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Review

Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands

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Abstract. Natural killer T (NKT) cells have been shown by a number of studies to play a protective role against cancers, autoimmune diseases and infectious diseases. Several glycolipids and phospholipids derived from mammalian, bacterial, protozoan and plant species have recently been identified as natural ligands (antigens) for NKT cells. Some of these glycolipid/phospholipid ligands have now been crystallized in forms bound to CD1d molecules, and the tertiary structure of these complexes has finally been revealed. This review is intended to list natural NKT cell ligands identified to date, and discuss how their structures relate to their propensity to bind CD1d molecules and, as a consequence, stimulate NKT cells.

Keywords. Glycolipid, phospholipid, CD1d, NKT cell, natural ligand.

Introduction

Natural killer T cells

Natural killer T (NKT) cells are lymphoid cells which are distinct from conventional T cells and natural killer (NK) cells. NKT cells are characterized by expression of both a T cell antigen receptor (TCR) and NK1.1 (NKR-P1 or CD161c), a C-lectin-type NK receptor [1, 2]. The definition of NKT cells is still vague and broad, and it appears to classify into three subgroups of NKT cells. First and foremost, a significant proportion of NKT cells express semi-invariant TCRs encoded by Vα14 and Jα18 gene segments in mice, and Vα24 and Jα18 gene segments in humans, and all of them recognize CD1d molecules (see below). This subgroup of CD1d-restricted NKT cells can be referred to as 'Vα14 invariant or Vα24 invariant NKT cells' (Vα14i or Vα24i NKT cells), and has been extensively studied to date. A second subgroup of CD1d-restricted NKT cells, however, utilizes other, more diversified TCRs to recognize CD1d molecules [3]. Finally, there is a population of NKT cells that does not depend on the recognition of CD1d molecules, and is called non-CD1d-restricted NKT cells.

NKT cells represent a large percentage of the lymphoid population in the liver and bone marrow of mice [1]. In the liver of normal mice, 20–30% of lymphoid cells are NKT cells, mostly Vα14i NKT cells. Recent studies have shown that 33% of lymphoid cells in the human liver are also NKT cells [4]. In contrast to murine NKT cells, however, the TCR repertoire of human hepatic NKT cells is not skewed to Vα24 [4]. Although a majority of murine NKT cells is either CD4+ or double negative, i.e. CD4–CD8–, human NKT cells are more heterogeneous and a significant percentage of human NKT cells are CD4–CD8+, both in the blood and the liver [4].

Upon activation, NKT cells rapidly secrete cytokines such as interleukin-4 (IL-4) and interferon-γ(IFN-γ) *in vivo*, and induce a series of cellular activation events leading to the activation of innate immune cells such as NK cells and dendritic cells (DCs), and the activation of adaptive immune cells such as B and T cells [5–11]. In addition, NKT cells, just like NK cells, display cytotoxic activity mediated by Fas, perforin, granzyme A/B and granulysin upon activation [12, 13]. NKT cells have been shown to display anti-tumor activity [14, 15], a therapeutic effect against autoimmune

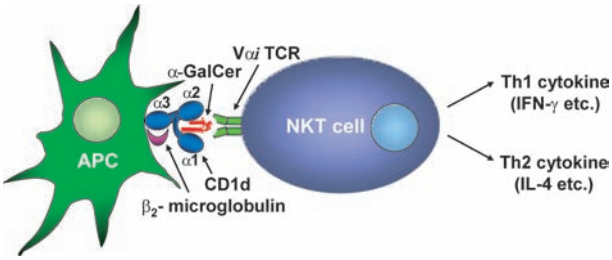

Figure 1. Mode of glycolipid presentation by CD1d to NKT cells. A CD1d molecule expressed by an antigen-presenting cell (APC) presents α-GalCer to an NKT cell through its Vαi TCR, resulting in rapid secretion of Th1 and Th2 cytokines.

diseases [16–19] and a protective effect against certain infectious agents [20–23]. Furthermore, rapid activation of NKT cells by a ligand for NKT cells has been shown to lead to the enhancement of adaptive immune responses when the ligand and an antigen are co-administered [8, 9, 24].

CD1d molecules

CD1 molecules are encoded by the CD1 locus comprising a family of non-polymorphic genes located on chromosome 1 in humans and chromosome 3 in mice, in contrast to those encoding major histocompatibility complex (MHC) molecules. CD1 molecules are structurally similar to MHC molecules, in particular MHC class I molecules, since both the heavy chains of CD1 and MHC class I associate non-convalently with β2-microglobulin [25, 26]. Therefore, CD1 molecules are also nicknamed non-classical MHC I or class I-like molecules. In humans, there are four CD1 isoforms, designated CD1a, CD1b, CD1c and CD1d, and these isoforms can be classified into group 1 (CD1a, b, c) and group 2 (CD1d), based on sequence homology. Important to note is that group 1 CD1 molecules exist in humans but not in mice, whereas CD1d molecules belonging to group 2 exist both in humans and in mice [25, 26].

Because of the structural similarity to MHC class I molecules, CD1 molecules have long been believed to present antigens to T cells. The discovery of α-galactosylceramide (α-GalCer) led to the remarkable finding that CD1d molecules can indeed present glycolipid antigens to non-conventional T cells, NKT cells (Fig. 1) [27–32]. CD1d is constitutively expressed by many cells, in particular antigen-presenting cells (APCs) including DCs and macrophages, and other cells including B and T lymphocytes in the thymus, liver and, to a lesser degree, in the spleen and lung [33, 34].

Discovery of α-GalCer: the first CD1d-binding ligand for NKT cells

Properties of α-GalCer

Glycolipids, which consist of an oligosaccharide and a lipid component that are linked, are typically found in plasma membranes of animal, bacterial and plant cells. Glycosphingolipids are complex glycolipids which contain ceramide as an extra-lipid component. In 1994, Kirin Pharmaceutical in Japan identified several glycosphingolipid compounds, named agelasphins, from an extract of the Okinawan marine sponge, *Agelas mauritianus* [35]. Some of the agelasphins were identified based on their strong anti-tumor activity in mice [35]. These agelasphins consist of D-galactose as the saccharide moiety and ceramide as the lipid moiety, and the two moieties are linked in an α-anomeric conformation (Fig. 2). Because of the minute amounts of agelasphins present in each sponge, and the difficulty of their scale-up synthesis, Kirin Pharmaceutical later modified the structure of agelasphins and synthesized α-GalCer, named KRN7000, as a candidate for clinical application [36]. Relative to the original agelasphins, α-GalCer lacks a hydroxyl group at the 2 position on the fatty acyl chain (Fig. 2). In addition, α-GalCer possesses a 4-hydroxyl group on the sphingosine chain, which is absent in some of the agelasphins (Fig. 2). α-GalCer has been, to date, the most extensively studied ligand for CD1d molecules and stimulant for NKT cells. However, it is puzzling why α-GalCer, a compound of marine origin, is such a potent agonist for NKT cells. The physiological significance of this compound in mammals has yet to be elucidated.

α-GalCer binds to CD1d molecules and stimulates rapid production of Th1 and Th2 cytokines, i.e. IFN-γ and IL-4, by Vα14i NKT cells in mice and homologous Vα24i NKT cells in humans [29–32]. These activities of α-GalCer are absent in both CDld- and Vα14i NKT-deficient mice, indicating that α-GalCer requires CD1d

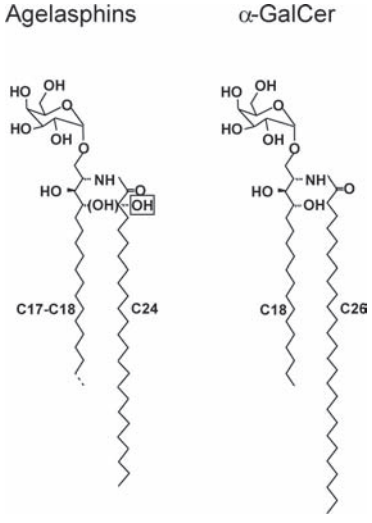

Figure 2. Structural comparison of agelasphins and α-GalCer. It is noteworthy that α-GalCer lacks the 2-hydroxyl group, which is boxed, on the fatty acyl chain relative to the original agelasphin. In addition, α-GalCer possesses a 4-hydroxyl group, which is in parentheses, on the sphingosine chain, unlike some agelasphins. Not shown are agelasphins with branches in the alkyl chain, which have a weak biological activity in mice.

molecules and Vα14i NKT cells to exhibit its activity *in vivo* [29, 30]. The mode of action of α-GalCer necessitates that it first binds to a CD1d molecule expressed by an APC, in particular a DC or macrophage depending on the organ [37], and the CD1d/α-GalCer complex is then in turn recognized by antigen receptors, Vα14i TCR for mice and Vα24i TCR for humans, on NKT cells (Fig. 1). Noteworthy is that human Vα24i NKT cells can recognize α-GalCer presented by mouse CD1d, and vice versa, i.e. murine Vα14i NKT cells recognize the human CD1d/α-GalCer complex [31]. A number of studies have documented that *in vivo* administration of α-GalCer triggers NKT cell activation through CD1d molecules and induces a myriad of activities against tumors [14, 15], as well as autoimmune [16–19] and infectious [20–23] diseases. α-GalCer has recently been shown to induce full maturation of DCs, as determined by an increased expression of co-stimulatory molecules on DCs, including CD40, CD80 and CD86, as well as MHC class II molecules [8]. The presence of NKT cells is required for α-GalCer to activate DCs.

Key structures of α-GalCer and its analogs for CD1d-binding and NKT cell activation

Two recent X-ray crystallography studies have revealed that the lipid portion of α-GalCer or its analogs fits tightly into a CD1d-binding groove formed by α1 and α2 helical domains (Fig. 3a) [38, 39]. The α1 and α2 helices of CD1d lie on top of six β strands and form a narrow but deep binding groove, which can be further divided into two large hydrophobic pockets, called A′ and F′. Both alkyl chains of the glycolipid are initially inserted perpendicular to the β sheet platform and then extended laterally toward the A′ and F′ pockets, respectively. Up to 18 carbons of the sphingosine chain are inserted into the F′ pocket, and up to 26 carbons of the longer acyl chain are anchored within the A′ pocket [38, 39]. The high degree of structural similarity in the lipid-binding groove between human and mouse CD1d molecules suggests that both CD1d grooves will accommodate α-GalCer in a similar orientation. The key hydrogen-bonding interactions between the glycolipid molecule and the CD1d structure include the bonding between the 2-hydroxyl group of the galactose head group and Asp 151 (Asp 153 for mouse) of the α2 helix of the CD1d, and the bonding between the 3-hydroxyl group of the phytosphingosine and Asp 80 of the α1 helix (Fig. 3a, b) [39]. These studies have also confirmed that a galactose ring extends above the surface of the lipid-binding groove, and thereby is exposed for recognition by the TCR of NKT cells.

Due to the limited number of α-GalCer analogs available, the structure-activity relationships (SARs) of these NKT cell ligands are still largely unknown. Nonetheless, the importance of the aforementioned galactose head group to α-GalCer function is evident, since eliminating or exchanging this moiety with various sugars either diminishes or abrogates activity, most likely due to the lack of recognition by the TCR of NKT cells [30]. Previous studies have demonstrated that the α-anomeric conformation of the glycolipid as well as the equatorial configuration of the 2-hydroxyl group of the sugar moiety and the 3–4 hydroxyl groups of the phytosphingosine are crucial for α-GalCer to bind CD1d molecules and to activate NKT cells through their TCR [28, 40]. The importance of the 2-hydroxyl group of the sugar moiety was confirmed by our recent study, which showed that any modification at the 2-hydroxyl group of the galactose completely abolishes activity, while some flexibility can be accommodated at the 3-hydroxyl position of the sugar head group [41, 42]. These results were recently corroborated by structural studies, demonstrating bonding between the 2-hydroxyl group of the galactose head group and the α2 helix of the CD1d, as described above. A sulfatide variant, 3-O-sulfo-α-galactosylceramide (3-O-sulfo-α-GalCer), with a sulfatide group instead of a free hydroxyl group at the 3-carbon position of the galactose, can strongly bind to CD1d and effectively

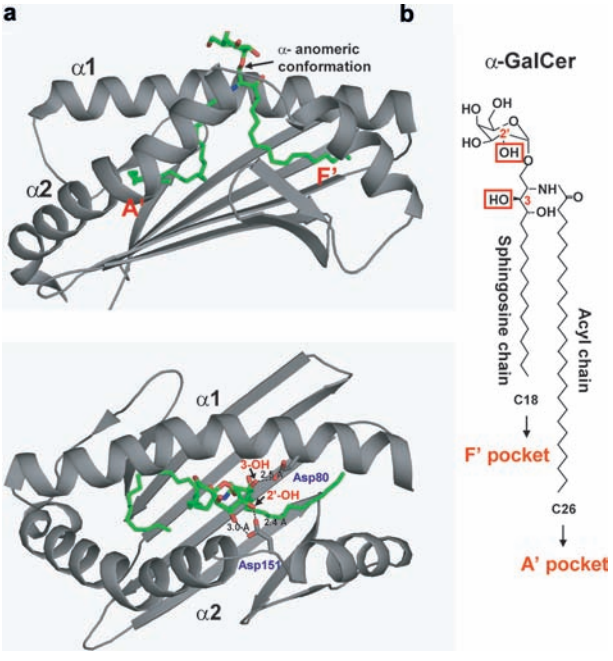

Figure 3. Overview of the structure of human CD1d and α-GalCer complex. Side and top views of crystallography, showing how α-GalCer fits into the groove of the human CD1d molecule (*a*) and chemical structure of α-GalCer (*b*). (*a*) The side view shows that the acyl chain of α-GalCer is inserted into the A′ pocket, whereas the sphingosine chain is anchored in the F′ pocket of the human CD1d molecule. The top view shows the bonding between the 2-hydroxyl group of the galactose head group (highlighted in red) and Asp 151 of the α2 helix of the human CD1d (highlighted in blue), as well as the bonding between the 3-hydroxyl group of the phytosphingosine (highlighted in red) and Asp 80 of the α1 helix (highlighted in blue). (*b*) The two key binding sites of α-GalCer to CD1d are boxed in red. Numbers in red indicate the carbon positions of the key binding sites.

stimulate both human and murine NKT cells (Fig. 4) [42]. Contrary to the key role played by the 2-hydroxyl group of this sugar moiety, several recent studies have found that the α-anomeric linkage of the glycolipid and the 4-hydroxyl group of the sphingosine chain may not be so crucial for CD1d binding and NKT cell stimulation. In these studies, β-GalCer (Fig. 4) was shown to effectively bind to CD1d [43] and, albeit to a much lower degree, β-anomeric GalCer could stimulate Vα14i NKT cells [44] and induce CD1d-dependent biological activities in mice [45].

Earlier research showed that α-GalCer is most amenable to modifications that do not affect function within the acyl tail portions of the molecule. In particular, work has been done in varying the hydrocarbon lengths and/or introducing unsaturation [46, 47] in the fatty acid chains. Brossay et al. [40] determined that the truncation of the fatty acid chain from 24 to 2 carbons did not significantly affect mouse NKT cell responses. However, one compound that has only 9 carbons in the sphingosine chain, an OCH compound, has been shown to skew the cytokine release profile toward Th2 cytokines (Fig. 4) [48]. Our previous work has found that a C-glycoside analog of α-GalCer, α-C-GalCer, also acts as an NKT cell ligand *in vivo* and preferentially stimulates a Th1-type response in mice (Fig. 4) [49, 50]. This analog, in comparison to α-GalCer, consistently stimulates prolonged production of IFN-γ, increased production of IL-12, and decreased production of the Th2 cytokine IL-4.

It is still unknown why α-GalCer induces both Th1 and Th2 cytokines, while some α-GalCer analogs, i.e. OCH, preferentially induce Th2 cytokines, and others, i.e. α-C-GalCer, induce Th1 cytokines [51, 52]. There are several possibilities to explain this phenomenon. First, these compounds have different binding affinity/avidity for CD1d molecules and/or TCRs of NKT cells. Second, these compounds may bind to different APCs expressing CD1d molecules, thereby activating NKT cells differently. Third, these compounds may stimulate different subsets of NKT cells, i.e. CD4+, CD8+ or double-negative NKT cells, thereby manifesting different cytokine production profiles. As suggested earlier, the elucidation of the precise mechanism of action of such compounds will likely require that we identify a larger number of α-GalCer analogs.

Microbe-derived glycolipids as natural CD1d-binding ligands for NKT cells

Sphingomonas species-derived glycosphingolipids

Two glycosphingolipids, resembling the chemical structure of α-GalCer, have been isolated from *Sphingomonas* species, Gram-negative bacteria lacking lipopolysccharide (LPS) and categorized as *Alphaproteobacteria* [53, 54]. α-Glucuronosylceramide (GSL-1) was isolated from the cell wall of *Sphingomonas capsulate*, whereas α-galacturonosylceramide (GSL-1′) was isolated from *S. yanoikuyae and S. wittichii* (Fig. 5) [55–57]. The abundant presence of a family of monoglycosylceramides in the cell wall of *Sphingomonas* species seems to substitute for LPS.

Recently, three independent studies identified these two glycosphingolipids as natural ligands for NKT cells [58–60]. In two studies, GSL-1 and GSL-1′ were synthesized and used to activate NKT cells [58, 59], whereas the third study successfully isolated GSL-1 from the extract of *S. paucimobilis* for use in *in vitro* experiments [60]. In all studies, the glycosphingolipids were shown to stimulate

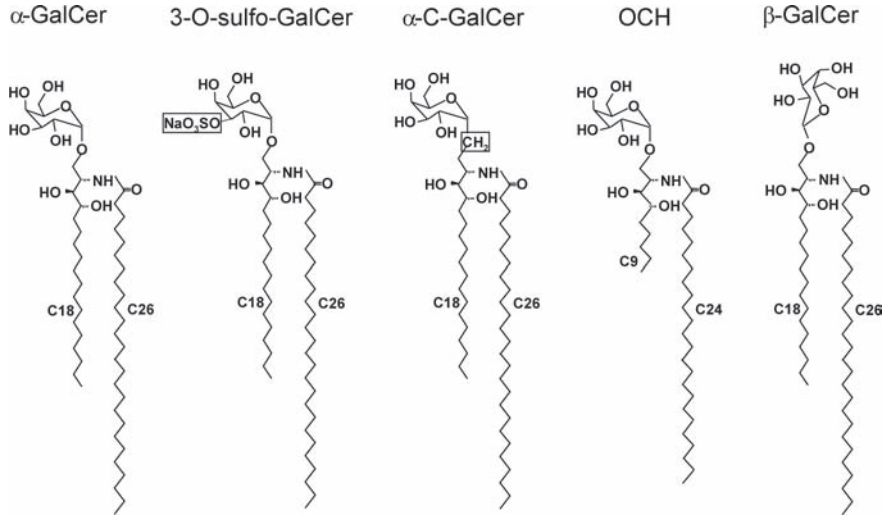

Figure 4. Structural comparison of α-GalCer and several synthetic analogs as CD1d-binding NKT cell ligands. 3-O-sulfo-α-GalCer has a sulfatide group (boxed) instead of a hydroxyl group at the 3-carbon position of the galactose, which does not alter NKT cell activation. α-C-GalCer, which is C-linked (boxed) between sugar and lipid moieties, stimulates NKT cells to secrete lower levels of Th2 and a prolonged output of Th1 cytokines. OCH has a shorter sphingosine chain and activates NKT cells to Th2-skewed secretion. β-GalCer, with a β-anomeric conformation between sugar and lipid, binds less to CD1d and stimulates NKT cells weakly.

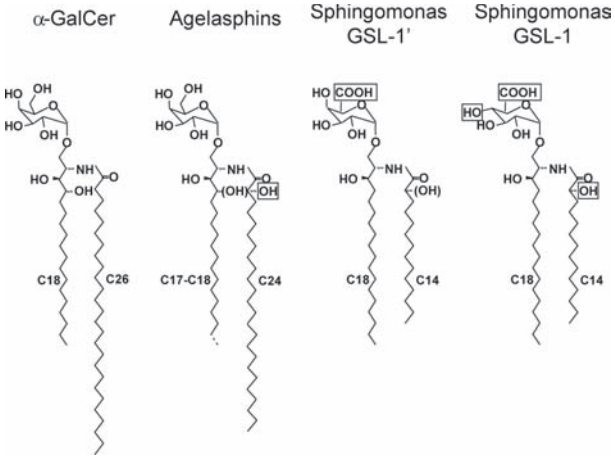

Figure 5. Structural comparison of α-GalCer, agelasphins and *Sphingomonas*-derived GSLs. Note that both GSL-1 and GSL-1′ have a carboxyl group (boxed) at the 6-carbon position compared with α-GalCer. Possession of a 2-hydroxyl group (boxed) on the fatty acyl chain and the lack of a 4-hydroxyl group on the sphingosine chain by GSLs resembles the structure of agelasphins rather than α-GalCer.

murine and human NKT cells *in vitro* in a CD1d-specific fashion, albeit to a lesser degree compared with α-GalCer. In addition, when CD1d tetramers and CD1d dimers were loaded with these glycolipids, the CD1d-glycolipid complex was shown to directly bind to NKT cells, as determined by a flow cytometric analysis, indicating that the *Sphingomonas*-derived glycolipids actually bind to CD1d molecules and are recognized by NKT cells. Although the direct role of these glycolipids in protection against *Sphingomonas* infection is as yet not proven, both CD1d-deficient mice and NKT-deficient mice have been shown to have a higher bacterial load than wild-type mice upon challenge with *Sphingomonas* [58, 59], suggesting that glycolipids in the context of CD1d molecules may have a protective role by activating CD1d-restricted NKT cells. However, one study demonstrated that upon challenge with lethal doses of *Sphingomonas*, CD1d-deficient mice showed, in contradistinction to other studies, lower mortality compared with wild-type mice [59]. This study suggests that NKT cells, activated by CD1d-glycolipid complexes, may cause sepsis-like shock in the mice by acutely precipitating the secretion of a considerable amount of cytokines such as IFN-γ.

If we scrutinize the structures of these *Sphingomonas*-derived glycosphingolipids and compare them with that of α-GalCer, both the glucuronic acid and galacturonic acid of GSL-1 and GSL-1′ respectively, have the carboxyl group at the 6-carbon position (Fig. 5). More interesting, these *Sphingomonas*-derived glycosphingolipids possess a hydroxyl group at the 2-carbon position in the fatty-acyl chain, and more closely resemble the original agelasphins derived from the marine sponge extract (Fig. 5). In addition, the absence of a 4-hydroxyl group at the sphingosine chain of *Sphingomonas*-derived glycosphingolipids is mirrored by the structure of some of the agelasphins. Noteworthy is in this regard, that *Sphingomonas alaskensis* (now termed *Sphingopyxis alaskensis*) was recently found in high abundance in the oligotrophic Pacific waters near the southern part of Japan [61]. This bacterium was isolated by limiting dilution of natural seawater samples, followed by monthly sub-culturing for 12 months. In view of this study, a plausible postulation is that the agelasphins may be derived from *Sphingomonas*-like bacteria that are either bound to or ingested by and contained within the Okinawan marine sponge, *A. mauritianus*, rather than the sponges themselves nascently producing the compounds. The structure of these *Sphingomonas*-derived glycosphingolipids has also revealed that the absence of the 4-hydroxyl group at the sphingosine chain slightly diminished but failed to abolish the NKT cell stimulation, indicating that the 4-hydroxyl group at phytosphingosine is not crucial for either CD1d binding or NKT cell recognition, as described above.

Borrelia species-derived glycolipids

Borrelia are bacteria that belong to the *Spirochaetaceae* family and lack LPS as well as peptidoglycan [62]. *Borrelia burgdorferi* is a spirochete that causes Lyme disease in humans. CD1d has previously been shown to be required to control *B. burgdorferi* infection, and the production of IgM by marginal zone B cells is impaired in the absence of CD1d [63, 64]. The cell wall of *Borrelia* was shown in the past to be comprised of two glycolipids and two phospholipids. The glycolipids were identified as α-galactosyl-diacylglycerolipids with two different fatty acid compositions [65]. More recently, the glycolipids were analyzed in detail and identified as cholesteryl 6-O-acyl-β-D-galactopyranoside (*B. burgdorferi* glycolipid 1, BbGL-I) and 1,2-di-O-acyl-3-O-α-D-galactopyranosyl-sn-glycerol (BbGL-II) (Fig. 6) [66]. BbGL-II, which is a monogalactosyl-diacylglycerol, consists of a D-galactose moiety with α-anomerically linked diacylglycerol (Fig. 6). The basic structure of BbGL-II not only resembles glycosphingolipids, but its lipid moiety is almost identical to phosphatidyl choline (PC) or phosphatidyl ethanolamine (PE) (described below) which can fit into the CD1d groove and bind to CD1d. The structural basis for CD1d binding and the fact that CD1d has been shown to mediate protection against *B. burgdorferi*-induced infection in mice [63] have prompted us to synthesize these two glycolipids and test their reactivity with NKT cells. BbGL-II appears to stimulate murine Vα14i NKT cells in a CD1d-specific fashion [M. Kronenberg, C.-H. Wong and M.Tsuji, unpublished data].

Mycobacteria species-derived phosphoglycolipids

The first indication of mycobacterial phosphatidylinositol mannoside (PIM) as a ligand for NKT cells came from

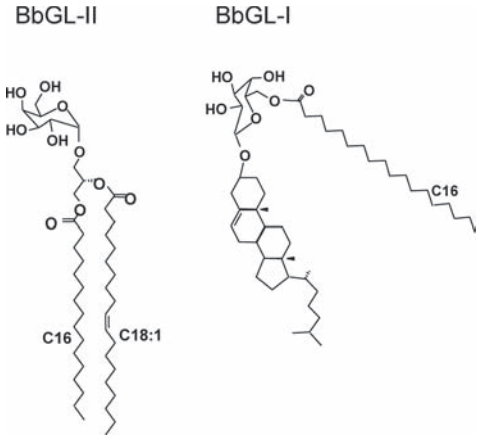

Figure 6. Structures of *Borrelia*-derived glycolipids, BbGL-I and BbGL-II. BbGL-II is a monogalactosyl-diacylglycerol, consisting of a D-galactose moiety with α-anomerically linked diacylglycerol.

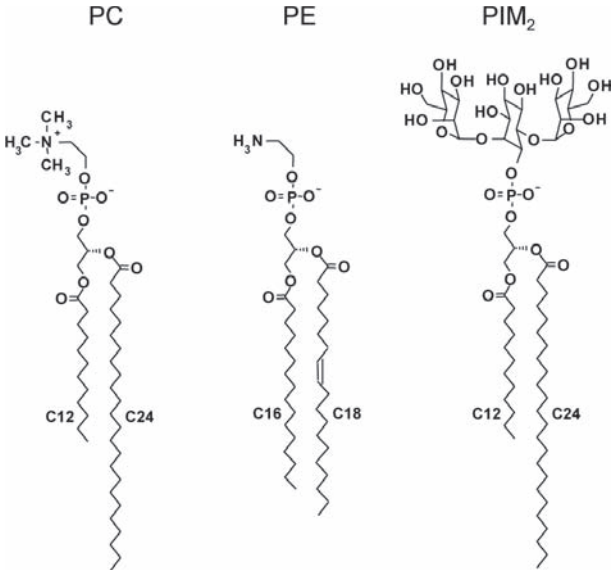

Figure 7. Structures of phospholipids as CD1d-binding NKT cell ligands. Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) share structural similarity and have diacylgycerol linked to phosphocholine and phosphoethanolamine, respectively. Both are derived either endogenously or from plants. Phosphatidylinositol dimannoside (PIM2), in which two mannose units are attached to a myo-inositol ring, is isolated from the cell wall of *M. bovis.*

a study in which dimannoside (PIM2) was isolated from the cell wall of *Mycobacterium bovis* bacillus Calmette-Guerin and injected into mice [67]. The study found that PIM2 could induce granuloma lesion formation that is largely infiltrated with Vα14i NKT cells, as determined by the expression of the mRNA of Vα14-Jα281 by semi-quantitative RT-PCR [67]. More direct evidence was presented by a study in which various lipid compounds, including PIM, lipoarabinomannan, lipomannan, diacyl trehalose, trehalose monomycolate and trehalose dimycolate, were isolated and purified from *M. bovis*. Only PIM could bind to CD1d and stimulate murine Vα14i NKT and human Vα24i NKT cells in a CD1d-specific fashion [68]. Although PIM possesses two fatty acyl chains that may fit into two hydrophobic pockets in the CD1d groove, how the three bulky sugar heads and the phosphate linkage can fit between the two α helices and yet also interact with the TCR of NKT cells remains to be determined (Fig. 7). In this regard, an alternative possibility for PIM-induced NKT cell activation is that, instead of PIM being directly recognized by NKT cells, it may up-regulate the expression of endogenous ligand in the context of CD1d through CD1d or other receptors, such as Toll-like receptors (TLRs), thereby stimulating Vα14i NKT cells in a CD1d-restricted fashion.

Protozoa-derived phosphoglycolipids

Phosphoglycolipids have been isolated from several protozoan parasites and have been shown to bind to CD1d molecules. In one study, mucin-like glycoproteins (GPI mucins) and glycoinositolphospholipids (GIPLs) have been isolated and purified from *Trypanosoma cruzi* and have been shown to bind to plate-bound CD1d molecules *in vitro* [69]. However, this study also demonstrated that these phosphoglycolipids were unable to stimulate NKT cells *in vitro* or *in vivo*, and that humoral responses to these phosphoglycolipids *in vivo* are elicited in an MHC class II-restricted fashion, independent of CD1d [69]. This indicates that, despite binding to CD1d, GPI mucins and GIPLs expressed by *T. cruzi* do not appear to evoke significant CD1d-restricted immune responses *in vivo*. Another recent study has shown that lipophosphoglycan (LPG) and GIPLs purified from *Leishmania donovani* not only bind with high affinity to CD1d, but also induce a CD1d-dependent IFN-γ response by a fraction of naive intrahepatic lymphocytes [70]. Since the binding of the CD1d/LPG or CD1d/GIPLs complex to the TCR of NKT cells was not determined in this study, it still remains possible that, by binding to CD1d or other receptors, i.e. TLRs, LPG and GIPLs may up-regulate the expression of CD1d that bears endogenous glycolipids (described in the next section), which may, in turn, stimulate CD1d-restricted NKT cells. Finally, it has been suggested that GPI anchors of *Plasmodium falciparum* and *T. brucei* induce a humoral response to the parasites in a CD1d-dependent fashion [71]. However, evidence for the role of GPI is controversial, because we and another group have independently shown that humoral responses to a plasmodial protein are restricted by MHC class II, rather than CD1d [72, 73].

Endogenous glycolipids as natural CD1d-binding ligands for NKT cells

To date, several endogenous CD1d-binding, NKT cell ligand glycolipids have been identified. The logical ap-

proach to search for such an endogenous glycolipid has led to the identification of a lysosomal glycosphingolipid, isoglobotrihexosylceramide (iGb3) [74]. In this study, iGb3 was shown to stimulate mouse and human NKT cells in a CD1d-dependent manner. In addition, impaired generation of lysosomal iGb3 in mice lacking β-hexosaminidase b resulted in severe NKT cell deficiency, suggesting that iGb3 mediates the development of NKT cells in mice. iGb3 consists of a ceramide and a sugar head group linked in a β-anomeric conformation. Its sugar head group consists of three sugar heads, composed of two galactose rings and one glucose, serially linked (Fig. 8). Structurally, iGb3 can fit well into the CD1d groove by virtue of two acyl chains filling the two hydrophobic pockets. However, the sugar head groups project out of the binding groove, perpendicular to the protein surface, due to the β-anomeric linkage. This presentation is quite different from that in α-GalCer which lies parallel to the protein surface and is, therefore, much less exposed. More recently, two studies have also implicated self glycolipids, a sulfatide (cis-tetracosenoyl sulfatide) derived from myelin and PC [75, 76]. Although both studies have convincingly presented the crystal structure of mouse CD1d bound to each glycolipid, the sulfatide was actually shown to bind to CD1d and stimulate NKT cells that include non-Vα14i NKT cells [75, 77]. A crystallographic analysis of a mouse CD1d/sulfatide complex revealed that the galactose headgroup of the sulfatide projects out perpendicular to the α1 and α2 helices and away from the binding groove, because of its β-anomeric linkage. From the structural point of view, both iGb3 and the sulfatide share similar features because of the β-anomeric linkage between the sugar moiety and ceramide (Fig. 8). In this regard, it would be interesting to determine how the TCR of NKT cells can still recognize the sugar head(s) which point away from the CD1d binding groove.

Two self glycolipids have been derived and identified from the extract of tumor cells. Disialoganglioside GD3, which is abundantly expressed on human tumors such as melanoma, but also expressed at low levels in some normal tissue in the human or mouse, has been shown to bind to a mouse CD1d molecule and stimulate mouse NKT cells *in vitro* [78]. Immunization of mice with human melanoma cells expressing GD3 but lacking CD1d resulted in the stimulation of mouse NKT cells, suggesting the *in vivo* cross-presentation of GD3 by mouse CD1d molecules. The study also showed that GD3-loaded, but not GM2-loaded APCs, stimulate NKT cells *in vitro*. Compared to GD3, which has four sugar heads serially linked (Fig. 8), GM2 carries four sugar heads in a para-position and, as a result, two bulky sugar heads out of four sugar heads in this conformation may make it difficult for NKT cells to recognize. In this regard, GM3, which has three sugar heads serially linked, one sugar head less than GD3, could be structurally more accessible than GD3 to the receptor of NKT cells.

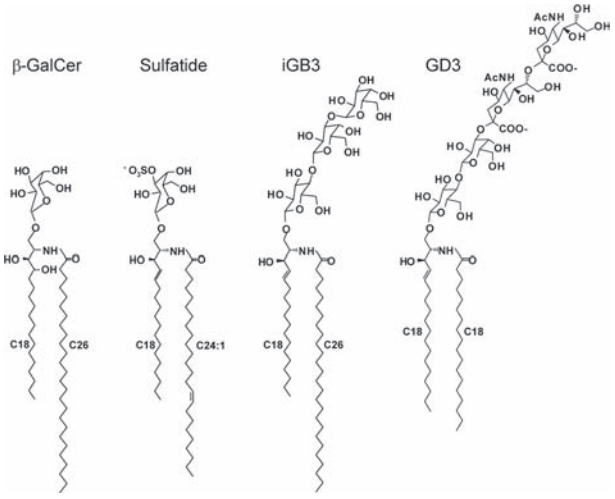

Figure 8. Structures of β-GalCer and its endogenous analogs as CD1d-binding NKT cell ligands. Like β-GalCer, all three endogenous glycolipids possess β-anomeric linkage between the sugar moiety and the ceramide. In their sugar moiety, sulfate has one galactose head, iGB3 has two galactose rings and one glucose ring serially linked, and GD3 has two galactose rings and two N-acetylneuraminic acids, also serially linked.

Alternatively, GD3 taken up by APCs may be cleaved during the intracellular processing and its sugar moieties may become accessible to the TCR of NKT cells.

PE was also purified from the polar lipid fraction of a tumor cell extract, and identified as an NKT cell ligand [79]. This study determined that increasing acyl chain unsaturation in the cis, but not trans, configuration correlated with increased binding of PE to CD1d molecules, as well as increased recognition by NKT cells. PE is structurally very similar to PC and has an NH_3 group after a phosphate linkage, instead of the $N(CH_3)_3$ group carried by PC (Fig. 7).

Plant-derived glycolipids as natural CD1d-binding ligands for NKT cells

There was no evidence that plant-derived glycolipids could act as ligands for CD1d-restricted NKT cells until a recent study showed that glycolipids extracted from cypress pollen bind to CD1d, as well as to CD1a molecules expressed by human DCs [80]. From the pollen extracts, PC and PE were identified as compounds that stimulate T cells from cypress-sensitive subjects in a CD1d- and CD1a-dependent fashion. Most human CD1d-restricted T cell lines are reactive to PE, while the majority of CD1a-restricted T cell lines respond to PC. Interestingly, among 20 CD1d-restricted T cell lines tested, only one was found to express the Vα24i TCR, which is an invariant TCR of NKT cells. Presumably CD1d molecules present plant-derived PE and PC to CD1d-restricted T cells in a manner analogous to the presentation of endogenous PC and PE as described above.

Concluding remarks

Since the discovery of α-GalCer as the first CD1d-binding NKT cell ligand, various natural glycolipids/phospholipids have been identified that bind to CD1d molecules. Some of these CD1d-binding natural compounds have been shown to stimulate NKT cells in a CD1d-specific fashion. Although the precise mode of NKT cell stimulation remains to be determined, the identification of a variety of CD1d-binding natural ligands that activate NKT cells, together with the structural analysis of CD1d/glycolipid complexes, will permit us to determine their SARs, and, hopefully, lead us to identify more potent NKT cell ligands in the future. Furthermore, in view of the fact that NKT cells have a function that forges a link between innate and adaptive immunity, natural NKT cell ligands could become useful as components of novel therapeutic and preventive strategies aimed at attenuating or eliminating disease.

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