

Chapter 2

Structure and Recognition of Antigens for Invariant NKT Cells

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Abstract It has been two decades since natural killer T (NKT) cells were identified and distinguished from conventional T cell populations by the invariant V α 14 rearrangement in their T cell antigen receptor (TCR). NKT cells recognize lipid antigens presented by CD1d, a member of a third family of antigen-presenting molecules. The first antigen known to activate NKT cells is a α -galactosyl ceramide (α GalCer), a highly potent synthetic glycosphingolipid (GSL) antigen closely related to a natural product, probably derived from a bacteria. Synthetic antigens related to α GalCer are being developed for clinical applications, and there is great interesting understanding why different variants cause different cytokine responses. Microbial glycosphingolipid antigens for NKT cells have been found in environmental microbes and also in pathogens such as *Borrelia burgdorferi*. For the microbial and synthetic antigens, when the TCR binds, it forces the sugar and CD1d into a fixed orientation. Self-antigens for NKT cells also have been defined, but these have diverse structures and it remains controversial if there is a single type of self-agonist responsible for the selection and peripheral activation of these cells.

1 Introduction

In vertebrates, antigen-presenting molecules play a critical role in host immune defense against microbial pathogens by presenting antigens (Ags) to T lymphocytes. Major histocompatibility complex (MHC)-encoded class I and class II molecules present peptides to T lymphocytes, while members of the CD1 family of molecules present lipid Ags (Kronenberg 2005; Rudolph et al. 2006; Bendelac et al. 2007).

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Considered as a third family of antigen-presenting molecules, in addition to MHC-encoded class I and class II proteins, CD1 polypeptides are expressed by a variety of cell types, including cortical thymocytes, myeloid cells, hepatocytes, intestinal epithelial cells, and keratinocytes. The highest amounts of CD1 expression, however, are found on marginal zone B (MZB) cells and various types of professional antigen-presenting cells (APC), including dendritic cells (DCs) and Langerhans cells (Silk et al. 2008a; De Libero et al. 2009).

Five human CD1 genes have been identified, with their protein products corresponding to CD1a, CD1b, CD1c, CD1d, and CD1e. Based on their sequence similarity, the antigen-presenting CD1 members are classified into two groups: CD1a, CD1b, and CD1c in group I, and CD1d in group II. CD1e does not present antigens but is instead localized to lysosomes where it binds glycolipids and is involved in assisting the processing of the carbohydrate portion of glycolipids (de la Salle et al. 2005). Mice and rats, by contrast, have only a CD1d ortholog (Brigl and Brenner 2004; Silk et al. 2008a; Cohen et al. 2009). Similar to MHC class I proteins, CD1 molecules are type I integral membrane proteins consisting of $\alpha 1$, $\alpha 2$, and $\alpha 3$ extracellular domains, non-covalently associated with β_2 -microglobulin (Brigl and Brenner 2004; Moody et al. 2005). However, CD1 proteins are relatively non-polymorphic with the exception of CD1e (Cohen et al. 2009; Salio et al. 2010).

The group I CD1 molecules present antigens to T lymphocytes that express diverse T cell antigen receptors (TCRs), and that appear to be similar in many ways to the general populations of T lymphocytes active in adaptive immunity. By contrast, in mice the majority of CD1d-reactive T cells express a semi-invariant TCR with an invariant α chain formed by a V $\alpha 14$ –J $\alpha 18$ rearrangement in mice (Godfrey et al. 2004). Humans have populations with a homologous V $\alpha 24$ –J $\alpha 18$ rearrangement (Porcelli et al. 1993; Dellabona et al. 1994), but it is much less certain that these cells constitute the majority population of CD1d-reactive cells. The invariant α chain is combined with a limited repertoire of β chains, V $\beta 8.2$, V $\beta 2$, and V $\beta 7$ in mice, and V $\beta 11$, a V $\beta 8$ homolog, in humans (Gumperz and Brenner 2001; Godfrey and Berzins 2007). Furthermore, the majority of the CD1d-reactive T cell population with the invariant TCR α chain was characterized as co-expressing C-type lectin family natural killer (NK) cell receptors, therefore they are referred to as invariant natural killer T (*i*NKT) cells. Once tetramers of CD1d loaded with glycolipid antigens were developed, it became apparent that not all cells with the semi-invariant TCR express typical NK receptors, such as NK1.1 (Kronenberg 2005; Bendelac et al. 2007; Matsuda et al. 2008). Furthermore, several populations of T lymphocytes express NK receptors that do not have the invariant TCR, including some cells that recognize CD1d but with diverse TCRs and others that do not recognize CD1d. Therefore, CD1d-reactive NKT cells with a semi-invariant TCR are often referred to as type I NKT cells (Godfrey et al. 2004), to distinguish them from CD1d-reactive cells with more diverse TCRs, sometimes called type II NKT cells, and from cells with other specificities. However, here we will refer to these cells simply as *i*NKT cells.

*i*NKT cells represent a separate T lymphocyte subset with an innate-like or natural memory phenotype and behavior. As a result of their unique differentiation pathway

in the thymus, they acquire an antigen-experienced phenotype and they respond immediately to antigenic stimulation. There is a synergy between the response to weaker Ags, some of which are presumably self-ligands, and cytokines, particularly IL-12 (Brigl et al. 2003; Sada-Ovalle et al. 2008). However, *i*NKT cells also respond to stimulation with cytokines alone, such as the combination of IL-12 and IL-18, in the absence of TCR engagement (Nagarajan and Kronenberg 2007), thereby functioning in this context similarly to cytokine-activated NK cells. After stimulation of their TCR with lipid Ags presented by CD1d, *i*NKT cells rapidly secrete a large amount of cytokines and chemokines, and consequently they activate various immune cells. By this means, *i*NKT cells amplify innate immune responses and provide a bridge response between the earliest innate responder cells and a full adaptive immune response (Parekh et al. 2005; Matsuda et al. 2008).

In the last decade, the origin and structure of the lipid Ags that stimulate *i*NKT cells has been an intense area of research. Several endogenous or self as well as foreign Ags have been identified, and in some cases their likely physiological role in host immune defense has been analyzed. Characterization of synthetic Ags also has provided insight into the nature of the trimolecular interaction between lipid Ags, CD1d, and the TCR of *i*NKT cells, and the consequent immune response elicited from these cells. This review will concentrate on these *i*NKT ligands, including their origin, structure, intracellular processing, and presentation by CD1d, recognition by the invariant TCR and the consequences when they activate *i*NKT cells.

2 Synthetic Lipid Ags

The first and most well-known lipid Ag that can be recognized by the TCR of *i*NKT cells is α -galactosylceramide (α GalCer) (Fig. 2.1). α GalCer is a derivative of natural agelasphins, a class of closely related glycolipids that differ only for the lipid portion. These compounds were found in a screen of natural substances for anti-tumor activity that was carried out by the Kirin Pharmaceutical Company (Natori et al. 1994), and they were purified originally from the marine sponge *Agelas mauritanicus*. α GalCer is a glycosphingolipid (GSL), a type of glycolipid with a ceramide constituting the lipid moiety. In α GalCer, the ceramide has a phytosphingosine base, meaning that the 18-carbon sphingosine is fully saturated. Kirin medicinal chemists modified the structure of the agelasphins by removing the acyl C2 hydroxyl group, which did not significantly affect anti-tumor activity, and by adding carbons to both the phytosphingosine to reach a C18 length and the acyl chain (= C26) to produce α GalCer, which they named KRN7000. In 1997, Kawano et al. showed that α GalCer was a potent ligand able to activate *i*NKT cells in a CD1d-dependent manner (Kawano et al. 1997).

A striking characteristic of α GalCer is that its hexose sugar has an α linkage between the ceramide moiety and carbohydrate head group. Mammalian cells have glycosphingolipids, but they can only synthesize ones with β -linked glycolipids. The α -anomeric conformation of the glycolipid is critical in most instances (see below) for

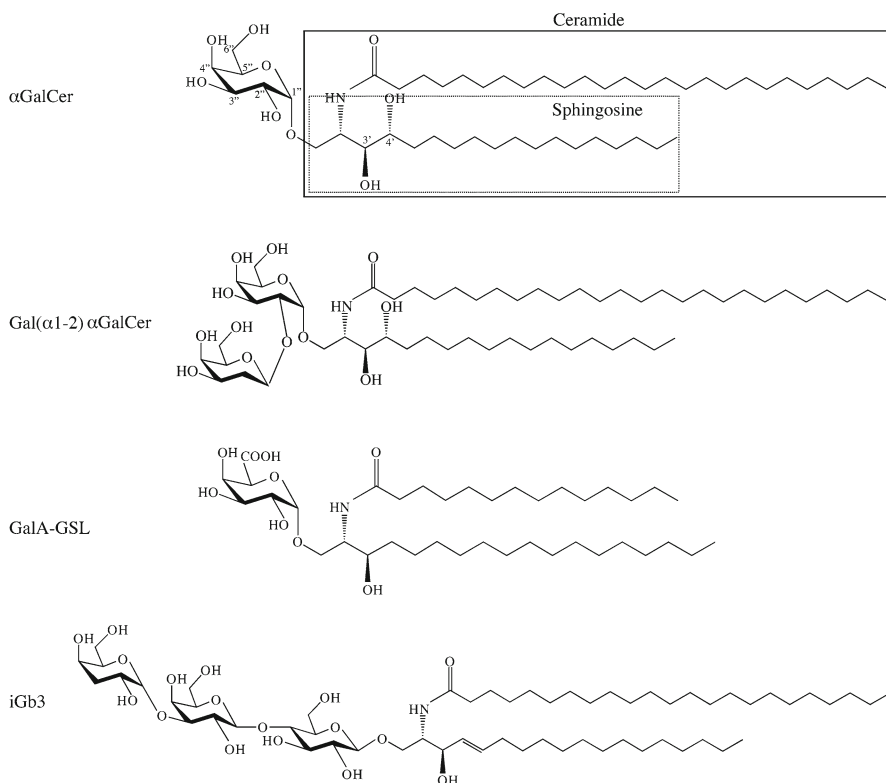


Fig. 2.1 Structures of some natural and synthetic GSL Ags that activate *i*NKT cells. Each of the Ags has a ceramide lipid (enclosed by the *larger box*) consisting of a sphingosine base (the lower of the two aliphatic carbon chains, enclosed by the *internal smaller box*) bound by a 1-1' linkage to hexose sugar

antigenic activity, however, as substitution to a β -anomeric conformation (β GalCer) resulted in complete abrogation of *i*NKT cell stimulation in most studies. Figure 2.2 shows how a GSL with a β -linked sugar would present a very different epitope to a TCR when bound to CD1d. In experiments based on surface plasmon resonance measurements, there was no measurable affinity of the invariant TCR for CD1d loaded with β GalCer (Sidobre et al. 2004). In other studies, however, it was reported that β GalCer also could induce *i*NKT cell stimulation, although it was much less potent than its counterpart with an α -linked sugar (Ortaldo et al. 2004; Parekh et al. 2004). Furthermore, it is possible that a minute amount of α GalCer contaminated some β GalCer preparations. It was also shown that α -glucosylceramide, in which galactose was replaced by glucose, was less antigenic and substitution of galactose with α -linked mannose completely abrogated *i*NKT cell activation (Kawano et al. 1997), demonstrating a high degree of carbohydrate specificity for the *i*NKT cell TCR. Surprisingly, it has recently been shown that β ManCer is an agonist for *i*NKT cells that causes a unique type of anti-tumor effector response in vivo (O'Konek et al. 2011).

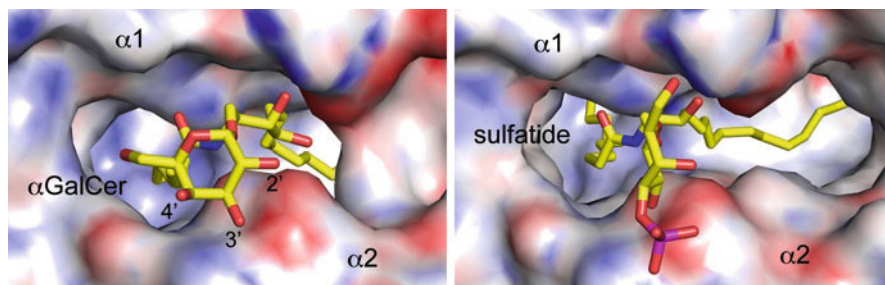


Fig. 2.2 Top view of the mouse CD1d binding groove in complex with α GalCer (left, PDB ID 1Z5L) or sulfatide (right, PDB ID 2AKR). The protein is shown as a molecular surface with electrostatic potential (electronegative in red and electropositive in blue from -30 to 30 kT/e). The ligands are shown in yellow. The hydroxyl groups on the protruding galactose sugar of α GalCer are indicated

More detailed structure–activity studies focusing on the sugar head group further demonstrated that the semi-invariant TCR could sense small modifications of the saccharide moiety. α GalCer analogs that were tested with any type of modification of the equatorial 2'' hydroxyl group (Fig. 2.1) of the galactose sugar, including 2''-amino, 2''-deoxy, or 2''-fluoro sugars, could not stimulate iNKT cells (Wu et al. 2005). The one exception is the disaccharide compound Gal(α 1-2) α GalCer (Fig. 2.1), in which the second or outer galactose is α linked to the 2'' position of the inner galactose, that in turn is bound to the ceramide lipid. In this particular modification, the second galactose needs to be removed by lysosomal carbohydrate antigen processing to generate a potent antigen (Miyamoto et al. 2001; Prigozy et al. 2001; Wu et al. 2005). Chemical modifications at the 3''- and 4''-hydroxyl positions of the sugar affected antigenic potency to some extent, but these could still be recognized (Wu et al. 2005; Xing et al. 2005). Also, an additional galactose at the 4''-hydroxyl position required antigenic processing to remove this saccharide to generate α GalCer before it became antigenic to iNKT cells (Zhou et al. 2004). By contrast, modifications at the 6''-position could be well tolerated for binding to CD1d and interaction with the TCR, without requiring lysosomal processing to remove them (Prigozy et al. 2001; Zhou et al. 2004). Therefore, these studies show that a portion of the hexose sugar ring contributes to antigenic activity, in particular, the 2'' position, with the 3'' and 4'' also contributing. A synthetic ceramide antigen with a trihydroxyl threitol moiety, rather than a hexose sugar, also can be recognized by the invariant TCR although this structure is believed to mimic the central portion of the hexose sugar (Silk et al. 2008b).

In parallel with structure activity studies of the hexose sugar, careful studies on the influence of modifications of the ceramide on the antigenic activity of α GalCer were also carried out. Compared with the original GSLs identified from the marine sponge (*A. mauritanicus*), α GalCer possessed a C4 hydroxyl group on the sphingosine base that is absent in the original GSLs, which enhanced the ability to stimulate iNKT cells (Brossay et al. 1998; Wu et al. 2005). Further analysis of the role of hydroxyl groups showed that removing both the 3' and 4'-hydroxyl groups on the

phytosphingosine base could lead to the inactivation of the compound, and that the 3'-hydroxyl group likely was more important than the 4'-position hydroxyl (Morita et al. 1995; Brossay et al. 1998; Sakai et al. 1999; Miyamoto et al. 2001; Ndonye et al. 2005).

Variation in the length and saturation of the hydrocarbon chains gave surprising results, because although these portions of the molecule are buried in the CD1d groove, they have a surprising degree of influence not only on antigenic potency, but also on the quality of the immune response they elicit. The phytosphingosine of α GalCer could be truncated from 18 to 11 carbons without a catastrophic loss of antigenic activity, although this compound was not as effective as α GalCer in stimulating *i*NKT cell hybridomas. A fatty acid chain is required, demonstrated by the finding that an α GalCer variant with its C26 fatty acid substituted with an aniline ring was not able to stimulate *i*NKT hybridoma cells for IL-2 synthesis (Brossay et al. 1998). However, unexpectedly, an acyl chain with even only two carbons could still activate *i*NKT cells (Morita et al. 1995; Brossay et al. 1998). A recent report showed that a synthetic GSL with a cyclopropyl sphingosine (C13-15) was significantly diminished in its antigenic activity; it retained some potency (Kinjo et al. 2008).

Several α GalCer variants with modifications of the ceramide lipid enhanced in vivo production of IL-4, the prototypical Th2 cytokine. Yamamura and co-workers initiated this line of work by synthesizing an α GalCer analog with a phytosphingosine chain reduced from 18 to 9 carbons, and the N-acyl chain from 26 to 24 carbons. While it had been well known that *i*NKT cells stimulated by α GalCer immediately produced both T helper type 1 (Th1) and T helper type 2 (Th2) cytokines (Kawano et al. 1997), they found that OCH induced a Th2 biased cytokine profile, with a quick and predominant release of IL-4 and a diminished IFN γ response (Miyamoto et al. 2001; Oki et al. 2004). Further investigations showed that the induction of biased cytokine profile was not limited to the compounds with modified phytosphingosine moiety, but in fact was observed with other modifications of the CD1d-buried lipid. For example, it was shown that α GalCer analogs in which the C26:0 N-acyl chain was substituted with shorter, unsaturated fatty acids, similarly modified the outcome of *i*NKT cell activation in favor of IL-4 synthesis (Yu et al. 2005). An α GalCer analog that has two unsaturated bonds in a fatty acid chain consisting of 20 carbons (C20:2) showed an obvious tendency to induce more IL-4 relative to IFN γ , with only a small effect on antigenic potency. These findings are consistent with another report in which the authors found that truncation of either the phytosphingosine or N-acyl chain endowed GSLs with the ability to bias *i*NKT cell-induced responses in favor of Th2 cytokines (Goff et al. 2004).

A different type of modification of the ceramide lipid could induce increased in vivo production of the prototypical Th1 cytokine, IFN γ . By replacing the oxygen atom linking and the galactose sugar with ceramide with a methylene group, Tsuji et al. synthesized and tested a C-glycosidic form of α GalCer, named C- α GalCer. After intravenous injection, C- α GalCer induced as much IFN γ as α GalCer, but it

induced much less IL-4. Interestingly, by stimulating Th1 responses, C- α GalCer could be shown to induce more potent anti-malarial and anti-cancer responses (Schmieg et al. 2003; Yang et al. 2004).

What is the mechanism for alterations in the *i*NKT cell response caused by modifying the ceramide lipid? By analogy with altered peptide ligands, it was attractive to propose that a reduced TCR affinity and/or a shorter half-life of the glycolipid/CD1d complexes on the cell surface, contributing to a reduced duration of TCR signaling, would cause a Th2 skewed cytokine profile from *i*NKT cells. Consistent with this, it was reported that a stronger and/or longer stimulation of *i*NKT cells in vitro caused more IFN γ release through an NF- κ B-mediated mechanism (Oki et al. 2004). Three findings, however, were not consistent with this direct mechanism. First, it was found that the immediate, in vivo response of *i*NKT cells to glycolipid antigen stimulation, when analyzed directly ex vivo, for example, by intracellular cytokine staining, was not Th2 skewed with a weaker antigenic stimulus (Matsuda et al. 2003; Stanic et al. 2003b; Sullivan et al. 2010). Second, the Th1 skewing Ag C- α GalCer is an even weaker antigen for the invariant TCR than OCH (Sullivan et al. 2010). Third, although glycolipid Ag-activated *i*NKT cells do immediately produce both IL-4 and IFN γ , in addition to other cytokines, after in vivo stimulation, most of the IFN γ produced results from the activation of NK cells stimulated downstream of *i*NKT cell activation (Carnaud et al. 1999; Eberl and MacDonald 2000; Matsuda et al. 2003; Sullivan et al. 2010), a phenomenon referred to as *trans* activation (Parekh et al. 2005). In fact the peak of this IFN γ synthesis, as measured by ELISA for serum cytokines, comes much later than the peak of IL-4 synthesis (Oki et al. 2004). Furthermore, C- α GalCer was shown to be more effective at this *trans* activation than OCH (Sullivan et al. 2010). The key explanatory factor for the ability of C- α GalCer for stimulating NK cell activation, thereby causing a Th1 cytokine skewing of the overall immune response, is apparently its pharmacokinetics, which allows it to carry out a more prolonged stimulation of *i*NKT cells. Therefore, although C- α GalCer-CD1d complexes have a lower affinity for the TCR, after C- α GalCer injection glycolipid plus CD1d complexes accumulate in vivo over time in APC. In fact, APC from mice injected 44 h earlier with C- α GalCer are as potent as APC from mice injected 44 h earlier with α GalCer, when tested ex vivo for their ability to stimulate *i*NKT cell hybridomas (Sullivan et al. 2010). Because C- α GalCer does not bind with more stability to CD1d, this accumulation of the compound in complex with CD1d must reflect its longer half-life in APC. By contrast, APC from mice injected even 17 h earlier with OCH no longer have stimulatory activity. Consistent with the importance of the biologic half-life of the glycolipid Ag, other studies showed that Th2 skewing compounds are less stable in cells due to lysosomal degradation (Bai et al. 2009). In fact, the synthetic chemist Richard Franck et al. designed C- α GalCer with the idea that replacement of the O-glycosidic bond would make the compound more stable. A portion of the CD1d molecules on the cell surface are found in lipid rafts (Lang et al. 2004; Park et al. 2005), and a recent paper reported that compounds that induce a Th2 cytokine skewing did not accumulate as glycolipid/CD1d complexes in the

raft portion of the plasma membrane (Im et al. 2009). It is not known, however, why the presence of glycolipid plus CD1d complexes in lipid rafts is correlated with an increased biologic half-life in APC, and moreover, why the *trans* activation of NK cells requires prolonged *i*NKT cell stimulation.

3 Natural Microbial Lipid Ags

Although α GalCer and similar compounds are useful tools for the study of *i*NKT cells, and α GalCer is a relatively high affinity antigen that is under development as an anti-cancer agent (Cui et al. 1997; Shimizu et al. 2007; Kunii et al. 2009; Motohashi et al. 2009), it was never regarded to be an important natural antigen for this population, given that the mammalian immune system is not selected in evolution for defense against marine sponges (Sandberg and Ljunggren 2005). The first report of a CD1d-dependent, microbial lipid antigen-mediated stimulation of *i*NKT cells was from the Schaible group in 2004 (Fischer et al. 2004). A series of lipid compounds, including phosphatidylinositoldimannoside (PIM₂), lipoarabinomannan, lipomannan, diacyltrehalose, trehalose monomycolate, and trehalose dimycolate, were isolated and purified from *Mycobacterium bovis*. Only PIM could bind to CD1d, but the binding was abolished by phospholipase A₂ (PLA₂), which degrades PIM to the lyso form by removing the fatty acid at the C2 position. CD1d loaded with PIM in a mouse B cell lymphoma line, A20, stimulated splenic *i*NKT cells to release IFN γ in a CD1d-specific manner. Furthermore, a PIM-loaded CD1d tetramer stained both mouse and human *i*NKT cell populations, although only a very small percentage of the *i*NKT cells were stained compared to α GalCer-loaded tetramer. Because *i*NKT cells differ for the complementarity determining region (CDR) 3 of the TCR β chain, it is possible that only a subset of this population, based upon particular CDR3 β sequences, has PIM reactivity.

In 2005, three groups independently found the first bacterial source for a glycolipid Ag that could activate essentially the entire population of *i*NKT cells from mice and humans. The Ags are GSLs, meaning that they have a ceramide lipid, and they were obtained from *Sphingomonas* spp., which are nearly ubiquitous, environmental Gram-negative bacteria that lack lipopolysaccharide (LPS) (Kinjo et al. 2005; Mattner et al. 2005; Sriram et al. 2005). Representative of this class of antigens are GSL-1 from *Sphingomonas paucimobilis* and GSL-1' from *Sphingomonas yanoikuyae*, which highly resemble α GalCer in structure, with a ceramide backbone employing an α -anomeric conformation of glycosidic bond. The major difference between GSL-1 and GSL-1', when compared to α GalCer, is that the *Sphingomonas* compounds use glucuronic acid and galacturonic acid, respectively, as the saccharide group, instead of the simple galactose in α GalCer. In later studies, GSL-1' has been referred to as GalA-GSL, standing for galacturonic acid (GalA)-containing glycosphingolipid (GSL) (Fig. 2.1). It is interesting that the hydroxyl group at the 2-carbon position in the fatty-acyl chain of GSL-1 and GSL-1' also appears in the original agelasphins that α GalCer was derived from (Natori et al. 1994). Based on the overall structural similarity and the presence of *Sphingomonas* bacteria

in sea water, it is possible that the agelasphins were a component of some *Sphingomonas*-like bacteria that were associated with the marine sponge *A. mauritanus* (Tsuji 2006). GSL-1 and GSL-1', and similar compounds found by others, have the ability to stimulate mouse and human *i*NKT cells both in vivo and in vitro, although these compounds are less potent than α GalCer (Kinjo et al. 2005; Mattner et al. 2005; Sriram et al. 2005), with a corresponding approximately 50-fold reduction in TCR affinity for the complex GalA–GSL with CD1d (Wang et al. 2010). Mice deficient for CD1d, or for the $J\alpha 18$ segment required to form the invariant TCR, had reduced clearance of the bacteria at early times, although by day 10 the bacteria were cleared even in *i*NKT cell deficient mice, with no evidence for liver damage when moderate doses of bacteria were used.

Not only do different *Sphingomonas* strains differ in their GSL components, but also a single strain can vary its GSL synthesis according to the nutritional supplements and other environmental changes (Kawahara et al. 2000, 2001, 2006). Besides GSL Ags containing monosaccharide sugars, antigens with tri- or tetrasaccharides also have been tested for antigenic activity. For the most part, those GSL Ags with more complex carbohydrates were not highly stimulatory, due to a failure by APC to efficiently degrade the more complex sugar head groups to a simple monosaccharide form that could be recognized (Long et al. 2007; Kinjo et al. 2008). One variant, known as GSL-4A, with a tandem tetrasaccharide α -mannose(1–2) α -galactose(1–6) α -glucosamine(1–4) α -glucuronosyl(1–1)ceramide, was able to stimulate *i*NKT cells, albeit to a much lesser degree compared with the monosaccharide-containing GSL. Unexpectedly, GSL4A could be recognized directly by the TCR of *i*NKT cells after presentation by CD1d, without a requirement for lysosomal processing of its tetrasaccharide head group (Kinjo et al. 2008). Regardless, the varying antigenic activity of the *Sphingomonas* GSL components, combined with the ability of these bacteria to modulate their GSL biosynthesis, might indicate a strategy by which these microorganisms could evade the immune response mediated by *i*NKT cells (Kinjo et al. 2008).

Although *Sphingomonas* bacteria are not highly pathogenic, and in fact there is evidence suggesting they may be a commensal organism (Wei et al. 2010), they potentially could be involved in the pathogenesis of infectious or inflammatory diseases. It has been reported that exposure to *Sphingomonas* bacteria is involved in the causation of a mouse model of primary biliary cirrhosis, an autoimmune disease in which the bile ducts are damaged (Hsueh et al. 1998; Perola et al. 2002; Selmi et al. 2003; Mattner et al. 2008).

A second type of microbe known to have glycolipid Ags that activate most *i*NKT cells is *Borrelia burgdorferi*, a spirochete that causes Lyme disease, currently the most common vector-borne disease in the USA (Bacon et al. 2008). Depending on the inbred strain background, mice infected by this pathogen can develop chronic inflammation of varying severity in skin, joints, heart, and the nervous system (Yang et al. 1994). Infected CD1d-deficient mice developed increased arthritis, and the secretion of pathogen-specific IgM antibodies by MZB cells was impaired in the absence of CD1d, coinciding with an elevated pathogen burden in the blood (Kumar et al. 2000; Belperron et al. 2005) and in the joint itself (Lee et al. 2010). These results indicated CD1d is required to control *Borrelia* infection. As noted

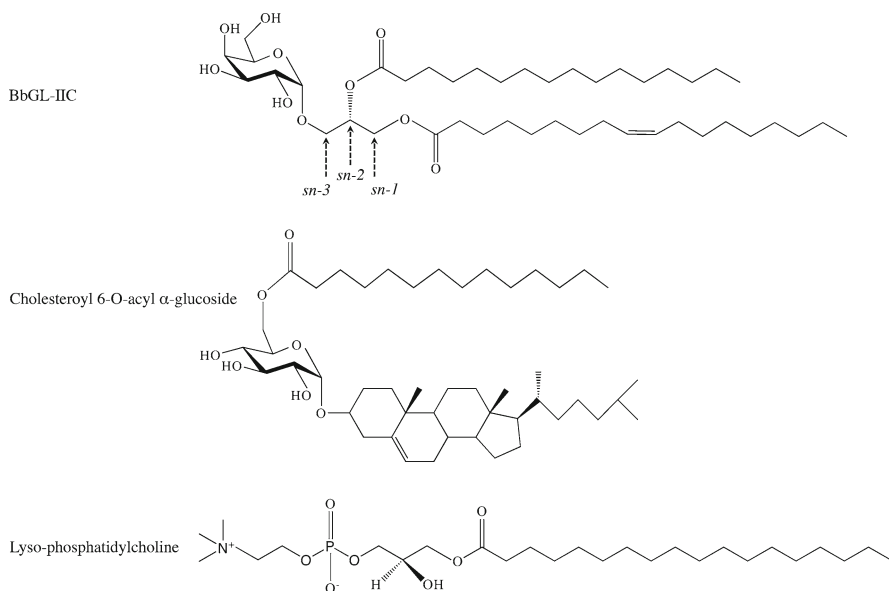


Fig. 2.3 Structures of synthetic and natural Ags that are not GSLs. *Top:* the *B. burgdorferi* glycosylated diacylglycerol Ag BbGL-IIc is depicted. *Middle:* cholesterol containing Ag from *H. pylori*. *Bottom:* lyso-phosphatidylcholine, a self-Ag for human *i*NKT cells

above, however, CD1d can stimulate cells in addition to *i*NKT cells, but in papers published recently, Jα18 knockout mice were shown to have defects in spirochete clearance and the prevention of arthritis when analyzed on the BALB/c background (Tupin et al. 2008), or carditis, when C57BL/6 mice were tested (Olson et al. 2009). These data indicate that *i*NKT cells have a critical role in the host defense against *Borrelia* pathogens.

Two abundant glycolipids were found in the cell wall of *B. burgdorferi*, and these are targets of an antibody response in infected individuals (Hossain et al. 2001). One of these is cholesteryl 6-*O*-acyl-β-D-galactopyranoside, called *B. burgdorferi* glycolipid 1 (BbGL-I), and the other is 1,2-di-*O*-acyl-3-*O*-α-D-galactopyranosyl-sn-glycerol (BbGL-II) (Ben-Menachem et al. 2003). BbGL-II was found to stimulate *i*NKT cells in a CD1d-dependent manner (Kinjo et al. 2006). It consists of a D-galactose moiety linked to diacylglycerol (DAG) (Fig. 2.3). The structure of BbGL-II is distinct from that of the GSL Ags, because its lipid moiety is identical to a typical glycerophospholipid, instead of a sphingolipid, with a ceramide backbone. Strikingly, however, this DAG *i*NKT cell Ag also has an α-anomeric glycosidic bond linking the hydrophilic saccharide portion to the hydrophobic lipid.

In fact, purified BbGL-II is not a uniform entity, as it contained a mixture of at least five different fatty acids that in principle could be linked in different ways to the *sn*-1 and *sn*-2 positions of the glycerol. Kinjo et al. analyzed eight synthetic versions of BbGL-II that differ only with regard to their fatty acid composition, and

interestingly, only one of these, BbGL-IIc, with a C18:1 oleic acid in the sn-1 position and a C16:0 palmitic acid in the sn-2 position was highly antigenic for *i*NKT cells. Human CD1d presented different BbGL-II variants to *i*NKT cells, preferring those with more unsaturated bonds in the fatty acids. The structural basis of this difference is uncertain, but human CD1d has a bulky tryptophan at position 153 in the $\alpha 2$ helix, near the opening of the groove where the sugar protrudes, compared to glycine in the equivalent position for mouse CD1d. Mutagenesis studies show that this position influences the preferential presentation of DAG Ags with certain fatty acids (Wang et al. 2010), perhaps because of differences in the conformation of the CD1d molecule when opened for lipid loading.

Recently it was shown that a cholesterol-containing antigen from *Helicobacter pylori*, which causes stomach ulcers, also could activate *i*NKT cells (Chang et al. 2011). The antigen, cholesteroyl 6-*O*-acyl α -glucoside, could activate *i*NKT cells in vitro in a CD1d-dependent fashion, and could modulate the function of *i*NKT cells in vivo in favor of IFN γ production, which was protective for asthma in young mice exposed to this compound (Chang et al. 2011). It is not certain, however, how the cholesterol moiety participates in the binding to CD1d and/or interactions with the semi-invariant TCR.

4 Endogenous Lipid Ags

In a variety of contexts, *i*NKT cells exhibit CD1d-dependent activation in the apparent absence of microbial Ags. Therefore, these cells are believed to be self-reactive, and it was further assumed that there is a single self-Ag, or a limited set of closely related and relatively high affinity self-Ags that can activate the invariant TCR. This hypothesis remains unproven (Gapin 2010), however, and several types of self-Ags have been identified, including GSLs and antigens that are not GSLs (Pei et al. 2011). Furthermore, none of the self-Ags have been shown convincingly to be required absolutely for the differentiation or stimulation of *i*NKT cell populations. An early analysis by mass spectrometry of the cellular lipids bound to CD1d found glycosylphosphatidylinositol (GPI) to be a major ligand (Joyce et al. 1998). However, there was no evidence showing this ligand can stimulate *i*NKT cells physiologically. Therefore, GPI was suggested to function as a “spacer” by filling the groove of CD1d to prevent its collapse when the protein is synthesized in the endoplasmic reticulum, with true stimulatory Ags acquired later as CD1d traffics to and recycles through endosomes. Subsequent chemical analyses of the lipids bound to CD1d found a variety of species, including phosphatidyl inositol (PI), phosphatidyl choline (PC), and the lyso forms of these antigens, i.e., forms lacking one of the fatty acid chains (Cox et al. 2009; Yuan et al. 2009).

The first demonstration that a self-derived cellular lipid had a stimulatory effect on *i*NKT cells was reported by Gumperz et al. (2000). They found that plate-bound mouse CD1d molecules presented lipid Ags from an extract of tumor cells to an *i*NKT cell hybridoma. The lipid Ags were identified to be phosphatidylinositol (PI)

and its derivatives, with PI having the highest antigenic potency. However, this *i*NKT cell hybridoma did not respond to α GalCer, and therefore it apparently was not representative of the *i*NKT cell population. Similarly, it was reported that disialoganglioside GD3, another self-derived cellular lipid provided an Ag for a subset of *i*NKT cells. GD3 immunization was required, however, in order to detect a GD3-reactive *i*NKT cell population (Wu et al. 2003). In both of these cases, a subset of cells with a particular V β TCR, that defines a particular specificity, may have been detected.

Because GSLs provide such high potency exogenous Ags for *i*NKT cells, it is perhaps reasonable to suppose that self-Ags also are different types of GSLs. Evidence consistent with GSLs providing self-Ags has been reported in several papers, but these did not identify a particular structure (Paget et al. 2007; Salio et al. 2007). Paget et al. found CpG ODN, a TLR9 ligand, activated DCs to synthesize a charged α -linked GSL(s), and also to produce type I IFN, both of which were required to stimulate *i*NKT cells (Paget et al. 2007). Also, Salio et al. showed Toll-like receptor (TLR) ligands could induce increased GSL biosynthesis in Ag presenting cells, coincident with enhanced recognition of CD1d-associated endogenous lipids by *i*NKT cells. Recently it was shown that APC from mice deficient for α -galactosidase A had increased stimulatory activity for *i*NKT cells. α -Galactosidase A removes terminal galactose sugars from certain oligosaccharide-containing GSLs, and together with other experimental results, the authors concluded that this finding reflects the ability of α -galactosidase A to degrade a major self-Ag for *i*NKT cells (Darmoisse et al. 2010).

Further identification of potentially important self-antigens was obtained by analyzing cells and mice deficient for enzymes important for GSL biosynthesis. In mammals, there are two GSL biosynthetic pathways: the major one synthesizes oligosaccharide-containing GSLs, including gangliosides, from β -glucosylceramide, and the minor one synthesizes GSLs from β -galactosylceramide. β -galactosylceramide deficiency did not cause a significant decrease in *i*NKT cell frequency (Stanic et al. 2003a). Because loss of ceramide glucosyltransferase is a lethal mutation, the effect of β -glucosylceramide deficiency was determined in the GM95 cell line, a mutant of the B16 melanoma deficient for this enzyme. GM95 transfectants with transient CD1d expression had defects in stimulating *i*NKT cells, while the control cells, which were rescued by introducing the cDNA encoding the wild-type enzyme, did not. This result indicated that β -glucosylceramide might be the biosynthetic precursor of the self-Ag, as β -glucosylceramide itself cannot stimulate *i*NKT cells directly. It is surprising, therefore, that stable CD1d transfectants of GM95 were able to stimulate *i*NKT cell autoreactivity (Pei et al. 2011).

An intriguing finding in the search for *i*NKT cell self-Ags was the identification of the GSL isoglobotrihexosyl ceramide (iGb3) as a putative self-Ag required not only for *i*NKT cell differentiation, but also for the stimulation of the autoreactive *i*NKT cells in the periphery (Zhou et al. 2004; Mattner et al. 2005). This identification was based in part on the finding that β -hexosaminidase b-deficient mice, a model of human Sandhoff's disease, have a profound reduction in *i*NKT cell number, while this was not found with several other GSL biosynthetic deficiencies. The conclusion

that iGb3 is the main thymic *i*NKT cell-selecting ligand subsequently has been challenged in different ways. Several reports have questioned if the defect in *i*NKT cells is related directly to Ag biosynthesis, as opposed to resulting from lipid storage disease, which would cause a more general defect in the lysosomal site of CD1d Ag loading (Gadola et al. 2006b; Schumann et al. 2007). Others have questioned if humans have a functional iGb3 synthase (Christiansen et al. 2008), and if mice have significant amounts of iGb3 in double positive thymocytes, the cells that positively select *i*NKT cells, or in the DCs that are most important for stimulating them in the periphery (Speak et al. 2007). The presence of iGb3 in these cell types remains controversial (Li et al. 2008). The most compelling evidence against iGb3 as the main positively selecting self-Ag, however, comes from the finding that *i*NKT cell number and function are normal in mice deficient for iGb3 synthase (Porubsky et al. 2007).

Regardless, iGb3 is a mammalian GSL, and it can stimulate *i*NKT cells. It consists of a ceramide, like all GSLs, and a trisaccharide head group, composed of two galactose rings and a glucose ring in tandem (Fig. 2.1). Consistent with other mammalian GSLs, but distinct from the microbial lipid Ags, the sugar portion and the lipid portion are linked with a β -anomeric conformation of the glycosidic bond. This raises the interesting question as to how this bulky trisaccharide is recognized by the invariant TCR, and with the β -glucose linked to the ceramide drastically out of position, which of the three sugars participates most in TCR recognition. The study of chemical analogies of iGb3 indicates that the terminal galactose of the trisaccharide is the most important for TCR recognition (Chen et al. 2007a), consistent with a model (Zajonc et al. 2008) in which the third sugar is squashed down into a position similar to the lipid-proximal sugar in monosaccharide-containing antigens.

Just as exogenous lipid Ags are not limited to GSLs, for example, DAG-containing Ags also have been found (Kinjo et al. 2006), a similar diversity in lipid structure has also been reported for the putative self-Ags. As mentioned above, a glycerophospholipid, PI, also is able to stimulate an *i*NKT cell hybridoma (Gumperz et al. 2000), but it is not the universal *i*NKT cell ligand. A recent study reported the surprising finding, however, that lyso-phosphatidylcholine (lyso-PC), eluted along with several other lipids from human CD1d, could stimulate the majority of human *i*NKT cell clones tested in a CD1d-dependent fashion (Cox et al. 2009; Fox et al. 2009). Lyso-PC, different from other phosphoacylglycerols like PI (Fig. 2.3), only contains a single hydrophobic acyl chain, yet is the most active compound compared to all of the others that were eluted from human CD1d. It is unknown why a glycerol Ag with a single fatty acid chain is more potent than one with two acyl chains, while the opposite is true for GSLs. Moreover, we found that lyso-PC could not stimulate mouse *i*NKT cells, although the data suggested that the self-Ags in some cell types could not be GSLs (Pei et al. 2011). This difference between mouse and human *i*NKT cell self-Ags is not surprising, however, as previously it was shown that presentation of the self-Ag for mouse *i*NKT cells required lysosomal localization of mouse CD1d, while a similar requirement was not found for human *i*NKT cell responses to self-Ag (Chen et al. 2007b). These data suggest that the two self-Ag recognition systems could be quite different, although this is curious considering the conservation of the invariant TCR, and its well-conserved recognition of microbial Ags.

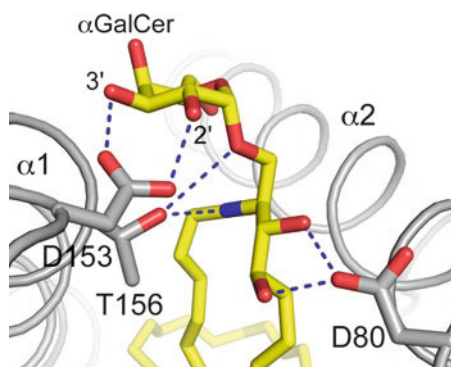
5 Structural Basis of Lipid Ag Recognition

In structure, CD1d is closely related to the MHC class I antigen presenting molecules, as it consists of a β_2m light chain and a heavy chain with Ag-binding $\alpha 1$ and $\alpha 2$ domains attached to a more membrane proximal immunoglobulin-like $\alpha 3$ domain. To the C terminal side of the $\alpha 3$ domain there is a *trans*membrane sequence and a short cytoplasmic tail (Brigl and Brenner 2004; Cohen et al. 2009). High-resolution three-dimensional structures of liganded CD1d, with and without the *i*NKT cell TCR bound, have provided important insights into the structural basis for lipid Ag presentation and recognition (Gadola et al. 2006a; Kjer-Nielsen et al. 2006; Borg et al. 2007; Pellicci et al. 2009). These have been complemented by site-directed mutagenesis studies of CD1d (Burdin et al. 2000; Kamada et al. 2001) and the TCR (Scott-Browne et al. 2007; Mallevaey et al. 2009). Compared with MHC class I or II molecules, the Ag binding groove of CD1d is deeper, narrower, and more hydrophobic. It is composed of two connected pockets, A' and F', that accommodate the hydrocarbon chains of lipids. The A' pocket is deeply buried and bigger with the capacity of accommodating up to a 26-carbon long hydrocarbon chain. The hydrocarbon chains must wind around a central pole formed by amino acids Cys12 and Phe70, and they do this in either a clockwise or counterclockwise direction, depending on the Ag bound. The F' pocket is smaller, and it can accommodate 18 carbon chains, or even longer, in a more or less linear orientation. The F' pocket is also more accessible to the solvent, at least when the TCR is not bound. A lipid Ag is accommodated in the CD1d groove with the hydrophobic lipid moiety anchored in the two pockets and the saccharide head group protruding from an opening in the surface of the CD1d protein. Therefore, the *i*NKT cell TCR contacts mainly the exposed carbohydrate moiety, and, to a lesser degree, the hydrophilic portion of lipid backbone, as well as amino acids on the top of CD1d.

The α -anomeric linkage of the sugar is a key factor that affects the recognition of lipid Ags by the invariant TCR. The crystal structures of α -linked GSL-CD1d complexes show that the carbohydrate portions of the Ag adopt a parallel orientation to the top of CD1d surface. The β -anomeric conformation renders the saccharide head group in a perpendicular orientation to the top of CD1d, and therefore pointing up toward the TCR, likely interfering with the TCR interaction. As noted above, iGb3 and β ManCer must constitute exceptions. For iGb3, perhaps the highly exposed trihexosyl head group is squashed by the invariant TCR to lie flat between the TCR and CD1d surface (Zajonc et al. 2005, 2008; Wu et al. 2006; Yin et al. 2009). While α ManCer, with the mannose sugar having a 2'' OH in the axial position, is not antigenic, it is possible that when bound to CD1d the β -linked sugar orients this hydroxyl in a more optimal position for contacting the CDR3 α region of the TCR, similar to the equatorial OH found in GSL Ags containing α -linked galactose or glucose.

Hydrogen bonds formed between CD1d and the hydrophilic portions of the glycolipid Ag, including the carbohydrate, and for GSL Ags the 3' hydroxyl of the sphingosine, are important for orienting the Ag for TCR recognition. All the GSL Ags adopt a similar mode of interaction with CD1d, with the N-acyl chain in the

Fig.2.4 Binding of GSL ligands in the mouse CD1d binding groove. Details of the interaction of α GalCer (ligand in yellow, PDB ID 1Z5L) with residues on the $\alpha 1$ and $\alpha 2$ helices of mouse CD1d are depicted. H bonds that stabilize the bound Ag are shown as blue dashed lines



A' pocket and the sphingosine in F' pocket. If the acyl chain is too short to fill the A' pocket, a spacer lipid, apparently is recruited to occupy the remainder of the pocket (Zajonc and Kronenberg 2007, 2009; Zajonc and Wilson 2007). However, when the sphingosine is short, as for OCH, the F' pocket tends to collapse at least partially, therefore leading to subtle structural changes at the CD1d surface above this pocket, and consequently affecting recognition by the *i*NKT TCR (McCarthy et al. 2007). The fixed mode of GSL Ag binding is based on the planar N-amide of the ceramide and hydrogen bonds between Asp80 of the $\alpha 1$ helix with the sphingosine hydroxyls, and Asp153 of the $\alpha 2$ helix with the 2' and 3' hydroxyls of the galactose sugar (Fig. 2.4).

DAG-containing Ags have a lipid backbone that is less rigid than the ceramide backbone of GSLs, and their different structure leads to two possible binding orientations to mouse CD1d for Ags in this category. Phosphatidylcholine (PC) is bound by CD1d with the sn-1 fatty acid in the F' pocket and sn-2 fatty acid in the A' pocket (Giabbai et al. 2005), while PIM₂ was bound in opposite orientation (Zajonc et al. 2006), which, as a result, will lead to a slight difference in head group positioning for the two lipids above the CD1d surface. For DAG containing glycolipid Ags with sugar head groups, the H bonding pattern is different from the GSLs. The galactose of BbGL-IIC is tilted up and away from the $\alpha 2$ helix of mouse CD1d, thereby losing the H bonds with Asp153, and there is a single H-bond between the galactose and Arg79 of mouse CD1d, a bond not observed with any GSL Ags.

A structural comparison of α GalCer to the two bacterial Ags, *S. yanoikuyae* GalA-GSL and *B. burgdorferi* BbGL-IIC, bound to mouse CD1d, with and without the TCR, is highly instructive of the principles governing synthetic and bacterial glycolipid Ag recognition. When α GalCer is bound to mouse CD1d, the sugar is protruding in the same orientation before and after TCR engagement, and the roof over the F' pocket is closed. Therefore, the antigen/CD1d complex is correctly oriented and no accommodation is required when the TCR binds. For GalA-GSL complexes with CD1d, which have a weaker affinity for the invariant TCR, the sugar is oriented correctly when the GSL Ag binds CD1d without the TCR, but the roof over the F' pocket is not closed. Closure does occur, however, in the complex

with the TCR. Therefore, in this case mouse CD1d accommodates the TCR by conformational change above the F' pocket. BbGL-IIc complexed with mouse CD1d is nearly tenfold weaker in binding affinity compared to GalA-GSL, and therefore very substantially weaker, approximately 500-fold weaker, than α GalCer. In this case, the sugar is rotated 60° counterclockwise from the optimal position when bound to mouse CD1d without the TCR, and the roof over the F' pocket also is not closed. Remarkably, when the invariant TCR is bound, the trimolecular complex that includes BbGL-IIc is quite similar to the one with α GalCer. Therefore, binding of the TCR involves not only conformational changes over the F' pocket of mouse CD1d but also movement of the galactose sugar by 60° in the clockwise direction.

These findings also shed some light on the mysterious selectivity for particular fatty acids for the recognition of the DAG-containing glycolipid Ags from *B. burgdorferi*. Although BbGL-IIf is not antigenic for mouse iNKT cells, it also binds to mouse CD1d (Wang et al. 2010). However, the different possible binding modes for the DAG-containing compounds causes the loss of antigenic activity in this case. For the antigenic BbGL-IIc, the C18:1 fatty acid linked to the sn-1 position of the glycerol is bound to the A' pocket of mouse CD1d. For the non-antigenic BbGL-IIf, it is the sn-2 fatty acid, also C18:1, that is bound in the A' pocket. While we do not know why the A' pocket strongly prefers a C18:1 fatty acid over both the C16:0 alternative in BbGL-IIc, or the C18:2 in BbGL-IIf, the difference in orientation of the glycerol backbone causes the sugar in BbGL-IIf to be rotated a further 60° counterclockwise compared to the complex of BbGL-IIc with mouse CD1d, or 120° compared to α GalCer complexes with mouse CD1d. This is apparently too far out of the optimal position for effective accommodation and invariant TCR binding. We have less information about how an Ag like BbGL-IIf is presented effectively by human CD1d, but as noted above, the evidence suggests that the presence of Trp153 in human CD1d compared to Gly155 in mouse CD1d is in part responsible.

The reciprocal cross-species reactivity of mouse and human iNKT TCRs (Brossay et al. 1998) and high-resolution crystal structures of mouse and human CD1d-glycolipid-TCR tri-molecular complexes show that the invariant TCR binds in a similar pattern to diverse antigens, reflecting the high degree of evolutionary conservation in their interactions. The main contacts of the TCR with CD1d antigen presenting molecule involve amino acids in CDR3 α with a contribution of amino acids in CDR2 β , particularly Tyr50 β . These interactions principally involve amino acids above the F' pocket. However, because of variability in the TCR β chain, both with regard to the presence of diverse CDR3 β regions, and the use in mice of three predominant V β segments, as noted above the TCR of iNKT cells is in effect semi-invariant. It is therefore not surprising that differences in the V β regions affect the interactions of the TCR with mouse CD1d- α GalCer complexes (Borg et al. 2007; Pellicci et al. 2009). For example, compared with the V β 8.2 chain in the invariant TCR, structural analysis of a V β 7-containing invariant TCR showed that this β chain contributed to a greater extent to interactions with the CD1d- α GalCer complex (Pellicci et al. 2009). Furthermore, the presence of V β 7 resulted in an altered interface between the α chain and CD1d, despite sharing of identical, invariant V α chains. The structural and mutagenesis studies also show that CDR3 β

sequences can participate in the trimolecular interaction to varying extents, thereby influencing TCR avidity (Mallevaey et al. 2009). These alterations also provide a structural basis not only for variability in avidity for common antigens for the entire population, but they also provide a rationale for the presence of subspecificities within the population, such as described for GD3 and perhaps other Ags.

6 Conclusion/Summary

Antigens for *i*NKT cells come from diverse sources, including synthetic Ags related to α GalCer, microbial Ags, and endogenous or self-Ags. Although much has been learned about the types of glycolipid Ags that are recognized by *i*NKT cells, how they bind to CD1d, and how the complexes interact with the TCR to achieve a conserved binding mode, the picture remains incomplete.

Considerable effort has been placed on the testing of synthetic Ags related to α GalCer, a highly potent Ag that was the first one discovered. Significant variations on the basic α GalCer structure, which alter either the sugar and/or the stereochemistry of its linkage to ceramide, the sphingosine base, or the fatty acid, can still be recognized by *i*NKT cells, and in some cases provide Ags equal to, or even greater in potency, than α GalCer (Schmiege et al. 2003). Variants of α GalCer can engender highly different cytokine responses, however, and while this involves a network of cellular responses initiated downstream of the initial *i*NKT cell activation, the molecular and cellular mechanisms for cytokine skewing by glycolipid Ags remain to be fully determined.

Microbial Ags that activate a large fraction of *i*NKT cells have been identified from three types of bacteria, and in two cases they share the common features of having a lipid with two acyl chains and an α -linked hexose sugar. Although the lipids are buried in the CD1d groove, studies have shown how the lipids can determine antigenic potency by influencing the orientation of the sugar protruding from the roof of CD1d. Furthermore, it has been demonstrated that these two types of microbes, *Sphingomonas* spp. and *B. burgdorferi*, produce mixtures of antigenic and non-antigenic glycolipids, which could constitute an immune evasion mechanism. For the third type of bacteria, *H. pylori*, the Ag has a very different structure that includes a cholesterol moiety derived from the eukaryotic host. The underlying biochemistry of the interactions between the *H. pylori*, Ag, CD1d, and the TCR is not known. Furthermore, although there is detailed knowledge of the structure of the Ags from these few species, the number of different types of bacteria having such Ags, the diversity of their structures, and the relationship of the antigen-specific response to the clearance of the bacterial infection all require further investigation.

Like the synthetic and microbial Ags, the self-Ags also have diverse structures. iGb3 clearly is a GSL self-Ag that activates *i*NKT cells, but it is highly unlikely that is required or the major self-Ag, and data from several groups suggest there could be other GSL self-Ags. In humans, an entirely different structure, lyso-PC, has been described as a self-Ag that activates many *i*NKT cells. Lyso-PC does not

activate mouse *i*NKT cells, however, which makes it difficult to show that this Ag is required for *i*NKT cell differentiation. Moreover, it would be surprising if mouse and human *i*NKT cells depended on entirely different self-Ags, considering the high degree of interspecies conservation of the *i*NKT cell TCR. Additionally, it remains uncertain if there is a single, major self-glycolipid required for the selection and/or activation of *i*NKT cells, or alternatively, if there are collections of relatively low affinity glycolipids bound to CD1d collectively that are sufficient for activation.

How is it that the semi-invariant TCR can recognize so many different types of Ags? Part of the answer lies in the extraordinarily high affinity of the TCR interaction with α GalCer-CD1d complexes, measured in some studies to be as low as 11 nM. It is therefore not difficult to measure an antigenic response to compounds with an affinity that is 500 times weaker than α GalCer, thereby allowing for considerable structural variation. Additionally, unlike for MHC class I and class II antigen presenting molecules, the roof over the CD1d groove is mostly closed, especially when the TCR is bound, and therefore many of the important contacts between the semi-invariant TCR and the glycolipid-CD1d complex involve CD1d. These stereotypical contacts, involving mostly CDR3 α and CDR2 β , dictate a particular binding mode, with the TCR oriented parallel to the CD1d α helices. Given the importance of contacts with CD1d, it is therefore possible that lipid-containing Ags with different structures can provide the marginal boost to TCR affinity that would allow for TCR engagement and *i*NKT cell activation.

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