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Antigen specificity of invariant natural killer T-cells

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ABSTRACT

Natural killer T-cells, with an invariant T-cell antigen receptor α -chain (iNKT cells), are unique and conserved subset of lymphocytes capable of altering the immune system through their rapid and potent cytokine responses. They are reactive to lipid antigens presented by the CD1d molecule, an antigen-presenting molecule that is not highly polymorphic. iNKT cell responses frequently involve mixtures of cytokines that work against each other, and therefore attempts are underway to develop synthetic antigens that elicit only strong interferon-gamma (IFN γ) or only strong interleukin-4 responses but not both. Strong IFN γ responses may correlate with tighter binding to CD1d and prolonged stimulation of iNKT cells, and this may be useful for vaccine adjuvants and for stimulating anti-tumor responses. iNKT cells are self-reactive although the structure of the endogenous antigen is controversial. By contrast, bacterial and fungal lipids that engage the T-cell receptor and activate IFN γ from iNKT cells have been identified from both pathogenic and commensal organisms and the responses are in some cases highly protective from pathogens in mice. It is possible that the expanding knowledge of iNKT cell antigens and iNKT cell activation will provide the basis for therapies for patients suffering from infectious and immune diseases and cancer.

Introduction

General background

Initially described in mice [1], natural killer T-cells (NKT cells) have characteristics of both innate and adaptive immune cells. In mice, they were originally identified as cells that

express NK 1.1, a marker for NK cells, which are a type of innate lymphocyte, expressed together with a $\alpha\beta$ T-cell receptor (TCR), characteristic of adaptive immune cells. It is now known that all NKT cells do not express NK 1.1, and a subset of these lymphocytes expresses an invariant TCR α -chain that imparts a particular glycolipid specificity that is described further below. Cells with this phenotype were later confirmed to be present also in humans [2]. As in the mouse, the human

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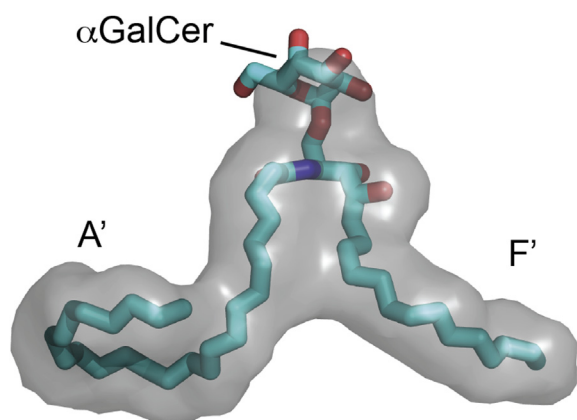


Fig. 1 – CD1d lipid binding pocket showing A' and F' grooves with α -galactosylceramide bound to CD1d for reference.

NKT cell population is marked by a NK cell receptor, in this case, CD161, and it also expresses the $\alpha\beta$ TCR with an invariant α -chain that shares significant homology with its mouse counterpart [3]. Comprising approximately 1% of peripheral lymphocytes in mice, NKT cells numbers in the peripheral blood mononuclear cells of humans tend to be less frequent, with a wide variation ranging from under 0.01% to 1% in healthy donors [4]; however, using CD161 and TCR expression as a definition of NKT cells, the frequency is much higher.

Because they express the $\alpha\beta$ TCR, it was originally assumed that NKT cells would recognize peptides, similar to conventional T-lymphocytes that recognize peptides presented by major histocompatibility complex (MHC)-encoded antigen presenting proteins. Although early data and some recent data [5,6] indicated that NKT cells may be able to recognize peptides, it has been widely confirmed that NKT cells recognize and respond to lipids, mostly glycolipids. These antigens are presented not by MHC-encoded molecules but by CD1d, a related protein encoded outside the MHC gene complex [7]. This review will provide a brief overview of NKT cells, some of their known antigens and properties of the CD1d molecule.

Type I and Type II natural killer T-cells

Although there are T-lymphocytes having different specificities that express NK receptors, NKT cells are now generally defined as T-cells that recognize CD1d. The most commonly studied NKT cells are invariant NKT cells (iNKT cells) or Type I NKT cells, which express a nearly fixed or invariant TCR α -chain encoded by a $V\alpha 14$ - $J\alpha 18$ rearrangement. This α -chain is co-expressed most frequently with a $V\beta 8.2$, $V\beta 7$, or $V\beta 2$ TCR β -chain in mice. In humans, the Type I NKT cell TCR is formed by a nearly fixed $V\alpha 24$ - $J\alpha 18$ (TRAV1-2-TRAJ18) rearrangement paired with a $V\beta 11$ β -chain. The mouse and human invariant α -chains are true homologs, as are human $V\beta 11$ and mouse $V\beta 8$. As a consequence; the specificity of mouse and human iNKT cells is highly conserved. This Type I NKT cell TCR has a conserved parallel binding motif radically different than the binding orientation of the TCR of mainstream CD4 or CD8 T-cells [8]. Type II NKT cells recognize CD1d as well; however, the α -chain is not highly restricted in diversity. Therefore, they do

not have a single specificity, and they have been much less studied [9]. Here, we will focus on Type I NKT cells or iNKT cells.

Biological response of invariant natural killer T-cells

iNKT cells are very rapid responders when stimulated through their TCR, providing a cytokine burst within 90 min of *in vivo* stimulation [10]. Though TCR recognition of a lipid antigen presented by CD1d [Fig. 1], iNKT cells can induce a wide range of cytokines including T-helper Type 1 (Th1), T-helper Type 2 (Th2), and other responses. Activated iNKT cells not only secrete these cytokines but also induce other cells to secrete cytokines. The results from a number of studies demonstrate that the totality of the iNKT cell-induced immune response is dependent on the structure of the lipid antigen that is presented and recognized. Certain lipid antigens cause the production of predominately Th1 cytokines such as interferon-gamma (IFN- γ) and tumor necrosis factor, and other lipids lead to a more Th2 skewed pattern of cytokines that includes interleukin (IL-4), IL-5, and IL-13 [11].

α -Galactosylceramide

The most studied glycolipid that activates iNKT cells, also the first discovered, is α -galactosylceramide (α GalCer) [Figs. 1 and 2]. This is sometimes considered Th0 skewing lipid as iNKT cells that respond to this lipid robustly produce both IFN- γ and IL-4. α GalCer was originally identified by the Kirin Pharmaceutical Company in a screen of natural extracts for substances that prevent metastases of the mouse B16 melanoma, and it was shown to reduce liver metastases [12]. The structure was then synthesized and optimized by medicinal chemistry using the tumor metastases assay [13,14]. α GalCer has α -linked galactose, a phytosphingoid base chain with 18 carbons, and an acyl chain containing 26 carbons. α GalCer has not yet proven highly successful in human cancer studies, which may be due to the fact that it leads to both Th1 and Th2 cytokine responses [15,16]. These opposing responses may not promote an optimal anti-tumor response, which is more Th1-dependent. There are other explanations for reduced efficacy including the lower affinity of α GalCer/CD1d complexes for the human TCR compared to the mouse TCR [17]. For these reasons, there have been extensive efforts to develop other iNKT cell-activating lipids that can skew the cytokine response, especially in a Th1 direction. In addition, the type of antigen-presenting cell (APC) targeted may be critical, and in clinical trials, transfer of dendritic cells (DC) incubated with α GalCer generated a more robust iNKT cell response than α GalCer alone [16,18,19]. Continuing efforts to develop more effective glycolipids, delivery systems, and cell-based therapies using α GalCer remain underway.

CD1d

CD1d antigen-presenting molecule is a member of the family of CD1 proteins. This family is divided into two groups: Group 1 CD1 proteins (CD1a, CD1b, and CD1c) and Group 2 CD1 (CD1d) [20]. There is also a third, intermediate group member (CD1e). Whereas CD1a, CD1b, CD1c, and CD1d are found on the cell surface; CD1e is an intracellular protein that facilitates

glycolipid processing and presentation [21]. These proteins are found in humans and most other mammals; however, the mouse genome contains only two copies of the CD1d gene and no Group 1 CD1 proteins.

CD1d has a heterodimeric structure similar to MHC Class I antigen presenting molecules, with a heavy chain having three extracellular domains and a conserved β_2 -microglobulin subunit [22]. However, whereas MHC Class I molecules have shallow binding grooves capable of binding peptides that are typically nine amino acids in length; CD1d has a much deeper, narrower, and more hydrophobic groove containing two pockets, delineated as A' and F' [Fig. 1]. This groove is perfectly suited to bind glycosphingolipids (GSLs) that have two hydrophobic chains that can anchor deeply within it. The phytosphingoid base chain of GSLs is localized to the smaller F' pocket, whereas the amide-linked fatty acid chain binds in the A' pocket. Within the A' pocket, the lipid chain must curl around a central point created by Cys12 and Phe70 [21]. The binding of the lipid chains within CD1d exposes the saccharide head group that is recognized and forced into a fixed orientation by the iNKT cell TCR [23].

CD1d is synthesized in the endoplasmic reticulum (ER) and binds to self-phospholipids that allow it to traffic to endosomal compartments and the cell surface [21]. It has a tyrosine-containing cytoplasmic tail motif that mediates internalization to endosomes and eventually to lysosomes before recycling back to the cell surface. Exchange of self-antigens obtained in the ER with exogenous glycolipids and with self-lipids involved in the positive selection of iNKT cells in the thymus occurs within endosomal compartments [24,25], and for some exogenous antigens, also on the cell surface [26]. CD1d is expressed on a wide variety of hematopoietic series cells including B-cells, DC, macrophages, Langerhans cells, monocytes, T-cells, and iNKT cells [27]. Some nonhematopoietic cells including hepatocytes and intestinal epithelial cells also express it [28,29]. Data indicate that DCs are the key activators of iNKT cells for exogenous glycolipids, except that various types of macrophages are important for presenting glycolipids in particulate form such as on beads or in liposomes [30]. Recently, the CD8 α^+ DEC-205 $^+$ DC subset was identified as the APC type responsible for inducing both Th1 and Th2 responses [31]. According to these findings, increased expression of cell surface markers Rae-1 and CD86 by APCs leads to a Th1 cytokine profile while increased APC expression of PD-L2 serves to promote a Th2 cytokine profile [31].

Synthetic antigens

Head group modifications

The hydrophobic chains of iNKT cell lipid antigens are usually bound deep within the pockets of CD1d, and a polar head group is exposed for recognition by the iNKT cell TCR. The CD1d molecule stabilizes this head group through interactions with $\alpha 1$ and $\alpha 2$ helices [22]. In humans, position 153 of the $\alpha 2$ helix is a tryptophan amino acid, instead of a glycine in the homologous position (155) of mouse CD1d. Tryptophan, being a much bulkier amino acid, shifts the sugar head group into a slightly different position [23,32]. This is the main distinction

between the structures of the GSL-CD1d complexes expressed by mice and humans.

Contacts between Asp153 and Thr156 of mouse CD1d and the 2' and 3'-hydroxyls of the α GalCer saccharide head group as well as the α GalCer O-glycosidic oxygen are important for ordering the sugar head group [33,34]. Because iNKT cell TCR recognizes the galactose head groups of GSLs, it is not surprising that altering this head group had deleterious effects on TCR binding. Modifications of the 2', 3', or 4' positions, particularly 2' diminished iNKT cell responses and TCR binding [35,36]. For example, while α GalCer and α -glucosyl ceramide (α GluCer) could both activate iNKT cells, which indicated that the axial versus equatorial orientation of the 4'-hydroxyl group is not critical; α GalCer was the most potent antigen. In contrast, α -mannosyl ceramide (α ManCer) did not activate iNKT cells, indicating that the equatorial 2'-hydroxyl is critical [37]. Additional studies demonstrated the importance of the 2'-hydroxyl group in TCR recognition, as synthetic analogs of α GalCer with modifications of this position lacked antigenic activity [38]. The 3' position is more permissive as modifications at this position decreased but did not completely diminish activity [38–40]. Modifications of the 6' position are the most tolerated. This makes sense as crystal structures of the CD1d-lipid-TCR complex show that the 6' position does not make contacts with the TCR [17,41–43]. Tolerance for modifications at the 6' position were revealed in a study that compared responses to Gal ($\alpha 1-2$) GalCer with Gal ($\alpha 1-6$) GalCer, which are both GSLs with a disaccharide as opposed to monosaccharide head groups. While Gal ($\alpha 1-2$) GalCer required carbohydrate processing to remove the terminal galactose in order for antigenic activity, Gal ($\alpha 1-6$) GalCer did not [44]. Indeed, modification of the 6'-hydroxyl proved to be beneficial when PBS57, a 6'-deoxy-6'-acetamide α GalCer analog, was synthesized [45]. PBS57 was not only more easily solubilized than α GalCer, and it also induced a more potent *in vivo* cytokine response [46]. The 6' position is permissive for the addition of other bulky chemical groups as exemplified by the naphthylurea α GalCer molecule (NU- α GC) [Figs. 2 and 3] [47]. NU- α GC causes a robust Th1 cytokine response and reduces tumor metastases in mice even more effectively than α GalCer. It is very likely that NU- α GC binds in a more stable fashion to CD1d than α GalCer as the NU group serves as a “third anchor” of the lipid for binding to the CD1d molecule. It binds on top instead of deep in the CD1d groove, but without impeding the TCR [48]. Other 6' modifications such as NC- α GalCer, 4ClPhC- α GalCer, and PyrC- α GalCer are also antigens that induce strong Th1 cytokine responses, correlated with a marked reduction of B16 melanoma metastasis to the liver [49]. PyrC- α GalCer, the most potent of these lipids, also shows novel and increased contacts with the both CD1d and the iNKT cell TCR. The 6' modifications have also served as tools for labeling GSLs with biotin or fluorophore, which has permitted analyses of their progress through endosomal compartments in cells [50].

Other synthetic alterations have been used to create α -carba-GalCer analogs [51]. In 2009, carbasugar and cyclitol analogs of α GalCer were generated, and another three analogs, RCAI-56 (a carba- α -D-galactose analog), RCAI-59 (a 1-deoxy-neo-inositol analog), and RCAI-92 (a 1-O-methylated analog), also were found to be Th1 skewing lipids [52]. The α -

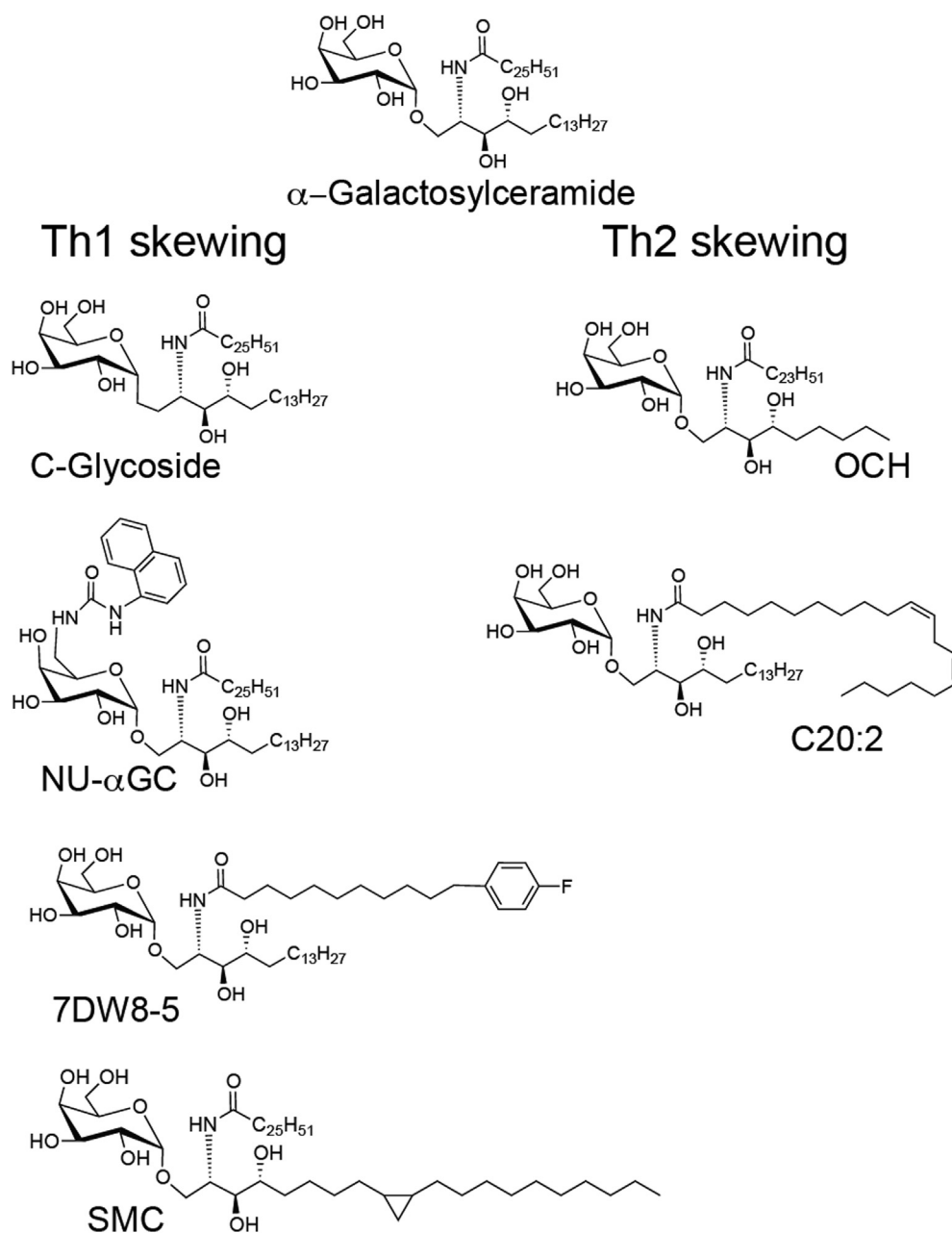


Fig. 2 – Some representative Th1 and Th2 cytokine skewing lipids compared to α -galactosylceramide.

carba-GalCer analog (RCAI-56), with the oxygen atom of D-galactose replaced with a methyl group, was shown to be an effective Th1 skewing molecule [53]. The authors proposed two mechanisms for this response. First, the methylene group replacing the oxygen atom interaction of the sugar head group makes the lipid less susceptible to degradation by hydroxylation, therefore allowing it to be presented for longer. Second, the oxygen of α GalCer may lead to a repulsive interaction with Pro28 of the TCR α -chain whereas the carbon of α -carba-GalCer would lead to an enhanced hydrophobic interaction [53]. This lipid also was tested in collagen-induced arthritis, an autoimmune model of rheumatoid arthritis in mice, and it was shown to provide protection against Th17-mediated

autoimmune arthritis through the induction of Th1 cytokines [54].

HS44 is another GSL with head group modifications of α GalCer that induces a strong Th1 cytokine response. HS44 is an aminocyclitol molecule in which the sugar head group is a carba cyclitol ring that mimics glucose instead of galactose, and which has the O-glycosidic linkage replaced with an amide group [55]. Structural analysis and binding assays by surface plasmon resonance showed that when bound to CD1d this compound had a 14-fold weaker interaction with the iNKT cell TCR compared to α GalCer. Despite this, it caused a strong Th1 cytokine response and was effective at preventing tumor metastases [56].

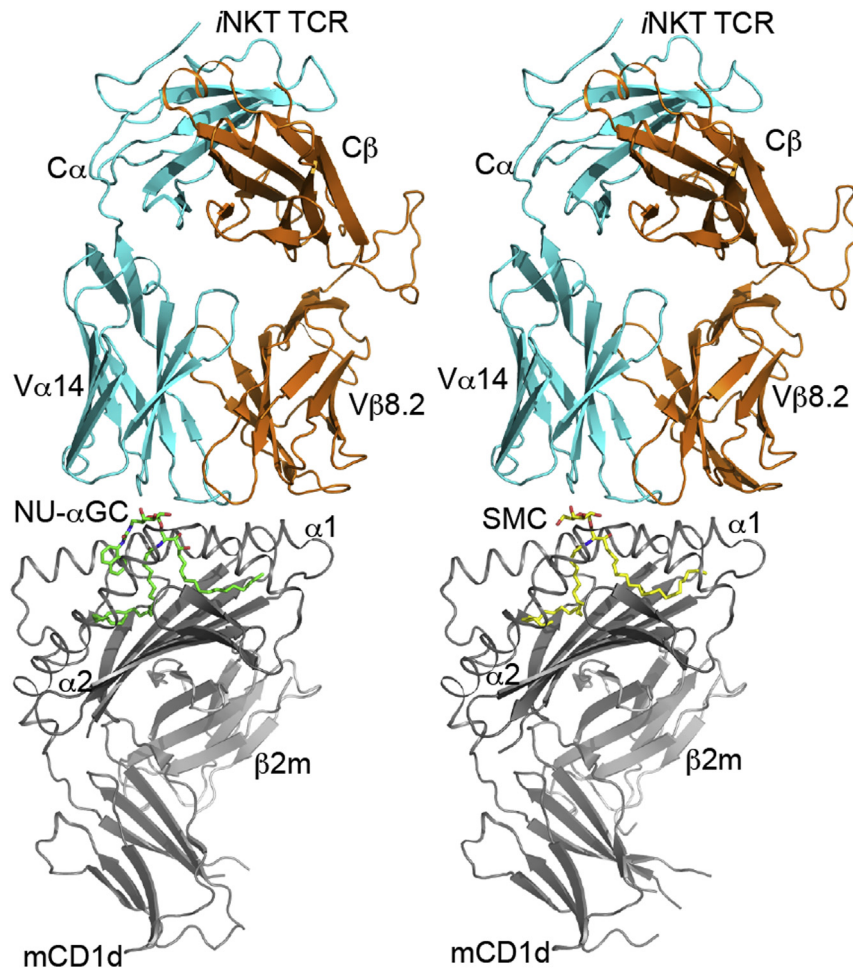


Fig. 3 – Two T-helper Type 1 skewing lipids crystallized in complex with mouse CD1d and the invariant Natural Killer T-cell receptor. PDB files: naphthylurea, α -galactosylceramide (PDB code: 3QUZ), SMC (PDB code: 3TVM).

Linkage modifications

As previously mentioned [55], the O-glycosidic linkage of α GalCer can be altered, and in doing so, can lead to some unique properties. In 2003, the oxygen bond was altered to a CH_2 , theorizing that this GSL would be more resistant to α -galactosidase-mediated catabolism [57,58]. This compound, referred as C-glycoside [Fig. 2], was the first glycolipid shown to induce a strong Th1 cytokine response in mice [59], but it did not stimulate human iNKT cells very strongly. The ternary structure of C-glycoside/CD1d complexes bound to the iNKT cell TCR demonstrated the importance of hydrogen bonding of the oxygen in the O-glycosidic linkage to CD1d [48,60]. Without this linkage, the galactose headgroup was in a sub-optimal orientation when bound to CD1d, resulting in a lower TCR affinity compared to α GalCer/CD1d complexes.

Several C-glycoside analogs with a double bond were synthesized to try to generate a glycolipid providing a more fixed orientation of the galactose headgroup, in the expectation that this would activate iNKT cells to produce a strong Th1 response [61]. One of these, GCK127 had an E-olefin linker instead of a CH_2 glycosidic linkage. This molecule activated both mouse and human iNKT cells, albeit it was less potent than α GalCer [61]. The O-glycosidic linkage has also been

modified by replacing the oxygen with a sulfur atom. The α -S-GC molecule induced a more Th2 skewed cytokine profile in mice and did not lead to increased survival in a tumor model [62]. A sulfur linkage was proposed to have a similar conformation to an oxygen bond, because although a sulfur atom is larger than oxygen, the bond angle is less than an O-glycosidic linkage and a thioglycosidic linkage would be less susceptible to enzymatic cleavage [63]. However, *in vivo* studies in mice indicated that α -S-GC did not lead to iNKT cell proliferation or cytokines. The authors proposed that the α -S-GC compound would lack some key hydrogen bonds when bound to CD1d, but this remains a conjecture. Another group synthesized the same compound and showed that although it did not stimulate mouse iNKT cells, it could activate human iNKT cells, causing cytokine secretion and iNKT cell-induced maturation signals in human DCs [64]. This is an intriguing counter example of species specificity of iNKT cell reactivity, compared to C-glycoside, which only works to activate mouse iNKT cells but not those from humans.

Lipid chain modifications

The sphingoid base is a defining property of the GSL antigens that activate iNKT cells. The most commonly found

sphingosine chain length in animal tissues is the aliphatic C: 18 chains; however, the number of carbon lengths can range from 14 to more than 27, and the sphingosine can have a wide variety of saturation levels and branched modifications [65]. The phytosphingosine of α GalCer [Fig. 2], with a hydroxyl group at C-4, differs from the more common natural sphingosine, which typically contains a *trans* double bond between C-4 and C-5. OCH is a compound with a phytosphingosine shortened by several carbon atoms [Fig. 2] [66]. This lipid antigen induced a more IL-4 dominated or Th2 profile. When injected into mice, OCH was shown to reduce the symptoms of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [66]. The further shortening of the sphingoid chain also led to a Th2 cytokine profile [67]. Different alterations of the sphingoid base can have an opposite polarizing effect on the cytokine response. For example, SMC124 is a synthetic Th1 skewing GSL similar to α GalCer but with a sphingoid base chain length increased to 22 linear carbons with the addition of a cyclopropyl group at C11-12 [Figs. 2 and 3]. It was designed to mimic partially a naturally occurring GSL called plakoside A [68]. The results from structural studies of OCH and SMC124 bound to mouse CD1d suggest that the length of the lipid chain packed within the F' pocket of CD1d may alter the presentation of the GSL [68]. The longer, bulkier chain of SMC124 may anchor the lipid more deeply within the antigen-presenting molecule, thereby prolonging antigen presentation, which may, for reasons that have not been completely elucidated, contribute to Th1 cytokine skewing. Conversely, OCH does not have a long chain to anchor CD1d and may have a reduced antigen presentation time.

The acyl chain of a number of synthetic GSL antigens is typically longer than the sphingoid base. For α GalCer, the acyl chain is an unbranched and fully saturated 26 carbons long. C20:2 is a GSL antigen containing a di-unsaturated and shorter, 20 carbon, and acyl chain [Fig. 2] [69]. This compound was identified in a screen of multiple α GalCer analogs with shorter acyl chains, all of which induced an enhanced Th2 cytokine profile [67]. The decrease in the carbon chains of the lipid, either in the acyl chain or in the sphingoid base, is proposed to destabilize the interactions between the lipid and CD1d. Indeed, when the lipid chains become too short, the interaction of the lipid and the CD1d is too unstable for any stimulation of iNKT cells [67]. Conversely, the elongation of the carbon chains, or the addition of bulky groups to the acyl chain, caused a Th1 profile that may be due to stabilization of the CD1d/lipid interaction. For example, the addition of an aromatic group to the terminus of the fatty acid created a potent IFN- γ inducing lipid in human cells [70]. 7DW8-5, which contains a C10 length fatty acyl chain with a fluorinated benzene ring at the end, is planned for use in clinical trials as an adjuvant for a malaria vaccine [Fig. 2] [71]. 7DW8-5 was shown to exhibit a higher binding affinity to both mouse and human CD1d molecules than α GalCer, resulting in a more potent stimulatory activity for iNKT cells [72]. Ultimately, 7DW8-5 could display a stronger adjuvant effect than α GalCer for malaria vaccine testing in mice [72] and nonhuman primates. EF77 is a companion lipid to SMC124 because it is also partially modeled after α GalCer and plakoside A. EF77 is identical to α GalCer but with an elongated acyl chain

containing a cyclopropyl group. Like SMC124, EF77 also induced a strong Th1 cytokine response in mice [68]. Both of these GSL antigens were shown to form more stable complexes *in vivo* with CD1d on the surface APCs, at least when compared to α GalCer, consistent with the hypothesis that stronger or prolonged antigen interactions with CD1d favor IFN- γ production *in vivo*. [68].

Endogenous ligands

When the initial antigens for iNKT cells were discovered, it was predicted that the linkage of the sugar to the lipid could only be α -anomeric, and a β -linkage would not lead to iNKT cell activation [37,73]. This was presumed as logical as the α -anomeric form as well as D-glycosylceramides were not thought to be detected in mammals and are therefore “foreign” epitopes. However, the analysis of iNKT cell differentiation in the thymus indicated that there had to be a self-ligand that could positively select these cells. A β -Linkage of sugar to the ceramide lipid does occur naturally in mammals, β -anomeric GSLs are part of cellular membranes [74,75]. β -linkages therefore were considered to be candidates for the major self-ligands [76]. Mice lacking β GalCer synthase, and thus lacking β GalCer, have normal iNKT cell development [77], and while mice treated with this compound have decreased iNKT cells *in vivo*, there was no detectable cytokine signal [78]. β GluCer, having glucose instead of galactose, is both an anabolic and catabolic GSL pathway metabolic intermediate [79], and it decreases *in vitro* iNKT cell proliferation [80] and is accumulated in patients with Gaucher's disease, which have an increase in iNKT cells [81]. Surprisingly, while α ManCer fails to activate iNKT cells, β ManCer is an iNKT cell ligand capable of inducing a protective anti-tumor response [82]. This anti-tumor ligand operates through the induction of TNF α and nitric oxide, without IFN- γ , and it does not induce anergy, a side effect of some other strong iNKT cell ligands [83].

Because iNKT cells originate in the thymus, it was assumed that there must be some endogenous ligand(s) that participate in their positive selection. The hunt for endogenous ligands turned up several candidates including, isoglobotrihexosylceramide (iGb3), plasmalogen lysophosphatidylethanolamine [84], lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, phosphatidylserine, phosphatidic acid β GluCer, β GalCer [85], and even a peptide from mouse collagen [6,75,81,86–89]. The structures of some of these are shown in [Fig. 4] iGb3, the first identified self-ligand [86], was crystallized, and it was shown that the mouse CD1d molecule can bind iGb3 [Fig. 5] [90]. When CD1d is loaded with iGb3, it forms a complex with the iNKT cell TCR in which the lipid-proximal sugar is moved into a conformation similar to α -linked ligands. The terminal sugar of iGb3 forming novel contacts with CD1d [Fig. 5] [91,92]. However, the validity of such a compound as an endogenous ligand has been clouded by reports that suggest mice lacking iGb3 synthase, essential for iGb3 formation, still have a normal iNKT cell population [93]. In addition, it has been claimed that human CD1d cannot present iGb3 because of the glycine-tryptophan difference mentioned above [94], and iGb3 is not present in

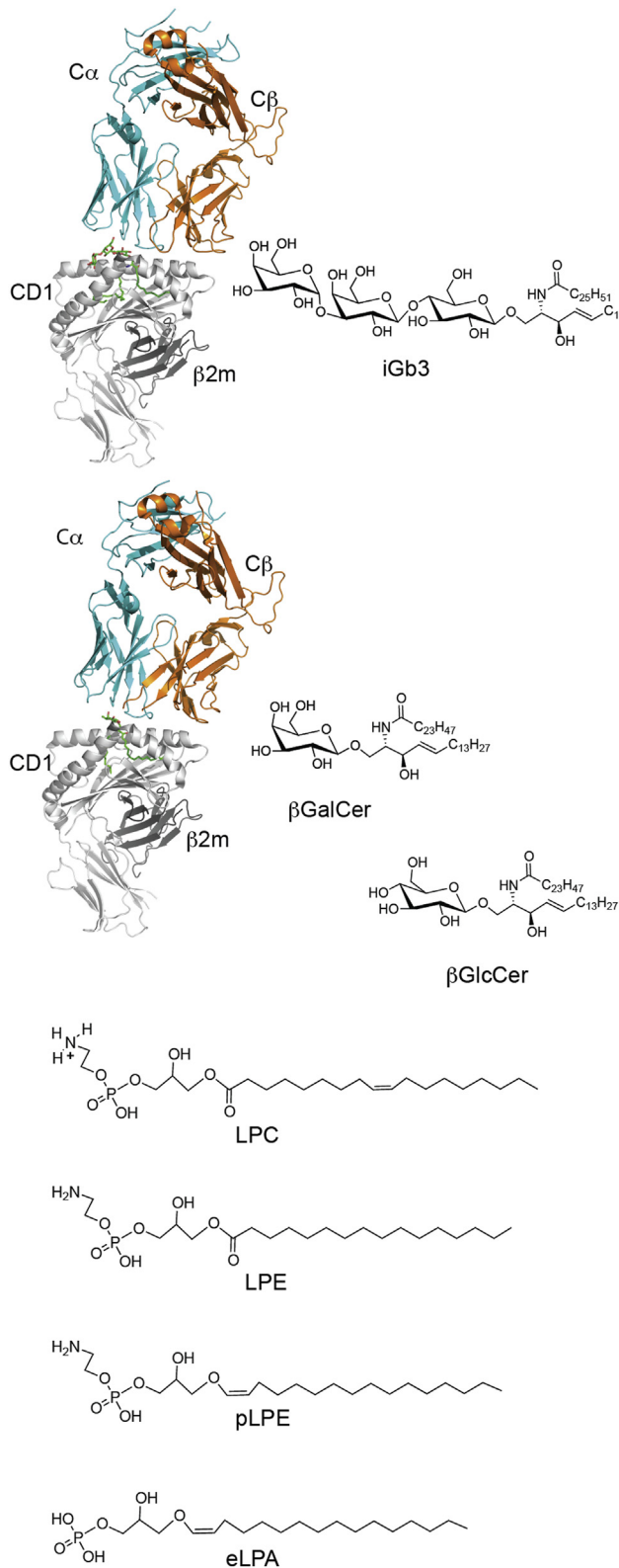


Fig. 4 – Proposed endogenous ligands for invariant natural killer T-cells. Top: Crystal structures of tri-molecular complexes and the corresponding glycosphingolipid antigen structures are indicated. Bottom: Phospholipid-containing putative self-antigens.

mouse or human thymus [95]. Therefore, iGb3 cannot be a required self-ligand for iNKT cells. β -Glucosyl ceramide was identified as another endogenous ligand for iNKT cells. With a structure very similar to α GalCer, and data indicating that this lipid accumulated in the presence of a microbial infection [96], as well as tumor prevention data [97], it seemed very logical that this ligand could activate iNKT cells. However, recent data has indicated that the activation of iNKT cells in this study could be due to contaminating trace α -anomeric lipids, possibly generated during synthesis of β -glycolipids [98,99]. Regardless of the antigen, it is entirely possible that these self-ligands serve as not only a selection mechanism but also a means to get over an activation threshold when iNKT cells are activated by cytokines such as IL-12, IL-18, or IFN-1 [100–103].

Bacterial antigens for invariant natural killer T-cells

Mycobacterium tuberculosis

The first lipid antigens identified for any T-lymphocytes were lipids from *Mycobacteria* presented by Group 1 CD1 molecules [104–106]. These bacteria have an unusual outer waxy coating, comprised of different lipids, which is used to evade immune clearance. A screen of CD1d binding mammalian and *Mycobacterium* lipids identified phosphatidylinositol tetramannoside (PIM₄) [107] isolated from a related bacterium, *Mycobacterium bovis* bacillus, a cause of bovine tuberculosis. The structure of a similar lipid, PIM₂, bound to CD1d allowed for modeling of the PIM₄ molecule in the CD1d groove [108]. However, a synthetic PIM₄ molecule was shown to not stimulate iNKT cells [109], highlighting the need to compare the outcomes with synthetic and purified lipids. Subsequent work showed that Type II NKT cells with diverse TCRs could recognize different phospholipids from *Mycobacterium tuberculosis*. [110].

Sphingomonas spp.

Sphingomonas spp. was the first bacteria unambiguously shown to have antigens for iNKT cells. *Sphingomonas* are commensal bacteria of the human intestine, and there is evidence that commensal bacteria affect the number and function of iNKT cells [73,111–113]. Although not highly pathogenic, *Sphingomonas* includes more than 30 species that live in a wide variety of environments including soil and seawater. *Sphingomonas paucimobilis* is the most pathogenic, and it may be an opportunistic pathogen associated with suppression of the immune system [114]. As early as 2000, purification of GSLs from *Sphingomonas* bacteria was performed and it was noted that bacterial synthesis of different lipids can change depending on environmental factors [115–117]. There is an unusually high presence of GSLs in this α -Proteobacteria, instead of the LPS that is otherwise commonly found in Gram-negative bacteria [118]. In 2005, using both purified and synthetic material, several strains of *Sphingomonas* bacteria were shown to have antigens for iNKT cells including *S. paucimobilis*, *Sphingomonas capsulate*, and *Sphingomonas yanoikuyae* [73,119]. From these organisms, GSLs were isolated that had very strong structural similarities to α GalCer. For example, from *S. paucimobilis*, GSL-1 was isolated α -glucuronosylceramide compound, and from *S. yanoikuyae* α -

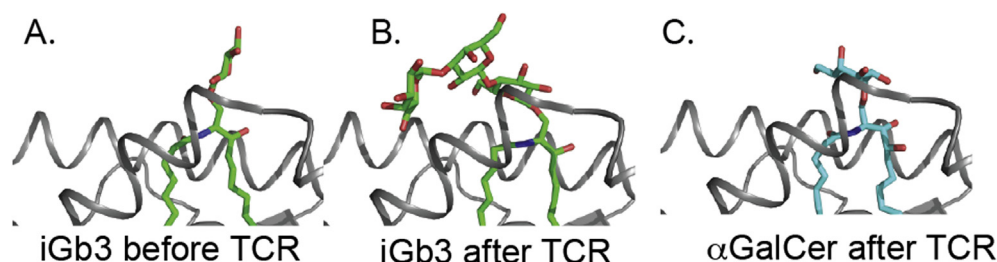
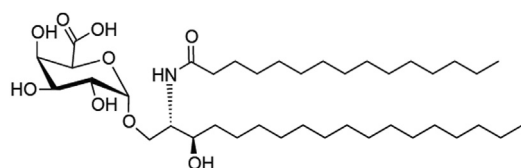
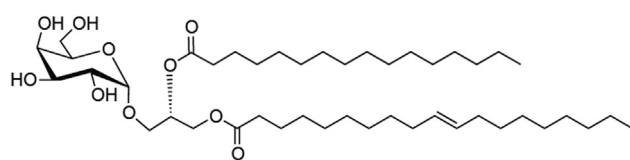


Fig. 5 – Induced fit by the invariant natural killer T-cell receptor with iGb3 showing the carbohydrate head group before and after T-cell receptor engagement. In A, only the sugar linked to the ceramide lipid could be resolved, presumably because the position of the two distal sugars was not fixed in the crystal. iGb3 before engagement (PDB code: 2Q7Y) and after engagement (PDB code: 3RZC) shown in comparison to α -galactosylceramide (PDB code: 3HE6).

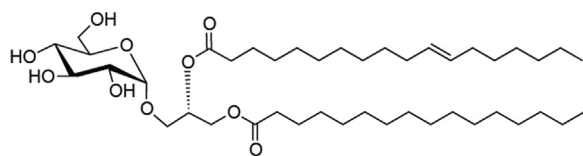
galacturonosylceramide originally called GSL-1'. These two lipids are highly similar but differ only in their carbohydrate head groups; having a glucuronic acid versus galacturonic acid (GalA). Since their discovery, GSL-1' has been renamed as GalA-GSL to indicate the presence of GalA [Fig. 6]. This family of *Sphingomonas*-derived compounds also contains some with additional saccharide groups including tri- and tetra-saccharides known as GSL-3 and GSL-4, respectively. While the monosaccharide-containing GSL antigens activate iNKT cells *in vivo* and *in vitro*, [73,119,120] they are all weaker than α GalCer. For example, the iNKT cell TCR affinity for GalA-GSL/CD1d is 50-fold weaker than for the α GalCer/CD1d complex [23]. The GSLs with oligosaccharide headgroups are not antigenic or do not activate as well as their monosaccharide counterparts, possibly due to a failure of the APCs to reduce the structures to a monosaccharide form in lysosomal compartments [46,121].



GalA-GSL (*Sphingomonas* spp.)



BbGL-2c (*B. burgdorferi*)



Glc-DAG (*S. pneumoniae*)

Fig. 6 – Invariant natural killer T-cell microbial lipids that have been crystallized in complex with mouse CD1d and the mouse invariant natural killer T-cell receptor.

Borrelia burgdorferi

Whereas *Sphingomonas* spp. is not considered pathogenic, another source of iNKT cell antigens is from the pathogenic spirochete *Borrelia burgdorferi*. These bacteria, transmitted to humans by *Ixodes scapularis* (deer tick) bites, are the cause of Lyme disease, which is currently the most common vector-borne disease in the United States [122]. The immune response of mice to *B. burgdorferi* infection is partially CD1d dependent as a strain of mice resistant to Lyme arthritis had symptoms when the gene encoding CD1d was deleted [123]. Furthermore, mice lacking CD1d had decreased pathogen-specific antibodies [124]. *B. burgdorferi*-induced pathogenesis was increased in *J α 18* deficient mice, which cannot form the invariant iNKT cell α -chain. In C57BL/6 mice, the absence of iNKT cells led to increased replication of spirochetes in the joint, delayed clearance, and arthritis [125,126] while *J α 18* deficient mice on the BALB/c background had increased carditis [127]. Analysis of lipids from *B. burgdorferi* detected a series of abundant galactosyl diacylglycerols (DAGs) including BbGL-2 [109]. BbGL-2 was shown to activate iNKT cells in purified and synthetic forms. BbGL-2 was the first reported iNKT cell antigen that is not a GSL; it contains a β -galactose saccharide group with an α -anomeric glycosidic bond and is a DAG lipid, with lipid chains that vary in length and degree of saturation. The most highly potent of these in mice was BbGL-2c [Fig. 6], with C18:1 oleic acid in the *sn*-1 position and C16:0 palmitic acid in the *sn*-2 position [109] [Fig. 6]. Structural characterization of multiple different DAG antigens showed how alteration of the lipid chains determines the binding mode. The *sn*-1 linked fatty acid is capable of binding to either A' or F' pockets of mouse CD1d, with C18:1 oleic acid preferring the A' pocket [23]. The different binding modes determine how the exposed saccharide group is oriented for recognition by the TCR. Structural analysis demonstrated that the orientation of the BbGL-2c allowed for an optimal configuration compared to another DAG lipid with two unsaturated fatty acid chains [23]. Interestingly, in humans, the optimal *sn*-1 and *sn*-2 fatty acids are different because of the role that the position 153 tryptophan in the human CD1d α 2 helix plays in orienting the exposed sugar.

Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram-positive bacterium that not only leads to pneumonia, but also causes potentially lethal

bacteremia, otitis media, and meningitis. iNKT cells were shown to be protective in mice following pulmonary *S. pneumonia* infection [128], and this protective response was linked to IFN- γ production by the iNKT cells [129]. The lipids of this bacterium were subjected to electrospray mass spectrometry analysis and two highly abundant DAGs were isolated: a monosaccharide linked DAG, α -glucosyldiacylglycerol (GlcDAG) [Fig. 6], and a disaccharide linked DAG, α -galactosyl GlcDAG (GalGlcDAG) [130]. Like the *B. burgdorferi* antigen, the DAG from *S. pneumoniae* has α -linked hexose sugar in the *sn*-3 position but is a glucose rather than galactose. This antigen has palmitic acid (C16:0) in the *sn*-1 position, and a vaccenic acid, a C18:1 fatty acid with a C11-C12 unsaturated bond, in the *sn*-2 position. The ternary crystal structure of GlcDAG bound to mouse CD1d with the iNKT cell TCR engaged shows that the vaccenic acid allows for a novel conformational change causing a re-orienting of the axial 4'-hydroxyl group of glucose to allow for the TCR to engage in a conserved binding motif [131].

Helicobacter pylori

Helicobacter pylori is a common, Gram-negative spiral bacterium that causes stomach problems in some individuals including gastritis, peptic ulcer, duodenal ulcer, lymphoma, and gastric cancer [132]. Certain *H. pylori* lipid extracts have been shown to expand iNKT cells and to play a beneficial role in clearing the bacterium [133,134]. This bacterium synthesizes cholesteryl α -glucosides using host-derived cholesterol. Although one study presented data indicating that cholesterol glucosylation allows *H. pylori* to evade phagocytosis by macrophages [135], these compounds also were shown to be recognized by iNKT cells, and the absence of iNKT cells in mice was correlated with increased *H. pylori* infection [134]. In a different study, mice were protected from asthma symptoms

by administering an *H. pylori*, cholesterol-containing, synthetic glycolipid called PI57 [Fig. 7] [133], although this compound activated iNKT cells in both mice and humans, as determined by immune assays, biochemical analysis of TCR interaction with the cholesteryl α -glucoside complex with CD1d, and structural analysis showing how a cholesterol-containing antigen binds to CD1d, have not been reported.

Fungal antigens for invariant natural killer T-cells

Recently, it was reported that *Aspergillus fumigatus* contains a glycolipid antigen that can activate iNKT cells. *Aspergillus* is a ubiquitous fungus, and although exposure through inhalation is almost a daily occurrence, the spores or conidia can lead to infections, most commonly in the lungs and sinuses, and furthermore, *Aspergillus* exposure can contribute to asthma [136]. Previously, it had been shown that iNKT cells were activated by β -1,3-glucans from *Aspergillus*, which triggered IL-12 secretion from APCs that mediated the activation of iNKT cells, but which also required recognition CD1d-presented self-antigens [137]. In the more recent study implicating a microbial lipid in iNKT cell activation, the lipids of the two most common strains of *Aspergillus*, *A. fumigatus*, and *Aspergillus niger* were fractionated and tested against primary iNKT cell lines, and the GSL asperamide B [Fig. 7] was identified as an antigen for mouse and human iNKT cells. Asperamide B is a β -linked glucosylceramide, which is different from any of the previously described α -linked exogenous ligands. It possesses a 9-methyl-4,8-sphingadienine chain, commonly found in fungi [65] and a β - γ unsaturated acyl chain with an α hydroxyl group [138]. Both purified and synthetic asperamide B were able to activate iNKT cells, and mice exposed to asperamide B experienced airway hyperreactivity, a feature of asthma, within 24 h [139].

Protozoan antigens for invariant natural killer T-cells

The glucosylphosphatidylinositol (GPI) anchors of surface proteins from *Plasmodium falciparum* and *Typanosoma brucei* could expand iNKT cells, and this expansion was CD1d dependent [140]. A subsequent study, however, contradicted the earlier finding that reported that the production of *Plasmodium* anti-circumsporozoite IgG was MHC Class II-independent, and by implication, iNKT cell dependent. These investigators also reported that GPI anchors could not induce iNKT cell activation [141]. More recently, a phosphoinositol antigen known as EhPI [Fig. 7] was isolated from a pool of lipopeptidophosphoglycans from *Entamoeba histolytica* trophozoites and was shown to activate iNKT cells [142]. *E. histolytica* can be fatal, especially in the developing world, as it leads to amoebiasis, a diarrheal disease [143]. There are two isoforms of EhPI, a and b, with the only difference between them being the acylation of inositol. Only the diacylated EhPIb [Fig. 7] stimulated iNKT cells. This lipid caused IFN- γ activation of iNKT cells that was dependent not only on CD1d but also on IL-12 production through Toll-like receptor signaling of APCs [142].

Summary

In conclusion, over the past two decades, knowledge about the specificity of iNKT cells has grown dramatically. Synthetic

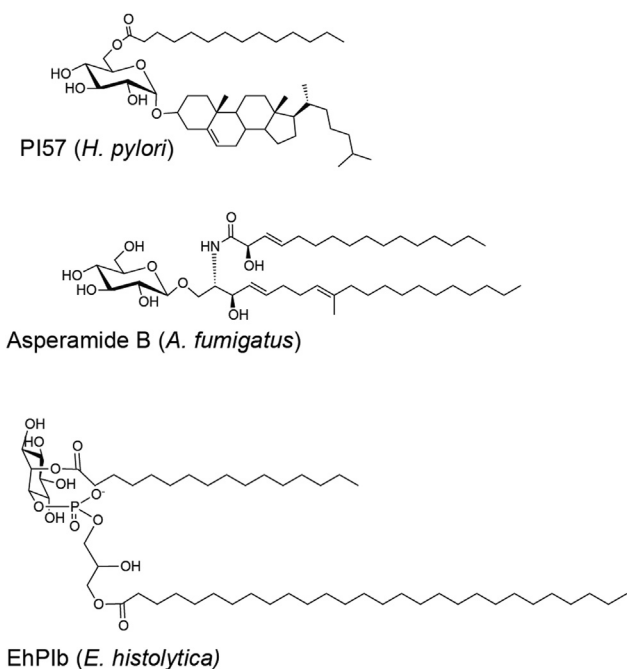


Fig. 7 – Invariant natural killer T-cell microbial lipids that currently have not been crystallized in complex with CD1d and the mouse invariant natural killer T-cell receptor.

agonists have been synthesized that bind with different degrees of stability to CD1d and that are able to preferentially skew the global immune response. Several natural antigens for iNKT cells have been identified, and they are both self-antigens that originate from antigen-presenting cells or foreign antigens from commensal or pathogenic microbes. It has been shown in several cases that the protective responses to pathogenic microbes depend on iNKT cell activation. The iNKT cell TCR can recognize these different structures, which are mostly glycolipids. A conserved docking motif of the iNKT cell TCR has been characterized through dozens of structural studies and is nearly always present, in some cases through a large accommodation of the antigen. Much work has been done to understand the mechanism of antigen recognition and the therapeutic potential of iNKT cells [36,144–146], and the hope is that this detailed biochemical knowledge can be harnessed to realize the therapeutic potential of these cells.

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