tested abrogated the antigenic activity of α GalCer, while modifications of the 3" or 4" hydroxyl groups were more tolerated, although

they did reduce antigenic potency to some extent [70–72] as well as having effects on TCR affinity [68]. The 6"-position, by contrast, can accommodate a number of chemical modifications without a requirement for lysosomal antigen processing to remove the modifications for recognition by the semi-invariant TCR [30, 73].

In addition to the carbohydrate moiety, the lipid backbone buried in the CD1d antigen binding groove also contributes to the antigenic potency. Removal of both 3' and 4'-hydroxyl groups on the phytosphingosine base resulted in the complete loss of activity for the compound [74–78], while removal of the 4'-hydroxyl alone led to some loss of antigenic potency and a reduced TCR affinity [65, 68, 74]. Truncation of the lipid chains, either the phytosphingosine base or the fatty acid, also reduced the ability of the compound to stimulate *N*KT cells [75].

Although the α -linkage of the sugar is required for a high degree of antigenic potency, several recent reports show that ceramide containing antigens with β -linked sugars, also can be recognized although they are weaker antigens. For β GalCer and isoglobotrihexosylceramide, structural studies show that TCR binding apparently squashes the sugar down so it is in close to an α conformation more parallel to the top of the CD1d molecule [79, 80]. It is surprising, however, that recent studies reported that β -glucosylceramide is a potent self-antigen, and that β -mannosylceramide is a novel *N*KT cell agonist that induces a potent anti-tumor response in vivo that is dominated by TNF secretion [81, 82]. The structural basis for the recognition of these latter two β -linked glycosphingolipid (GSL) antigens by the semi-invariant TCR remains to be determined.

Glycosphingolipid antigens derived from *Sphingomonas* bacteria

The first chemically well-defined microbial antigens that activate *N*KT cells were obtained from *Sphingomonas* *ssp.*, which are Gram-negative bacteria widely distributed in nature, especially in water and soil. *Sphingomonas* may be commensal organisms in the intestines of mice and some humans [83, 84] and although these bacterial species usually are not human pathogens, they have been implicated in causing a number of nosocomial and community-acquired infections, for example, bacteremia, meningitis and peritonitis [83, 85–87]. It also was proposed that these bacteria could contribute to the cause of primary biliary cirrhosis, an autoimmune disease leading to small bile duct damage. According to these studies, *Sphingomonas* infection induced antibodies against the microbial pyruvate dehydrogenase complex E2 enzyme that are cross-reactive with the host mitochondrial counterparts as well as inducing chronic T-cell mediated autoimmunity [83, 88]. Also, in another mouse disease model, administration of a *Sphingomonas* derived synthetic glycolipid antigen rapidly induced airway hyperreactivity by activating *N*KT cells [89], although there is no evidence yet that bacterial infection would lead to the same outcome.

Of Gram-negative bacteria, *Sphingomonas* are different because they do not express lipopolysaccharide (LPS), but instead employ GSLs to construct their cell walls. The main GSL components of these cell walls are heterogenous GSLs that include α -glucuronosylceramide, α -galacturonosylceramide and GSLs with more complex carbohydrates containing up to four saccharide units, in addition to variations in the ceramide lipid [90–92]. Mattner and co-workers using both purified material from *Sphingomonas capsulata* and synthetic material based on this, and Kinjo and colleagues taking a similar path with materials from *Sphingomonas paucimobilis* and *Sphingomonas yanoikuyae*, identified the main glycolipid antigens that stimulate *N*KT cells [93, 94]. Almost at the same time, Sriram et al. found crude glycolipid extracts from *S. paucimobilis* were able to activate several *N*KT cell hybridomas [95]. The GSLs purified from these *Sphingomonas* strains have minimal variations in structure, with a glucuronic acid (GlcA) sugar in *S. paucimobilis* to

form GlcA-GSL-1 and a mixture of glucuronic and galacturonic acids (GalA, to form GalA-GSL-1) in *S. yanoikuyae* (Fig. 1). All of these antigens, however, have an α -linkage to connect the carbohydrate to the ceramide [93]. CD1d tetramers loaded with the *Sphingomonas* compounds identified the λ NKT cell population in wild type splenocytes or liver mononuclear cells, but cells were not stained from Ja18^{-/-} mice, which cannot form the invariant TCR α chain because of a deficiency for the required Ja segment. Furthermore, CD1d deficient or Ja18^{-/-} mice had a significantly higher burden of bacteria at early times after *Sphingomonas* infection, pointing to a defect in bacterial clearance due to the absence of λ NKT cells [93, 94].

While monosaccharide-containing GSLs are the main glycolipid components of the cell walls of *Sphingomonas* species, they are not the only ones. Not only do different strains express distinct GSL profiles, but also according to the environmental conditions these bacteria are able to synthesize various GSLs with mono- or oligo- saccharides linked to ceramides consisting of different sphingosine bases [90–92]. *Sphingomonas* GSLs with more complex carbohydrate head groups include trisaccharide and tetrasaccharide glycosylceramides, originally designated as GSL-3 and GSL-4 respectively (Fig. 1). Both of these compounds contain glucuronic acid linked to the lipid. The antigenic activity of the GSL-4 variants for λ NKT cells were found to be not detectable or very weak, probably because mammalian APCs do not have glycosidases that can degrade the carbohydrate head groups to a monosaccharide form containing glucuronic acid [96, 97]. However, it is notable that a variant designated as GSL-4A (Fig. 1), comprised of a tandem tetrasaccharide of α -mannose(1–2) α -galactose(1–6) α -glucosamine(1–4) α -glucuronosyl linked to a ceramide with an α -anomeric conformation, not only was able to stimulate λ NKT cells, although to a lesser extent compared with than a synthetic *Sphingomonas*-based antigen GlcA-GSL, but also did not require lysosomal processing to trim the relatively complex oligosaccharide head group.

Besides variations in the sugar, the sphingosine chains of *Sphingomonas* bacterial GSLs also vary in the length and unsaturation, including a C18:0, C20:1 and a cyclopropyl-C21:0 [90, 92, 98, 99]. A synthetic GSL with a single galactosyl group and a cyclopropyl-C21:0 sphingosine chain, named as GalGSL-C21cycl (Fig. 1), showed antigenic activity intermediate between GalA-GSL and α GalCer, which further confirms the previous conclusion that the hydrophobic glycolipid backbone also contributes to the ability of glycolipid antigens to activate λ NKT cells.

The synthetic GalA-GSL compound (Fig. 1) was analyzed in an X-ray crystallographic structural study for its interactions with CD1d and in a trimolecular complex with CD1d and the semi-invariant TCR. One important difference distinguishing GalA-GSL from α GalCer is the sugar moiety, as the 6'-OH in galactose is substituted with a carboxylate in GalA-GSL. This compound also has a much shorter fatty acyl chain composed, of only 14 carbons versus 26 carbons for α GalCer, and the GalA-GSL sphingosine lacks the 4'-OH of the phytosphingosine of α GalCer. The hydrophobic CD1d binding groove has two large pockets, designated A' and F' [100]. Analysis of the crystal structure of the GalA-GSL-CD1d complex showed that similar to the α GalCer complex, the fatty acyl chain of the antigen inserted into the A' pocket of CD1d with the sphingosine chain in the F' pocket and the sugar head group protruded from the center of the binding groove at the CD1d surface [101]. However, lack of the 4'-OH led to a lower orientation of sphingosine in the pocket in order to make optimal hydrogen bonds with Asp at CD1d position 80, which resulted in GalA-GSL more deeply inserted into the CD1d groove. Moreover, the hydrogen bond between Arg-79 and the 3'-OH in the α GalCer-CD1d complex was lost, and therefore Arg-79 orients differently and may interfere partially with the interaction between CD1d and the TCR. A shortened fatty acyl chain was not able to fully occupy the A' pocket, and a so-

called spacer lipid, a fatty acid with 16 carbons, also bound in this pocket and presumably stabilized the GalA-GSL-CD1d complex. Overall, these structural alternations caused the galacturonosyl group of GalA-GSL to shift laterally compared with the galactosyl group of α GalCer in the complex with CD1d [101]. There were no hydrogen bonds linking the 6'-COOH in the galacturonic head group to CD1d consistent with the ability of this sugar position to tolerate variation in chemical structure [101].

Glycophospholipid antigens derived from *Borrelia* species

Borrelia spp. are Gram-negative spirochetes that do not express LPS in their cell wall. Twelve of the 36 known species are able to cause Lyme disease, also called borreliosis, a zoonotic, vector-borne illness transmitted by the bite of infected ticks. According to a report from the United States Centers for Disease Control and Prevention, the rate of this disease is 7.9 cases out of 100,000 persons and the number of reported cases has been increasing, making it the most common vector borne disease in the United States. In North America, *B. burgdorferi* is the predominant one of the three major *Borrelia* species that cause Lyme disease. Several *Borrelia* strains, including *B. hermsii*, *B. parkeri*, and *B. recurrentis*, can cause relapsing fever, often together with severe bacteremia.

CD1d deficiency rendered mice that are highly resistant to *Borrelia* susceptible to arthritis, a principal disease manifestation in the mouse model of Lyme disease [102], suggesting a role for λ NKT cells in host protection. Compared with wild-type mice, after infection CD1d^{-/-} mice had a dramatically increased level of spirochete DNA in tissues and a higher titer of *Borrelia* specific IgG2a in serum, the increased IgG2a might reflect an increased bacterial load in the absence of λ NKT cells. A subsequent study analyzed the role of splenic marginal zone B (MZB) cells that express the highest amount of CD1d level [103] on the clearance of *Borrelia* bacteria. It was found that MZB cells secreted *Borrelia* specific IgM and helped to reduce bacterial load in wild-type mice by 24 hours post-infection, whereas by 96 hours the MZB cells in CD1d knockout mice only produced minimal pathogen-specific IgM and the bacterial burden remained elevated [104]. CD1d has multiple functions, however, as it activates type II as well as type I λ NKT cells, and potentially it could have other roles. Tupin *et al.* therefore analyzed Ja.18^{-/-} mice to demonstrate the involvement of λ NKT cells in the clearance of *Borrelia* bacteria more directly. They showed increased joint inflammation and effects on spirochete clearance in BALB/c background Ja.18^{-/-} mice [105]. They also demonstrated λ NKT cell activation *in vivo*, including the secretion of cytokines, at one week after infection. In a subsequent study, bacterial load in the joints was increased 25-fold at day 3 in the absence of λ NKT cells, and following infection, alteration in λ NKT cell migration in the liver sinusoids and interactions with Kupffer cells, liver macrophages, were visualized by intravital microscopy [106]. In C57BL/6 mice, however, there was increased carditis more prominently than arthritis in the absence of λ NKT cells, as well as evidence for a local accumulation of λ NKT cells in the heart and increased local IFN- γ [107].

Analytical chemistry was required in order to determine if *B. burgdorferi* has a glycolipid antigen that can activate λ NKT cells. There are two major glycolipids in this pathogen. One of these, designated as *B. burgdorferi* glycolipid 1 (BbGL-I), is a cholesteryl 6-O-acyl- β -galactoside, and it accounts for about 23% of the total glycolipids. A second one, BbGL-II (Fig. 1 and Table 1), is a 1,2-diacyl-3-O- α -galactosyl-*sn*-glycerol, accounting for approximately 12% of the total glycolipids [108]. When plated on the microwells pre-coated with purified mouse CD1d protein, the diacylglycerol (DAG)-containing BbGL-II, but not BbGL-I, was able to stimulate λ NKT cell hybridomas to secrete IL-2, indicative of TCR engagement. Natural BbGL-II is a mixture of glyco-DAG compounds with C14:0, C16:0, C18:0, C18:1 and C18:2 fatty acid chains, with C16:0 and C18:1 as the most abundant ones (Table 1). Based on this, eight BbGL-II compounds comprised of different combination of

C16:0, C18:1, and C18:2 fatty acids were synthesized and their antigenic activity was determined. A compound designated as BbGL-IIc, with a C18:1 oleic acid in the *sn*-1 position and C16:0 in the *sn*-2 position of the glycerol backbone, was the optimal one for stimulating mouse λ NKT cells. BbGL-IIc, by contrast, with the C18:1 fatty acid in the *sn*-2 position, could bind to mouse CD1d but was not antigenic. Structural analysis showed that the two compounds bound to CD1d differently. BbGL-IIc could be accommodated by mouse CD1d with the kinked *sn*-1 oleoyl chain, caused by a *cis* double bond at the nine position, inserted into A' pocket, while the saturated C16:0 palmitoyl chain resided in the F' pocket (Fig. 2). This permitted the formation of polar interactions between the galactosyl head group of BbGL-IIc and the amino acids Asp153 and Thr156 in the CD1d α 2-helix, similar to that of α GalCer. However, different from α GalCer, there were no hydrogen bonds between Arg-79 or Asp-80 with BbGL-IIc because of the different structures of the diacylglycerol and ceramide backbones [109]. In the case of BbGL-IIc, the C18:1 oleic acid also was bound to the A' pocket of CD1d, but since in BbGL-IIc this fatty acid is in the *sn*-2 rather than the *sn*-1 position, the galactose sugar protruding from the top of CD1d was oriented differently and in a suboptimal position compared to the BbGL-IIc-CD1d complex. This illustrates a more general property of the two major classes of microbial glycolipid antigens. Glycosphingolipids are bound tightly in a single orientation with the sphingoid base in the F' pocket due to a network of hydrogen bonds that hold this category of glycolipids rigidly in place. The DAG containing antigens, by contrast, have reduced hydrogen binding and therefore can bind more flexibly in two orientations [110].

Glycolipids from Gram-positive pathogens also engage the semi-invariant TCR

Streptococcus pneumoniae (*S. pneumoniae*, also known as pneumococcus) is an agent of pneumonia, bloodstream infections and meningitis in children and the elderly. Neurologic sequelae and/or learning disabilities can occur in meningitis patients. Hearing impairment can result from recurrent otitis media, now estimated to cause 11% of all deaths in children from 1 month to 5 years of age [111]. Group B *Streptococcus* (GBS) is a cause of life-threatening bacterial infections such as sepsis and meningitis in human newborns. Both of these Gram-positive pathogens have antigens that activate λ NKT cells. We focused on *S. pneumoniae* because mice lacking λ NKT cells previously were shown to be highly sensitive to intratracheal infection with these bacteria [112, 113]. The previous work showed that during an infection with *S. pneumoniae*, IFN- γ derived from liver mononuclear cells, likely λ NKT cells, plays a role in clearance through stimulating increased production of TNF- α and MIP-2, which recruits neutrophils to the lung by 12 h [112]. Subsequent research showed more directly that λ NKT were activated in an antigen-dependent fashion nearly immediately following infection. λ NKT cells in the lungs of mice infected with *S. pneumoniae* expressed IFN- γ and IL-17 by 13 hours, and treatment with a CD1d blocking antibody substantially reduced IFN- γ production and caused a higher bacterial burden in the lung. Furthermore, dendritic cells (DCs) from taken directly infected mice could activate λ NKT cell hybridomas in vitro to secrete IL-2, indicating that the DC presented an antigen that could activate λ NKT cells.

Biochemical evidence for a glycolipid antigen in *S. pneumoniae* was first obtained from λ NKT cell hybridomas that were activated by CD1d coated plates exposed to total bacterial sonicates in a cell free antigen presentation assay. Subsequent analyses demonstrated that the major antigen in *S. pneumoniae* and GBS contained a glucose as opposed to a galactose sugar α -linked to the *sn*-3 position of a DAG antigen with palmitic acid (C16:0) in *sn*-1 and *cis*-vaccenic acid (C18:1, n-7) linked at *sn*-2 (Fig. 2) [114]. This single species, which we refer to as *S. pneumoniae* glucosylated diacylglycerol (SPN Glc-DAG) consisted of up to 43% of the total glycolipid of some *S. pneumoniae* strains. Analysis of variants created by

chemical synthesis indicated that the uncommon *cis*-vaccenic acid was required solely at the *sn*-2 position, a variant we called SPN Glc-DAG-s2. Four other variants tested, including a glycolipid lacking *cis*-vaccenic acid, one with *cis*-vaccenic acid at the *sn*-1 position, or one with *cis*-vaccenic acid at both glycerol positions all failed to activate mouse or human α NKT cells [114].

As noted above, GSL bacterial antigens were shown to be more potent with a galactose or galacturonic acid compared to glucose or glucuronic acid, the difference between the two types of sugars being the orientation of the hydroxyl in the 4' position of the hexose sugar. A recent structural analysis illustrates why the glucose sugar is preferred with DAG antigens containing *cis*-vaccenic acid, providing a vivid illustration of the interplay between the sugar and the CD1d-buried lipid in forming an epitope for the semi-invariant TCR. The glucose in Glc-DAG-s2 leads to a loss of a hydrogen bond with Asn at position 30 of CDR1 of the TCR α chain compared to binding the galactose from BbGL-IIc, but once the TCR is bound a new contact is made between the equatorial 4' hydroxyl of the glucose with Gly155 on the α 2 helix of mouse CD1d. This change does not lead to a large difference in the affinity of the semi-invariant TCR for glycolipid mouse CD1d complexes, which is 4.4 μ M for SPN Glc-DAG-s2 and 6.2 μ M for BbGL-IIc [110, 115]. However, the glucose is important in the context of the Glc-DAG-s2 lipid, because the axial hydroxyl at the 4' position of galactose, meaning the hydroxyl is facing up from rather than parallel to the hexose ring, would not permit the contact with position 155 of mouse CD1d to form. In fact, when galactose is combined in a synthetic antigen in α linkage with the *S. pneumoniae* DAG containing vaccenic acid, it does not stimulate α NKT cells [114].

Interestingly, although the equilibrium binding constants are similar, when binding the *S. pneumoniae* antigen, the TCR has both a slower association rate and a slower dissociation rate compared to binding to the *Borrelia* antigen. This fits our model that the TCR first contacts the exposed sugar, with the ability to contact the sugar affecting primarily the association rate. Subsequent contacts with and accommodation of CD1d affect primarily the dissociation rate, with the glucose interaction with position 155 proposed to be responsible for the more stable binding to complexes of Glc-DAG-s2 by the semi-invariant TCR compared to BbGL-IIc, once the trimolecular complex has formed. Also consistent with this hypothesis are the changes observed over the F' roof of the CD1d groove. Figure 2 shows that in the absence of TCR engagement only α GalCer binding causes a closing of the roof over the F' pocket. Even for the GalA-GSL antigen, this F' roof is open, exposing part of the lipid tail in the groove. Although the association rates of the α NKT cell TCR for the two GSL antigens are similar, the TCR dissociation rate for GalA-GSL-CD1d complexes is faster. We attribute this faster rate to the opening of the F' pocket and a possible flexing of the CD1d molecule even after the TCR is bound. Therefore, the ability of α GalCer to lock CD1d into the optimal conformation accounts in part for its extraordinary ability of α GalCer-CD1d complexes bind to the semi-invariant TCR.

Summary, conclusion, and perspectives

From the discovery of the first antigen in a marine sponge, which in fact was most likely associated with a *Sphingomonas*-like microorganism that produced the GSL antigen that was originally isolated, much has been learned in recent years about the diverse microbial sources of the glycolipid antigens that activate α NKT cells. Although such antigens may not be found in all bacteria, they can be derived from either Gram-negative or Gram-positive organisms, and from pathogens, commensals, or environmental bacteria. They have an α -linked hexose sugar and primarily contain one of two types of lipids: GSLs or DAGs. Recently, however, a cholesterol-containing glycolipid that activates α NKT cells has been obtained from *Helicobacter pylori*, the organism that causes stomach ulcers [116].

Therefore, it is likely that the diversity of antigens recognized by the semi-invariant TCR has not been exhausted.

Much has learned as well about the biochemical basis for the specificity of the semi-invariant TCR, including the factors determining the high affinity reactivity for complexes of α GalCer bound to CD1d, how the binding of GSLs and DAG antigens to CD1d differs, and how the kinetic parameters of TCR binding are determined by the position of the sugar for the association rate, and the flexibility of CD1d for the dissociation rate. While ultimately most of the critical features of antigen recognition by the semi-invariant TCR relate to the specific binding to the monosaccharides displayed on the surface of the CD1d antigen presenting protein, critically important as well is the role that the CD1d-bound lipid plays in positioning this sugar.

Years ago Janeway proposed that pattern recognition receptors would allow cells of the innate immune system to recognize critical or conserved molecular features of microbes that are not present in the host [117]. Considering how abundant the microbial glycolipid antigens are, the likelihood they are critical for microbe survival, and the absence of α -linked GSL or DAG antigens in mammalian hosts, when bound to CD1d these glycolipids constitute a kind of microbial associated molecular pattern that stimulates a subpopulation of T cells that carry out innate-like responses. The analogy to pattern recognition receptors may not be perfect, as self-GSL compounds are able to activate λ NKT cells to some degree, although this requires a TCR-induced accommodation of the β -linked sugar to a position similar to the α -linked saccharides. However, this does not invalidate the comparison, as toll like receptors, the paradigm of innate cell pattern recognition receptors, are also known to bind to self as well as microbial components. It should be noted that λ NKT cells also can be activated by cytokines, particularly IL-12 [44], or by self-antigens presented by CD1d plus IL-12. In a recent study, the authors found that the responses to bacteria that are known to have antigens, such *S. pneumoniae*, and those not known to have antigens, such as *E. coli*, are similar in intensity, their requirement for CD1d expression, and their requirement for IL-12 [118]. From these data, the authors concluded that even for microbes expressing a foreign antigen, the responses to self-antigens are predominant. However, this conclusion remains speculative, in part because removal of either the self antigen(s) or the bacterial antigens is not yet possible for technical reasons, making it difficult to weight their relative importance for λ NKT cell stimulation. In our view, it seems unlikely that a glycolipid antigen comprising more than 40% of the total glycolipid, as is the case for *S. pneumoniae*, does not contribute to λ NKT cell activation, although this does not exclude a role for self-antigen.

In addition to further exploration of microbial antigens, future work likely will explore in depth the structural basis for the recognition of different glycolipids by human λ NKT cells. Furthermore, as α GalCer was first developed as an anti-cancer agent, research undoubtedly will focus on the design of antigens that might be even more effective in clinical settings, such as in the treatment of cancer.

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Fig. 1.

Structures of synthetic α GalCer and natural microbial lipid Ags. α GalCer has a galactosyl sugar head group and a ceramide (enclosed by a larger solid line box) lipid backbone containing a sphingosine base (enclosed in a dot line internal box), connected by a 1-1' α linkage. The lipid moiety of *B. burgdorferi* Ag BbGL-II is a diacylglycerol, containing two fatty acid chains, R1 and R2, which are described in Table 1. GlcA-GSL-I, GalA-GSL-I, GSL-4A, and BbGL-II are the names given to purified antigens, which in some cases have heterogeneity.

Fig. 2.

Comparison of α GalCer, GalA-GSL, BbGL-IIc, and Glc-DAG-s2 binding to mCD1d before and after TCR engagement. (Top) Glycolipid ligand presentation shown in “TCR view” before TCR binding. Note how the galactose of BbGL-IIc and the glucose of Glc-DAG-s2 lose intimate contacts with α 2-helix of mouse CD1d but instead rotate counter-clockwise ($\sim 60^\circ$) toward Asp80 (D80) of α 1-helix, in contrast to α GalCer. (Bottom) Glycolipid bound in the TCR containing ternary complexes shown in the same view as the top, but with the bound TCR removed for clarity. Note that the TCR forces both the galactose of BbGL-IIc as well as the glucose of Glc-DAG-s2 into a position similar to that of α GalCer and GalA GSL before TCR engagement. This structural change induced upon TCR binding is necessary to allow for the conserved TCR binding footprint on CD1d. Also, note how the TCR is able to induce the formation of the F' roof in all CD1d-glycolipid complexes that do not have a preformed F' roof (e.g. α GalCer, top panel).

Table 1

Fatty acid components of synthetic BbGL-II compounds

BbGL-II compounds	R1	R2
BbGL-IIa	C16:0	C18:1
BbGL-IIb	C16:0	C18:2
BbGL-IIc	C18:1	C16:0
BbGL-IId	C18:1	C18:2
BbGL-IIe	C18:2	C16:0
BbGL-IIf	C18:2	C18:1
BbGL-IIg	C16:0	C18:0
BbGL-IIh	C16:0	C16:0

R1 and R2 represent *sn*-1 and *sn*-2 positions of glycerol, respectively (Fig. 1). The length of fatty acyl chains and the number of unsaturated bonds are indicated.