MINIREVIEW

The Alpha and Omega of Galactosylceramides in T Cell Immune Function[*]

Alysia M. Birkholz[‡§], Amy R. Howell[¶], and Mitchell Kronenberg[‡§1]

From the [‡]Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, California 92037, the [§]Division of Biological Sciences, University of California, San Diego, La Jolla, California 92037, and the [¶]Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269

Glycosphingolipids are a subgroup of glycolipids that contain an amino alcohol sphingoid base linked to sugars. They are found in the membranes of cells ranging from bacteria to vertebrates. This group of lipids is known to stimulate the immune system through activation of a type of white blood cell known as natural killer T cell (NKT cell). Here we summarize the extensive research that has been done to identify the structures of natural glycolipids that stimulate NKT cells and to determine how these antigens are recognized. We also review studies designed to understand how glycolipid variants, both natural and synthetic, can alter the responses of NKT cells, leading to dramatic changes in the global immune response.

T lymphocytes are important cells of the adaptive immune response, and natural killer T cells (NKT cells)[2] are a type of T lymphocyte. NKT cells were originally characterized as having cell surface markers expressed by innate immune cells such as NK1.1 in mice (1) and CD161, CD57, and CD56 in humans (2–4), as well as a T cell antigen receptor (TCR), a protein expressed by adaptive immune cells (5, 6). However, recent studies have indicated that only a portion of the NKT cells express NK1.1 in mice or CD161 and the other markers in humans, but all NKT cells are defined by a particular TCR specificity (7). Although most T lymphocytes recognize peptide fragments bound to or presented by MHC-encoded class I molecules, a subset of T cells, described below, recognizes glycosphingolipids (GSLs) and some other types of glycolipids. These GSLs are recognized when they are presented by CD1d, a cell surface protein homologous to MHC class I molecule (7, 8). The GSLs fall within the larger family of sphingolipids, or lipids that have ceramide as a core entity (9). Ceramides contain a sphingoid base with an amide linkage to a fatty acid chain (10) (Fig.

1A). Both of these chains can vary in saturation, length, branching, and hydroxylation. Natural sphingoid bases consist of sphingosines, sphinganines, or phytosphingosines (Fig. 1B); however, synthetic variants can deviate from these structures. The most common, natural fatty acid chain lengths are C:16 and C:18, but the lengths of natural and synthetic GSL fatty chains range from very few carbons to greater than C:30. The sphingoid base of GSLs has a 1″-1 glycosidic linkage to the carbohydrate head group, which is most commonly galactose or glucose, with gangliosides having more complex oligosaccharide structures (11). Because the 1″ carbon is asymmetric, it might be oriented in either an α or a β linkage (Fig. 1, C and D); however, the β orientation is the dominant linkage in mammals (12).

There are several types of lipid-reactive NKT cells in mammals that can respond within hours of GSL stimulation by producing cytokines (7). The kinetic properties of this response are similar to innate immune cells. Type I, or invariant NKT (iNKT) cells, are one type of NKT cell. The moniker "invariant" for iNKT cells arises because these cells have an essentially identical TCR α gene rearrangement, using the Vα14 segment in mice and the homologous Vα24 (TRAV10) in humans (8). The invariant α chain is co-expressed with a restricted diversity of β chains, Vβ11 (TRBV25-1) in humans and Vβ8.2, Vβ7, and Vβ2 in mice (8). Type II NKT cells have a wider diversity of α and β chains. Because of their diverse TCRs and the relative lack of reagents to detect them, Type II NKT cells have been less studied (13). Here we will discuss the knowledge (alpha to omega) of studies on GSL recognition by both Type I and Type II NKT cells.

Presentation of GSLs by CD1d

The first GSL antigen for iNKT cells with a defined structure was α-galactosylceramide (αGalCer), which has a galactose in 1″-1 α linkage to a phytosphingosine base (Fig. 1C). To date, it remains the most studied antigen for iNKT cells, and it is among the most potent that have been identified. αGalCer was identified from structure activity relationship studies around Agelasphin 9b (Fig. 1E) by Kirin Pharmaceuticals in a screen for naturally occurring molecules that prevented tumor metastases in mice (14). This synthetic version, also known as KRN7000, retains the activity of Agelasphin 9b while being much easier to synthesize (15). The strategic simplifications included removing the fatty acid C2 hydroxyl group and the terminal branching of the sphingoid base. By also elongating the sphingoid base chain to C:18 and the fatty acid chain to C:26, the stimulatory activity of Agelasphin 9b was maintained for αGalCer.

GSLs such as αGalCer bind to the CD1d antigen-presenting molecule in a defined orientation (16). CD1d has two hydrophobic pockets termed A′ and F′. The sphingoid base chain localizes to the F′ pocket in the CD1d hydrophobic groove, and the fatty acid localizes to the A′ pocket (17) (Fig. 2A). This allows for optimal hydrogen bonding as well as optimal orientation of the saccharide head group for recognition by the iNKT cell TCR. The sugar linked to the sphingoid base plays a prom-

[1] To whom correspondence should be addressed: Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037. E-mail: mitch@lji.org.

[2] The abbreviations used are: NKT, natural killer T cell; iNKT, invariant natural killer T cell; αGalCer, α-galactosylceramide; βGalCer, β-galactosylceramide; iGB3, isoglobotrihexosylceramide; TCR, T cell receptor; Th1, T helper type 1; Th2, T helper type 2; GSL, glycosphingolipid; APC, antigen-presenting cell.
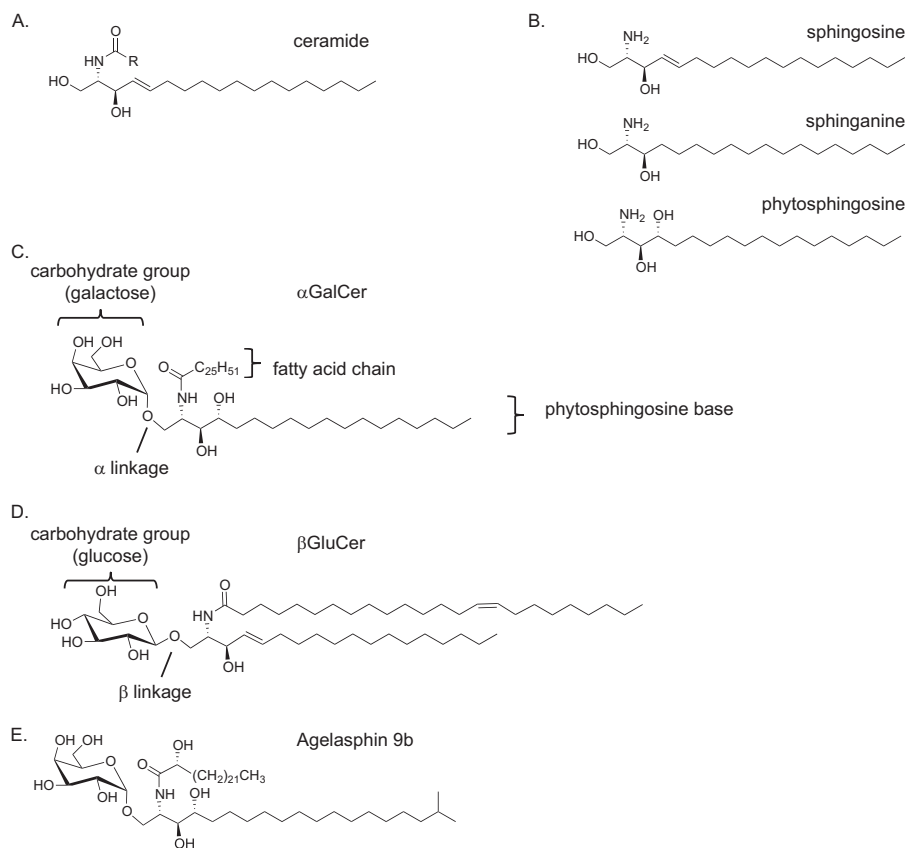
FIGURE 1. **Components and structures of some GSL antigens.** *A*, ceramide. *B*, common types of sphingoid bases that differ at the C4 position *C*, αGalCer. *D*, β-D-glucopyranosyl ceramide (β*GluCer*). *E*, Agelasphin 9b.

inent role in the molecular determinant recognized by the iNKT cell TCR, with a galactose, in most cases, being the most potent moiety (Fig. 1*C*) (18). Although α-linked sugars (Fig. 1*C*) provide a much more potent activation of iNKT cells, the glycosidic bond to lipids is likely a β-linkage in mammals (Fig. 1*D*) (19, 20). Recent evidence suggests, however, that there are some GSLs with α-linked sugars in mammals (21), although their complete structure has not been determined and these GSLs are not abundant (22).

GSL Activation of iNKT Cells Alters the Immune Response

Relatively minor changes in the structure of the activating GSL antigen can cause very different types of immune responses (17). For example, certain GSLs can lead to a Th1 immune response (23). The Th1 response is characterized by the secretion of cytokines such as IFN-γ. IFN-γ is crucial for defense against intracellular pathogens, and it is important in the response against cancers. Conversely, other GSLs can cause the immune system to skew more toward a Th2 response (24), which is responsible for extracellular pathogen destruction and is characterized by secretion of the cytokine IL-4 and other cytokines. An excessive Th1 response can lead to autoimmunity or chronic inflammation, and an excessive Th2 response can lead to allergies and asthma (25). Therefore, both of these immune responses must be tightly regulated for immune homeostasis, and interestingly, Th1 and Th2 responses inhibit one another. The diverse outcomes following immunization with particular GSLs make them potential therapeutic agents

for regulating immune responses and preventing immune-mediated disorders.

Although it is not known how subtle variations in GSL structure affect the immune response, several alternative hypotheses have been proposed. Some data suggest that Th1 responses depend on prolonged antigenic stimulation of iNKT cells, and this may be due to several factors, including enhanced GSL chemical stability *in vivo*, more stable binding of the GSL to CD1d, or increased TCR affinity for the GSL complex with CD1d (17). Alternatively, GSLs may have differential effects on antigen-presenting cells (APCs), for example, by trafficking to different components of the cell and inducing the expression of different cell surface molecules that influence immunity (27).

Among the factors that might contribute to prolonged antigenic stimulation, increased TCR affinity for the GSL-CD1d complex is not a good predictor of the type of immune response that will result (28, 29). Multiple studies have demonstrated that it is difficult to obtain a GSL with a higher affinity than αGalCer for the iNKT cell TCR by altering the sugar head group or by modifying the ceramide lipid in either the sphingoid base or the carboxylic acid. Crystallization studies have identified a similar docking motif of the iNKT TCR on the GSL-CD1d complex regardless of the modifications that have been analyzed (Fig. 2*A*) (30, 31). The iNKT cell TCR, in each case, is oriented over the F′ pocket of CD1d in a parallel orientation with the CD1d α helices, and the TCR uses germline-encoded residues in the CDR1α, CDR3α, and CDR2β loops to recognize
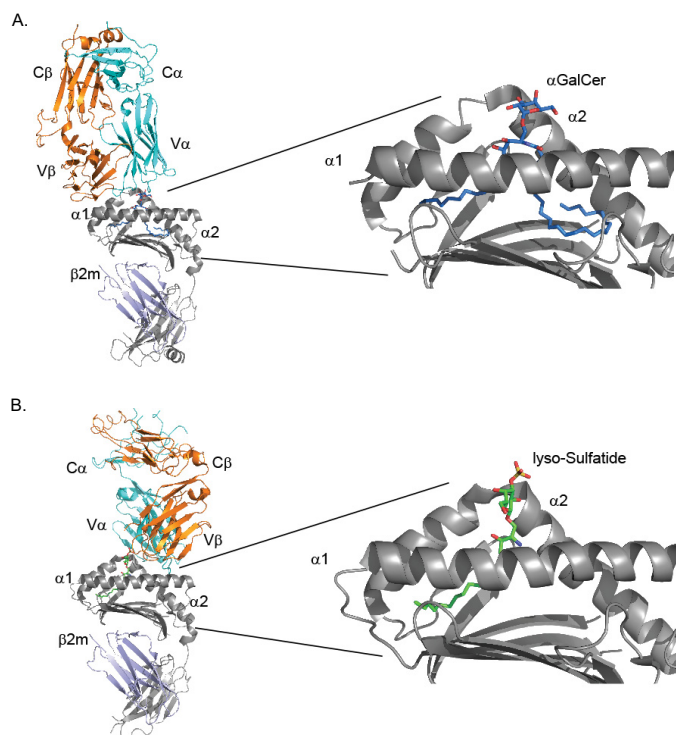
FIGURE 2. **Structures of GSL-mouse CD1d complexes and trimolecular structures with the TCR.** *A*, *left*, structure of the iNKT cell TCR binding to αGalCer-CD1d. The TCR α chain is in *cyan*, and the β chain is *orange*. The CD1d heavy chain is in *gray*, and the associated β2-microglobulin light chain is in *violet*. *Right*, expanded view of αGalCer (*blue* and *red*) in the CD1d binding groove (*gray*). The α1 and α2 helices of CD1d are labeled; the F′ pocket binding the phytosphingosine is on the *left*, and the acyl chain-binding A′ pocket is on the *right*. Taken from PDB code 3HE6. *B*, *left*, structure of a Type II NKT cell TCR binding to lyso-sulfatide-CD1d. Color scheme is the same as in *panel A* with lysosulfatide hydrocarbons in *green*. *Right*, expanded view of lysosulfatide (*green*, *red*, and *yellow*) in the CD1d binding groove (*gray*). Taken from PDB code 4ELM.

the CD1d-presented lipid. A version of αGalCer derivatized at the 6″ carbon, α-GalCer-6″-(pyridin-4-yl) carbamate, promoted a very strong Th1 response as compared with αGalCer, and crystallographic analysis showed that this GSL bound to CD1d made extra contacts with the TCR. Despite this, SPR measurement of TCR affinity for this GSL-CD1d complex did not demonstrate more avid binding as compared with αGalCer-CD1d complexes (32).

As compared with TCR affinity for GSL-CD1d, there is a stronger correlation of a Th1 immune response with the strength of the interaction of the lipid with CD1d. GSL antigens that have more contacts with CD1d, and/or that are packed more tightly within the A′ and F′ pockets, are likely to be presented for a longer time by APCs. For example, the alteration of the sphingoid base chain to contain a bulkier cyclopropyl group (compound SMC124, Fig. 3*A*) (33) or phenyl group (7DW8-5) (34) led to a stronger Th1 response in mouse or human models, respectively. CD1d presentation of SMC124 by APC *in vivo* was more stable over 22 h as compared with αGalCer, perhaps due to an enhanced GSL-CD1d interaction. Crystal structure analysis of the SMC124-CD1d complex indicated that this lipid may bind in a more compact orientation within the F′ groove of CD1d. Another example of a GSL promoting a Th1 cytokine response that might be related to enhanced interaction with
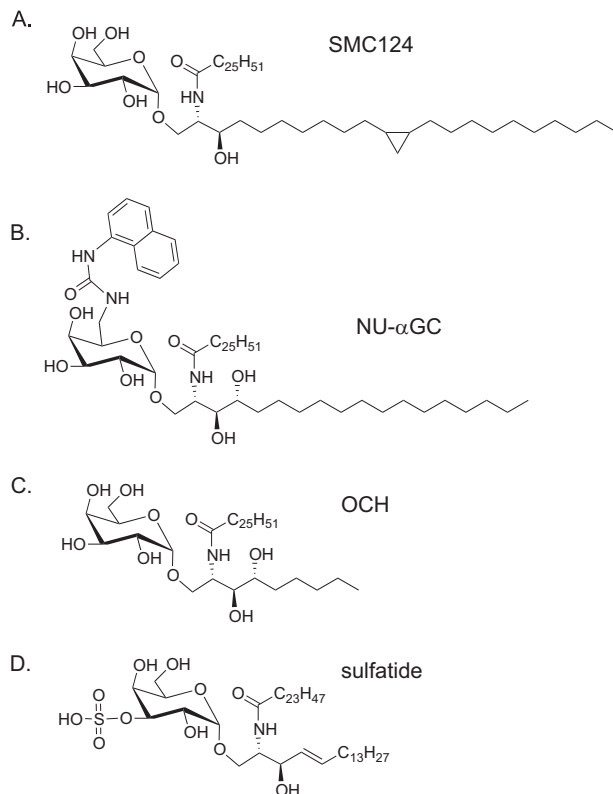

FIGURE 3. **Structures of iNKT activating GSLs.** *A*, SMC124. *B*, naphthyl-urea-αGalCer (*NU-αGC*). *C*, OCH. *D*, sulfatide.

CD1d is provided by the lipid naphthyl-urea-αGalCer, which has a naphthyl urea group linked to the 6″ position of the saccharide (Fig. 3*B*). Crystal structure analysis demonstrated that the naphthyl urea group folds over and makes additional contacts with the surface of the CD1d molecule when the TCR is engaged (35). Conversely, the GSL OCH (Fig. 3*C*), which causes primarily a Th2 response, has a shortened sphingoid base chain and thus lacks some GSL-CD1d contacts that could stabilize the complex (24). Although these results are persuasive with regard to the important role of the GSL-CD1d interactions, the ability to promote a Th1 response depends on the interactions of iNKT cells with multiple cell types, and the causes of a Th1 cytokine response are likely to be multifactorial.

Microbial GSL Antigens for iNKT Cells

Several microbial GSLs have been shown to activate iNKT cells. The microbiome has been a highly researched area in recent years, and we are only beginning to understand the role that commensal bacteria play in the immune system. Research has shown that the development of iNKT cells is influenced by the microbiome directly or indirectly through other cell types (36, 37). Germ-free mice had a slightly reduced iNKT cell population in the liver, spleen, and thymus as compared with normal mice, and iNKT cells from germ-free mice were hypo-responsive, whereas mice colonized with a restricted flora had an even more significant decrease in iNKT cells (36). These data suggest that the large differences in the frequency of iNKT cells in the peripheral blood of humans could be related to microflora. In contrast, germ-free mice had increased numbers of iNKT cells in the colon, lamina propria, and lungs (37, 38), and

these cells are hyper-responsive, which led to exacerbated inflammation in models of inflammatory bowel disease and allergic asthma. Early life exposure to microbes could reverse the increased number and hyper-reactivity of iNKT cells and the susceptibility to inflammatory disease. These data lend support to theories that relate early childhood exposure to microbes to a decrease in immune-mediated diseases, the so-called hygiene hypothesis (39).

The commensal bacteria *Sphingomonas* spp. and *Bacteroides fragilis* are two microbial species that have GSL antigens that activate iNKT cells. *Sphingomonas* spp., which are α-proteobacteria, were discovered to have two GSL antigens for iNKT cells, GSL-1 and GSL-1′, which have either a glucuronic or a galacturonic saccharide, respectively, linked to a ceramide backbone having a sphinganine base (40, 41). Different *Sphingomonas* species produce variable GSLs, in some cases with oligosaccharide moieties containing three or four sugars, but GSLs with more complex sugars do not strongly activate iNKT cells (42, 43).

B. fragilis have an assortment of membrane phospholipids including sphingolipids. When the repertoire of sphingolipids was assessed, an isoform of αGalCer with methyl branches in the lipid chains was identified. This compound can activate both mouse and human iNKT cells (44), although in another study, it was reported that this GSL can serve as an antagonist (45).

Mammalian GSL Antigens for iNKT Cells

Mammalian GSLs represent potential self-antigens. Like other T lymphocytes, the TCR of iNKT cells must interact with ligands in the thymus to survive (46). Unlike other T cells, iNKT cells also are self-reactive as mature cells, but this self-reactivity is controlled, in part, through the expression of inhibitory receptors (47). The nature of the thymic self-ligands and stimulating self-antigens for mature iNKT cells is controversial, but some data suggest that they include both GSLs and other types of lipids (19, 20, 48). Nonetheless, certain mammalian or self-GSLs stimulate iNKT cells. Although initially it was thought that only GSLs with α-anomeric lipids could be antigens for iNKT cells, β-linked GSLs were also shown to activate them (49, 50), although they are weaker antigens than their α-anomeric counterparts. The crystal structure of β-galactosylceramide (βGalCer) bound to mouse CD1d in complex with the iNKT cell TCR revealed that the TCR was able to squash or push the orientation of the β-linked galactose to a similar orientation as the galactose in the αGalCer CD1d-GSL-iNKT cell TCR trimolecular complex (51). The closely related β-D-glucopyranosylceramide, a sphingosine containing GSL with a C24:1 fatty acid (Fig. 1D), may activate both human and mouse iNKT cells (52), although recent studies indicate that this activation is due to a possible natural α-anomeric GSL (21). The GSL isoglobotrihexosylceramide (iGb3), a trisaccharide containing GSL with glucose in β-1″-1 linkage to the sphingosine base, also activated iNKT cells. This antigen was discovered after noting that mice lacking β-hexosaminidase b, which removes the terminal β-linked GalNAc residue of isoglobotetrahexosylceramide (iGb4) to make iGb3, had a reduced number of iNKT cells (53). Although iGb3 can participate along

with other self-antigens, the analysis of mice deficient for iGb3 synthase indicates that it is not essential for iNKT cells (54).

Type II NKT Cells and the Sulfatide GSLs

Type II NKT cells, as mentioned earlier, do not express an invariant TCR α chain, and consequently, they have diverse specificities. However, a number of Type II NKT cells recognize sulfatide (Fig. 3D), a GSL composed of βGalCer with the galactose sulfated at the 3″ position. In a mouse model of multiple sclerosis, sulfatide-reactive Type II NKT cells were specifically recruited to the central nervous system (55). Natural isoforms of sulfatide differ with regard to the fatty acid and sphingoid base, and it was a lyso-sulfatide that showed the greatest antigenic potency when tested with a particular Type II NKT cell hybridoma (56). Interestingly, the Type II NKT cell TCR, which has been crystalized in complex with sulfatide antigen bound to CD1d, has a completely different binding mode as compared with the iNKT cell TCR. The sulfatide-reactive Type II NKT cell TCR is oriented over the A′ pocket of the CD1d molecule, with predominant binding interactions with the TCR β chain (26) (Fig. 2B).

Conclusions

The relationship of GSLs and the T cell branch of the immune system has been studied extensively through studies of Type I and Type II NKT cells. Many synthetic, microbial, and mammalian GSLs have been tested, and some have been shown to activate one or the other type of NKT cells and influence the overall immune response. The exact mechanism whereby activated iNKT cells can skew the immune response in either the Th1 or the Th2 direction is not completely understood, but efforts are underway to develop compounds that give a strong and predictable cytokine response in humans so that GSLs can be used in clinical settings.

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