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Immune and Structural Studies of Synthetic Invariant Natural Killer T cell Glycosphingolipid Activators

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Alysia Marie Birkholz

Committee in charge:

Professor Mitchell Kronenberg, Chair
Professor Ananda Goldrath, Co-Chair
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Professor Stephen Hedrick
Professor Gentry Patrick
Professor Dirk Zajonc

2015

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University of California, San Diego

2015

DEDICATION

This document is dedicated to all who made me who I am, who made sure that I was okay and who stood by me no matter how crazy I got. Thank you for being part of my significant population.

EPIGRAPH

The Grand Illusion
By: STYX

"Welcome to the Grand illusion
Come on in and see what's happening
Pay the price, get your tickets for the show
The stage is set, the band starts playing
Suddenly your heart is pounding
Wishing secretly you were a star.

But don't be fooled by the radio
The TV or the magazines
They show you photographs of how your life should be
But they're just someone else's fantasy
So if you think your life is complete confusion
Because you never win the game
Just remember that it's a Grand illusion
And deep inside we're all the same.
We're all the same...

So if you think your life is complete confusion
Because your neighbors got it made
Just remember that it's a Grand illusion
And deep inside we're all the same.
We're all the same...

America spells competition, join us in our blind ambition
Get yourself a brand new motor car
Someday soon we'll stop to ponder what on Earth's this spell we're under
We made the grade and still we wonder who the hell we are"

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LIST OF ABBREVIATIONS AND SYMBOLS

α: alpha
Å : angstrom
αGalCer or αGC: α-galactosylceramide
αGluCer: α-glucosyl ceramide
αManCer: α-mannosyl ceramide
Ag: antigen
APC: antigen presenting cell
Asp: aspartic acid
β: beta
βGalCer: β-galactosylceramide
βGluCer: β-glucosylceramide
CD1d-TD A20: transfected B cell line expressing CD1d lacking a tyrosine motif in its cytoplasmic tail
CD1d-WT A20: transfected B cell line expressing CD1d
Cys: cysteine
DC: dendritic cell
EAE: experimental autoimmune encephalomyelitis
ER: endoplasmic reticulum
γ: gamma
Gal: galactose
Glu: glucose
GSL: glycosphingolipid
LPC: lysophosphatidylcholine
LPE: lysophosphatidylethanolamine
Man: mannose
MCII: mouse collagen II
MHC: major histocompatibility complex
NK: natural killer
NKT: natural killer T-cell
NU-αGC: napthyl-urea αGC
IFN-γ: Interferon gamma
IL-4: interleukin 4
IL-5: interleukin 5
IL-12: interleukin 12
IL-13: interleukin 13
*i*NKT: invariant natural killer T-cell
iGB3: isoglobotrihexosylceramide
PA: phosphatidic acid
PC: phosphatidylcholine
Phe: phenylalanine
PI: phosphatidylinositol
PIM_4: phosphatidylinositol tetramannoside
pLPA: plasmalogen lysophosphatidylethanolamine
Pro: proline
PS: phosphatidylserine
SPR: surface plasmon resonance
TCR: T cell receptor
TD: tail deleted
Th1: T helper type 1
Th2: T helper type 2
Thr: threonine
TNFα: tumor necrosis factor α
WT: wild type

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PREFACE

In this dissertation I have included an introduction chapter (Chapter 1) that is in preparation for publication as a review article. This introduction covers the general background of invariant NKT cells, the antigen-presenting molecule CD1d and the glycosphingolipids that bring these two proteins together. Chapter 2 is work in process for submission for publication and is a look at a biological, biochemical and crystallographic details from a variety of different glycosphingolipids as they engage with invariant NKT cells and CD1d. Chapter 3 was published in the *Journal of Chemistry & Biology* and probes in more detail at one of the glycosphingolipids mentioned in Chapter 2 and it's sister ligand. This chapter addresses why specific interactions may lead to the Th1 skewed immune response. Chapter 4 has been submitted for publication and is work on a particular glycosphingolipid that has a small alteration allowing for significant immune responses. The contribution of this work and suggestions for future inquiry are highlighted in Chapter 5.

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ABSTRACT OF THE DISSERTATION

Immune and Structural Studies of Synthetic Invariant Natural Killer T cell Glycosphingolipid Activators

by

Alysia Marie Birkholz

Doctor of Philosophy in Biology

University of California, San Diego, 2015

Professor Mitchell Kronenberg, Chair
Professor Ananda Goldrath, Co-Chair

Invariant Natural Killer T (*i*NKT) cells comprise a small fraction of immune cells capable of responding within hours when stimulated by glycosphingolipid (GSL) antigens (Ags) and can impact the immune system months later. These *i*NKT cells respond to GSLs that are presented by the antigen presenting molecule CD1d. The *i*NKT cell driven immune cytokine response can alter depending on the GSL that is presented by CD1d. The GSL can lead to the production of a T helper type 1 (Th1) or a T helper type 2 (Th2) response characterized by IFN-γ and IL-4 cytokines. Many synthetic GSL

analogs of the most common *i*NKT cell antigen, α-Galactosylceramide (αGalCer) were used in this study to determine immunological and biochemical properties of Ags capable of activating *i*NKT cells. Studies consisted of *in vitro* Ag binding models, crystallographic structural models and *in vivo* immunological studies. Ags that are categorized as Th1 *i*NKT cell cytokine skewers have properties correlated with this response. The Th1 Ags studied are presented by CD1d on dendritic antigen presenting cells (APCs) and the CD1d and GSL seem to form more contacts according to structural studies and the CD1d-GSL complexes are more biologically stable *in vivo*. The work from these studies adds the to growing information in the field regarding the nature of *i*NKT cell immune system skewing.

Chapter 1:

Introduction to invariant NKT cells, CD1d and glycosphingolipids

1.1 GENERAL BACKGROUND

Natural Killer T cells (NKT) are a unique subset of lymphocytes in the immune system. They were initially characterized in the late 1980's (Yankelevich et al., 1989) and were identified to be reactive to the antigen presenting molecule CD1d in the mid 1990's (Bendelac et al., 1995). Since then, many studies have tried to uncover the enigmas of NKT cells.

Initially described in mice, NKT cells are unique in that they have characteristics of both innate and adaptive immune cells. In mice, they were originally identified by expression of NK1.1, a marker for Natural Killer (NK) cells, which are a type of innate lymphocyte. On NKT cells, this marker is expressed together with an αβ T cell receptor (TCR) that is a characteristic of adaptive immune cells. The majority of these $NK1.1^+$ $TCR\beta^+$ lymphocytes express an invariant TCR α chain that imparts a particular glycolipid specificity, which is described further below. Cells with this phenotype were later confirmed to also be present in humans (Spada et al., 1998). Like the mouse, the human cell population is marked by an NK cell receptor, in this case CD161, and it also expresses an αβ TCR with an invariant α chain that shares significant homology with its mouse counterpart (Exley et al., 2010). 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, (Lee et al., 2002) however using the CD161 definition, the frequency can be much higher. Because of their αβ 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, (Tangri et al., 1998; Liu et al., 2011) indicate 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 locus. This dissertation analyzes these NKT cells, some of their lipid antigens, and the CD1d molecule.

1.2 TYPE I VERSUS TYPE II NKT 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 (*i*NKT) cells or Type I NKT cells, which express a nearly fixed or invariant TCR α chain encoded by a Vα14-Jα18 rearrangement. This α chain is co-expressed most frequently with a Vβ8.2, 7 or 2 TCR β chain in mice. In humans, the Type I NKT cell TCR is formed by a fixed Vα24Jα18 (TRAV1-2-TRAJ18) rearrangement paired with a Vβ11 beta chain. The mouse and human invariant α chains are true homologs, as are human Vβ11 and mouse Vβ8.2. As a consequence, the specificity of mouse and human NKT cells is highly conserved. This *i*NKT cell TCR has a conserved parallel binding motif radically different than the binding orientation of TCR of classical T cells.

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. Here we will focus on Type I NKT cells or *i*NKT cells.

1.3 BIOLOGICAL RESPONSES OF *i*NKT CELLS

*i*NKT cells are very rapid responders when stimulated through their TCR, providing a cytokine burst within 90 minutes of *in vivo* stimulation (Matsuda et al., 2000). Through TCR recognition of a lipid antigen presented by CD1d (Figure 1.1), *i*NKT cells can induce a wide range of cytokines, including T helper type 1 (Th1), T helper type 2 (Th2) and other responses. Not only do activated *i*NKT cells secrete these cytokines, but also they induce other cells to secrete them. The results from a number of studies demonstrate that the totality of the *i*NKT 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 type I cytokines, like IFN-γ and TNFα. Other lipids lead to a more Th2 skewed pattern of cytokines that includes IL-4, IL-5, and IL-13.

1.3.1 αGalCer

The most studied glycolipid that activates *i*NKT cells, also the first discovered, is alpha-galactosylceramide (αGalCer) (Figure 1.1 and Figure 1.2). This is sometimes considered a Th0 skewing lipid, as *i*NKT cells that respond to this lipid robustly produce both IFN-γ and IL-4. αGalCer was originally identified by 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 (Natori et al., 1994). The structure was then synthesized and optimized in the tumor metastases assay (Akimoto et al., 1993; Natori et al., 1994). αGalCer has an alpha-linked galactose, a phytosphingoid base chain with 18 carbons and an acyl chain containing 26 carbons. This GSL 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 (Ishikawa et al., 2005; Uchida et al., 2008). 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 αGalCer/CD1d complexes exhibited by the human TCR compared to mouse (Gadola, 2006). For these reasons, there have been extensive efforts to develop other *i*NKT cell-activating lipids that can skew the human cytokine response, especially in a Th1 direction. Additionally, the type of antigen presenting cell (APC) targeted may be critical and, in clinical trails, transfer of dendritic cells (DCs) incubated with αGalCer generated a more robust *i*NKT cell response than αGalCer alone (Chang et al., 2005; Ishikawa et al., 2005; Nieda et al., 2004). Continuing efforts to develop more effective glycolipids, delivery systems and cell-based therapies using αGalCer remain underway, including clinical trials that include patients enrolled at the UCSD medical center.

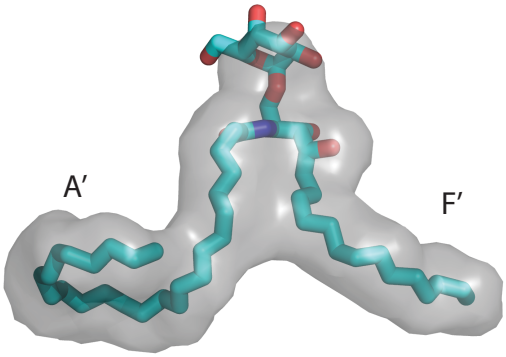


Figure 1.1. Hydrophobic pockets of CD1d. A' and F' labeled showing binding orientation of αGalCer**.**

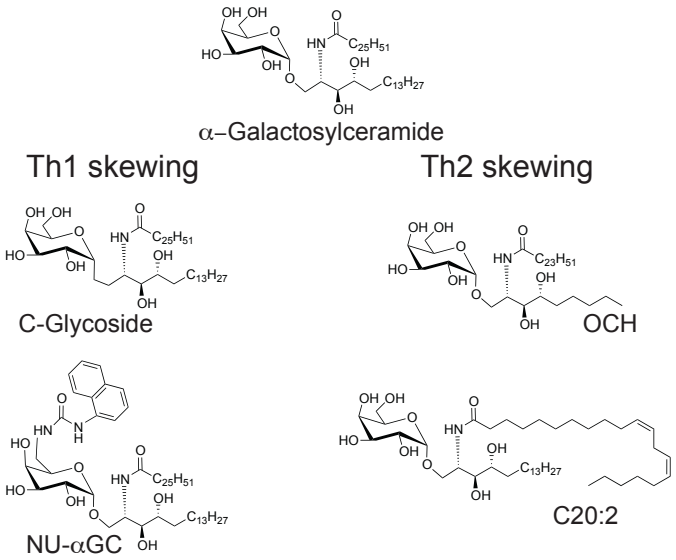


Figure 1.2. Chemical formation of αGalCer and select Th1 and Th2 skewing GSLs. Chemical formations of αGalCer, Th1 GSLs C-Glycoside and NU-αGC, Th2 GSLs OCH and C20:2.

1.3.2 CD1d

The CD1d antigen-presenting molecule is a member of the family of CD1 proteins. This family is divided into two main groups; group 1 CD1 proteins (CD1a, CD1b, and CD1c) and group 2 CD1 (CD1d) (Cohen et al., 2009). There is also a third, intermediate group (CD1e). Whereas CD1a, CD1b, CD1c and CD1d are found on the cell surface, CD1e is an intracellular protein that facilitates glycolipid processing and presentation (Barral and Brenner, 2007). 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 basic heterodimer structure similar to MHC class I antigen presenting molecules, with a heavy chain having three extracellular domains and a conserved β2-microglobulin subunit (Zajonc and Kronenberg, 2007). 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' (Figure 1.1). 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 (Barral and Brenner, 2007). The binding of the lipid chains within CD1d exposes the saccharide head group that is recognized and forced into a fixed orientation by the *i*NKT cell TCR (Wang et al., 2010).

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 (Barral and Brenner, 2007). 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 positive selection of *i*NKT cells in the thymus, occurs within endosomal compartments, (Cernadas et al., 2003; Zhou et al., 2004b) and for some exogenous antigens, also on the cell surface (Im et al., 2009). CD1d is expressed on a wide variety of hematopoietic series cells including B cells, DCs, macrophages, Langerhans cells, monocytes, T cells, and *i*NKT cells themselves (Dougan et al., 2007). Some non-hematopoietic cells including hepatocytes and intestinal epithelial cells also express it (Canchis et al., 1993; Blumberg et al., 1991). Data indicate that DCs are the key activators of *i*NKT 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 (Courtney et al., 2012). Recently, the $CD8\alpha^{+}$ $DEC\text{-}205^{+}$ DC subset was identified as the antigen presenting cell type responsible for inducing both Th1 and Th2 responses (Arora et al., 2014). According to these findings, up-regulation 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 profile (Arora et al., 2014).

1.4 LIPID ANTIGENS, WORKING DOWN THE GLYCOSPHINGOLIPID

1.4.1 Head group

The hydrophobic chains of *i*NKT cell lipid antigens are usually bound deep within the pockets of CD1d and a polar head group is exposed for recognition by the *i*NKT cell

TCR. The CD1d molecule stabilizes this head group through interactions with the α1 and α2 helices (Zajonc and Kronenberg, 2007). In humans, position 153 of the α2 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 (Wang et al., 2010; Koch et al., 2005). This is a main distinction between the GSL structures presented by mouse and human CD1d.

Contacts between Asp153 and Thr156 of mouse CD1d and the 2' and 3' hydroxyls of the αGalCer saccharide head group, as well as the αGalCer O-glycosidic oxygen, are important for ordering the sugar head group (Kamada et al., 2001). Because the *i*NKT 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 the 2', diminishes *i*NKT cell responses and TCR binding (Trappeniers et al., 2008; Girardi and Zajonc, 2012). For example, while αGalCer and α-glucosyl ceramide (αGluCer) could both activate *i*NKT cells, which indicated that the axial versus equatorial orientation of the 4' hydroxyl group is not critical, αGalCer was the more potent antigen. In contrast, α-mannosyl ceramide (αManCer) did not activate *i*NKT cells, indicating that the equatorial 2' hydroxyl is critical (Kawano et al., 1997). Additional studies demonstrated the importance of the 2' hydroxyl group in TCR recognition, as synthetic analogs of αGalCer with modifications of this position lacked antigenic activity (Wu et al., 2005). The 3' position is more permissive as modifications at this position decreased, but did not completely diminish activity (Franchini et al., 2007; Wu et al., 2005). Modifications of the 6' position are tolerated the best. This makes sense, as crystal structures of the CD1d-lipid-TCR complex show that the 6' position does not

make contacts with the TCR. (Gadola, 2006; Kjer-Nielsen, 2006). Tolerance for modifications at the 6' position were revealed in a study that compared responses to Gal(α1-2)GalCer with Gal(α1-6)GalCer, which are both GSLs with a disaccharide as opposed to monosaccharide head group. While Gal(α1-2)GalCer (GGC) required carbohydrate processing to remove the terminal galactose in order for antigenic activity, Gal(α1-6)GalCer did not (Prigozy et al., 2001). Indeed, modification of the 6' hydroxyl proved to be very beneficial when PBS57, a 6'-deoxy-6'-acetamide αGalCer analog was tested (Liu et al., 2006). PBS57 was not only more easily solubilized than αGalCer, it also induced a more potent *in vivo* cytokine response (Long et al., 2007). The 6' position is permissive for the addition of other bulky chemical groups, as exemplified by the napthylurea αGalCer molecule (NU-αGC) (Pauwels et al., 2011). NU-αGC causes a robust Th1 cytokine response and reduces tumor metastases in mice even more effectively than αGalCer. It is very likely that NU-αGC binds in a more stable fashion to CD1d than αGalCer, as the napthylurea group serves as a "third anchor" of the lipid for binding to the CD1d molecule. It binds on top of the CD1d protein instead of deep in the groove, but does not impede the TCR interaction (Aspeslagh et al., 2011). Other 6' modifications, such as 4ClPhC-αGalCer and PyrC-αGalCer are also antigens that induce strong Th1 cytokine responses, correlated with a marked reduction of B16 melanoma metastasis to the liver (Aspeslagh et al., 2013). PyrC-αGalCer, the most potent of these lipids, also shows novel and increased contacts with the both CD1d and the *i*NKT cell TCR. The 6' modifications have also served as tools for labeling GSLs with biotin or fluorophores, which has permitted analyses of their progress through endosomal compartments in cells (Zhou et al., 2002).

Other synthetic alterations have been used to create α-carba-GalCer analogs (Yu et al., 2006). In 2009, carba- sugar and cyclitol analogs of αGalCer were generated and three analogs, RCAI-56 (a carba-α-D-galactose analog), RCAI-59 (a 1-deoxy-neo-inositol analog), and RCAI-92 (1-O-methylated analog) also were found to be Th1 skewing lipids (Tashiro et al., 2009). The α-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 (Tashiro et al., 2010). 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 αGalCer 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 (Tashiro et al., 2010). 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 (Yoshiga et al., 2011).

HS44 is another GSL with αGalCer head group modifications 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 has the O-glycosidic linkage replaced with an amide group (Harrak et al., 2009). Structural analysis and binding assays by surface plasmon resonance (SPR) showed that when bound to CD1d, this compound had a 14-fold weaker interaction with the NKT cell TCR compared to αGalCer. Despite this, it caused a strong Th1 cytokine response and was effective at preventing tumor metastases (Kerzerho et al., 2012).

1.4.2 Linkages

As previously mentioned (Harrak et al., 2009), the O-glycosidic linkage of αGalCer can be altered, and in doing so, can lead to some unique properties. In 2003, the oxygen bond was altered to a CH_2, based on the theory that this GSL would be more resistant to α-galactosidase-mediated catabolism (Franck and Tsuji, 2006; Schmieg et al., 2003). This compound, referred to as C-glycoside, was the first glycolipid shown to induce a strong Th1 cytokine response in mice, (Fujii et al., 2006) but it did not stimulate human *i*NKT cells. The ternary structure of C-glycoside-CD1d complexes bound to the *i*NKT cell TCR demonstrated the importance of hydrogen binding of the oxygen in the O-glycosidic linkage to CD1d (Aspeslagh et al., 2011). Without this linkage, the galactose head group was in a suboptimal orientation when bound to CD1d, resulting in a lower TCR affinity compared to αGalCer-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 head group, in the expectation that this would activate *i*NKT cells to produce a strong Th1 response (Li et al., 2009). The O-glycosidic linkage has also been modified by replacing the oxygen with a sulfur atom. An α-S-GalCer molecule, induced a more Th2 skewed cytokine profile in mice, and did not lead to increased survival in a tumor model (Chang et al., 2007). 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 (Blauvelt et al., 2008). However, *in vivo* studies in mice indicated that α-S-GalCer did not lead to *i*NKT cell proliferation or cytokines. The authors proposed that the

α-S-GalCer 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 *i*NKT cells, it could activate human *i*NKT cells, causing cytokine secretion and *i*NKT cell-induced maturation signals to human DC (Hogan et al., 2011). This is an intriguing counter example of species specificity, compared to C-glycoside, which only works to activate mouse *i*NKT cells but not those in humans.

1.4.3 Alpha versus Beta linkages and Endogenous ligands

When the initial ligands for *i*NKT cells were first discovered, it was predicted that the linkage could only be of an α-anomeric nature, and a β linkage would not lead to *i*NKT cell activation (Kawano et al., 1997). This was presumed as logical as the α-anomeric form as well as D-glycosylceramides are not detected in mammals and are therefore "foreign" epitopes. However, the selection of *i*NKT cells in the thymus indicated that there had to be a self-ligand that could positively select these cells. Since a β linkage occurs naturally in mammals, β-anomeric glycolipids are part of cellular membranes, (Goni and Alonso, 2006; Sonnino et al., 2006) β linkages were reviewed much more thoroughly (Parekh et al., 2004). It was noted that mice lacking β-Galactosylceramide (βGalCer) synthase, and thus βGalCer, have normal *i*NKT cell development (Stanic et al., 2003) and mice treated with this compound have decreased *in vivo i*NKT cells, however, there is no detectable cytokine signal when mice were treated with this GSL (Ortaldo et al., 2004). β-Glucosylceramide (βGlcCer) (Figure 1.3), both an anabolic and catabolic GSL pathway metabolic intermediate, (Lalazar et al., 2006) seems to decrease *in vitro i*NKT cell proliferation (Margalit, 2005) and is accumulated in

patients with Gaucher's disease, which seem to have an increase of *i*NKT cells (Balreira et al., 2005). With a structure very similar to αGalCer and data indicating that this lipid accumulates in the presence of a microbial infection (Brennan et al., 2011) as well as tumor prevention data (Inafuku et al., 2012), it seems very logical that βGlcCer can activate *i*NKT cells and therefore is a likely endogenous ligand candidate. However, there is a possibility that this ligand could have been contaminated with an α linked GSL (Brennan et al., 2014). Strangely enough, while αManCer fails to activate *i*NKT cells, βManCer is suggested to be an *i*NKT ligand capable of tumor protection (O'Konek et al., 2011). Stranger still, this antitumor ligand appears to operate through an entirely different mechanism independent of IFN-γ and instead relying on TNFα and nitric oxide and doesn't induce anergy, a side effect of other strong NKT ligands (O'Konek et al., 2013). The hunt for endogenous ligands turned up several other candidates including ligands such as isoglobotrihexosylceramide (iGB3), sulfatide, plasmalogen lysophosphatidylethanolamine (pLPA), lysophosphatidylcholine, (LPC), lysophosphatidylethanolamine (LPE), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA), (Figure 1.3) (Mallevaey et al., 2011; Fox et al., 2009; Parekh et al., 2004; Zhou et al., 2004a) and even mouse collagen II (MCII), a collagen peptide (O'Konek et al., 2011). The validation of these ligands has been difficult. For instance, iGB3, the first identified self-ligand (Zhou et al., 2004a) was crystalized and it was proven that the mouse CD1d molecule can bind iGB3 (Figure 1.3 and Figure 1.4) (Zajonc et al., 2008). Later it was noted that CD1d loaded iGB3 could form a complex with the *i*NKT cell TCR resembling a conformation similar to α-linked ligands with the terminal sugar of iGb3 forming novel contacts with CD1d (Figure 1.4)

(Pellicci et al., 2011; Yu et al., 2011). However, the validity this endogenous ligand has been clouded by reports that suggest mice lacking iGB3 synthase, essential for iGB3 formation, still have normal *i*NKT cells (Porubsky et al., 2007). Also, it has been noted that human CD1d cannot present iGB3 (Sanderson et al., 2013) and iGb3 is not present in mouse or human thymus, (Speak et al., 2007) rendering this ligand as an unlikely important self-ligand of NKT cells. It is also possible that these self-ligands serve, not only in positive selection, but also as a means to get over an activation threshold when NKT cells are activated by cytokines such as IL-12, IL-18 or IFN-1 (Nagarajan and Kronenberg, 2007; Paget et al., 2007; Tyznik et al., 2008; Wesley et al., 2008).

1.4.4 Sphingoid base chain

The sphingoid base is a defining property of many antigens that activate NKT cells. The most commonly found sphingosine chain length in animal tissues is the aliphatic C:18 chain, 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 (Christie, 2014). The phytosphingosine of αGalCer (Figure 1.2), 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 GSL with a phytosphingosine shortened by several carbon atoms (Figure 1.2) (Miyamoto et al., 2001). This lipid antigen induced a more IL-4, or Th2 profile. When injected into mice, OCH was shown to reduce the symptoms of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (Miyamoto et al., 2001). The further shortening of the sphingoid chain also led to a Th2 cytokine profile (Goff et al., 2004). Different alterations of the sphingoid base can have an opposite polarizing effect on the

cytokine response. For example, OCH doesn't have a long chain to anchor CD1d and may reduce antigen presentation time.

1.4.5 Acyl Chain

The acyl chain of a number of synthetic GSL antigens is typically longer than the sphingoid base. For αGalCer, 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, acyl chain (Yu et al., 2005). This compound was identified in a screen of multiple αGalCer analogs with shorter acyl chains, all of which induced an enhanced Th2 cytokine profile (Goff et al., 2004). The decrease in the carbon chains of the lipid, either in the acyl chain or 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 *i*NKT cells (Goff et al., 2004). Conversely, the elongation of the carbon chains, or the addition of bulky groups to the acyl chain, seem to cause a Th1 profile that may be due to stabilization of the GSL-CD1d 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 (Fujio et al., 2006). 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 (Padte et al., 2013). There is likely a limit to chain length as the hydrophobic pockets of CD1d only have a certain depth.

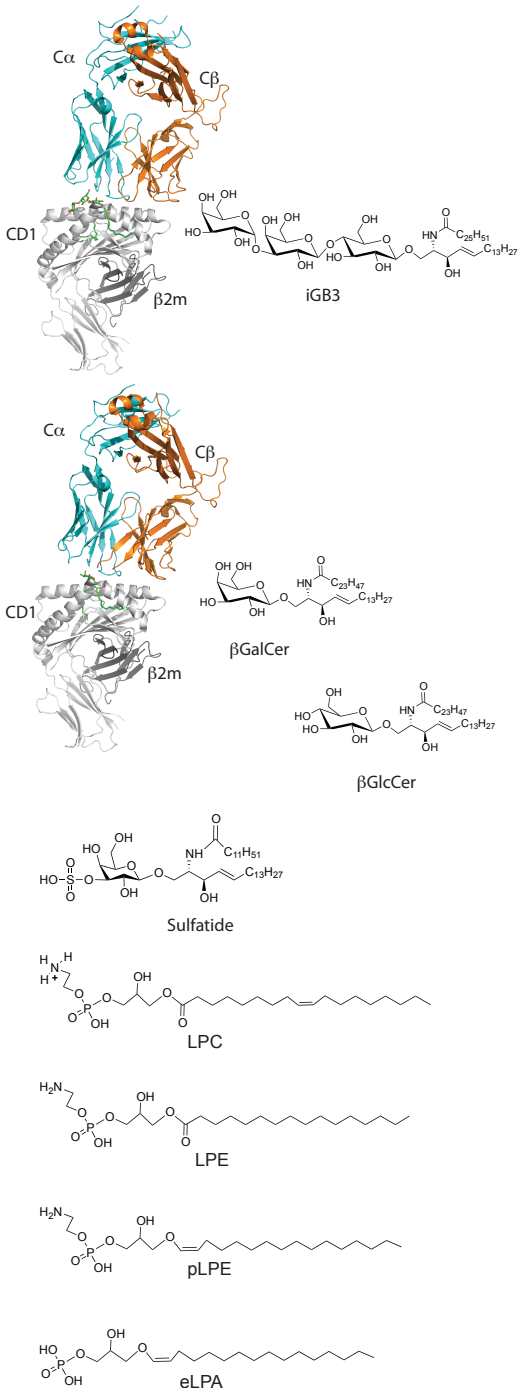


Figure 1.3. Proposed endogenous ligands for *i*NKT cells. Endogenous ligands shown in chemical formation. Ligands iGB3 and βGalCer in their ternary mouse CD1d-GSL-*i*NKT cell TCR crystal structure format.

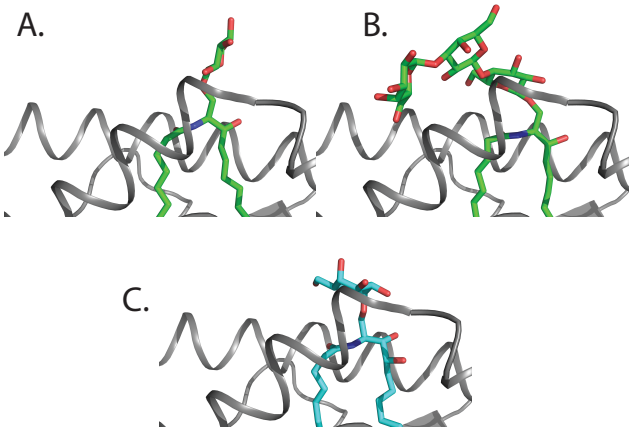


Figure 1.4. Impact of TCR binding. A.) iGB3 before TCR binding B.) iGB3 after TCR binding C.) αGalCer binding

1.5 CONCLUSION

In conclusion, over the past two decades our understanding of *i*NKT cells and the activation of this intriguing subset has greatly improved. We have generated synthetic agonists that bind with different degrees of intensity to CD1d. We have identified the conserved docking motif of the *i*NKT cell TCR. And we have also identified several natural activators of *i*NKT cells, both endogenous and exogenous. Although much work has been done and recently reviewed elsewhere (Anderson et al., 2013; Banchet-Cadeddu et al., 2011; Girardi and Zajonc, 2012; Laurent et al., 2014) these cells still remain a confounding subset of the immune system and much work is still being done to understand the mechanism, development and therapeutic potential of the interaction between GSLs presented by CD1d and *i*NKT cells. The work summarized in this dissertation analyzes a variety of different GSLs and explores the theories behind why certain GSLs can lead to a preferential Th1 immune response.

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Chapter 2:

Structural modifications of αGalCer in both lipid and carbohydrate moiety influence activation of murine and human *i*NKT cells

2.1 INTRODUCTION

Natural Killer T (NKT) cells are a distinctive population of T lymphocytes with the capacity to impact the immune system in a wide array of functions, ranging from protection against tumors to autoimmune regulation (Robertson et al., 2014; Roozbeh et al., 2014). They accomplish this feat through their ability to secrete Th1 and Th2 cytokines, most notably IFN-γ and IL-4, respectively, and their ability to impact bystander cells such as NK cells, dendritic cells and B cells (Bendelac et al., 2007; Brennan et al., 2013). Although much work has been done to analyze these skewing responses, the exact mechanism of polarization is not completely elucidated. NKT cells, specifically invariant NKT cells (*i*NKT cells) have a semi-invariant T-cell receptor (TCR) that has an evolutionarily conserved α chain, Vα14-Jα18 in mice and Vα24/Jα18 in humans. The TCR α chain, pairs with a less restricted β chain repertoire, to recognize glycosphingolipids (GSLs) presented by CD1d. CD1d is a member of the CD1 family of antigen presenting molecules (CD1a-e in humans, CD1d in mice) that is structurally similar to the peptide presenting major histocompatibility complex (MHC) class I molecules. The CD1d heavy chain is composed of three domains, α1, α2 and α3. While the α3 domain non-covalently associates with β2-microglobulin, the α1-α2 superdomain forms central, hydrophobic antigen binding groove (Zajonc and Wilson, 2007). This groove further segregates into two connected pockets called A′ and F′. Each pocket binds one chain of a dual alkyl chain lipid antigen. In case of glycosphingolipids, the

sphingosine chain binds in the F′ pocket, while the A′ pockets bind the fatty acid. This lipid binding orientation allows the sugar head group to be exposed in the center for recognition by the *i*NKT cell TCR, specifically the α chain of the TCR (Joyce et al., 2011). Many GSLs have been studied to understand the activation of *i*NKT cell TCRs, and the most commonly studied is alpha-galactosylceramide (αGalCer) (Banchet-Cadeddu et al., 2011). This prototypical glycolipid activates *i*NKT cells *in vivo* within 90 minutes to secrete both IL-4 and IFN-γ. Since this initial discovery, many glycolipids have been studied that sway the response of the immune system predominately toward either a Th1 or a Th2 response (Anderson et al., 2013). One of the earliest Th1 skewing lipid studied to date is C-glycoside where the O-glycosidic linkage of αGalCer is replaced with a carbon molecule, predicted to stabilize this lipid (Godfrey and Kronenberg, 2004). Although this lipid is a Th1 skewer in mice, it is unable to activate human *i*NKT cells. The importance of Th1 skewing in a mammalian system is that this response is important to drive the system towards an inflammatory response essential for tumor clearance (Osherovich, 2011) and vaccine adjuvant activity (Petrovsky and Aguilar, 2004). Thus *i*NKT cell lipids have been studied in the context of possible therapeutics. In this study, we looked at 5 different lipids that have been previously demonstrated to activate *i*NKT cells and tested their activation of human and mouse *i*NKT cells side-by-side. We have further assessed their TCR binding affinities and crystallized the mouse ternary CD1d-GSL-TCR complexes to look at the molecular interactions that underlie TCR triggering.

2.2 MATERIALS AND METHODS

2.2.1 GSL synthesis

The GSLs used in this study have all been described previously: EF77 (Tyznik et al., 2011), GCK127 and 152 (Li et al., 2009), NC-αGC (Aspeslagh et al., 2013), 7DW8-5 (Li et al., 2010a; Padte et al., 2011; 2013; Venkataswamy et al., 2014; Xu et al., 2014).

2.2.2 *in vitro* GSL presentation assay

The GSL presentation assay has been described previously, briefly, APCs (1×10^5 per well) were pulsed with 100 ng of the indicated lipid and were incubated overnight. The cells were then combined with 5×10^4 *i*NKT cell hybridomas for 24 h. The DN3A4-1.2 (1.2) Vα14 *i*NKT cell hybridomas cells have been described previously (Brossay et al., 1998). TCR engagement was measured using a sandwich ELISA for IL-2 in the supernatant of hybridoma cultures.

2.2.3 Human NKT cell Assay

The isolation and expansion of human Vα24$^+$ *i*NKT cells has been published previously (Rogers et al., 2004). Human donor PBMCs ($1–1.5 \times10^6$/ml) were isolated and cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) Pen-Strep-Glutamine (10,000 U/ml penicillin, 10,000 g/ml streptomycin, 29.2 mg/ml L-glutamine; Invitrogen) and cultures were expanded by weekly re-stimulation with αGalCer-pulsed, irradiated PBMCs and recombinant human IL-2. PBMCs (1×10^5 per well) were pulsed with GSLs and were seeded in 96 well plates and cultured in the presence of 5×10^4 Vα24$^+$ human *i*NKT cells for 24 h. GM-CSF release was evaluated in a sandwich ELISA following the manufacturer's instructions (R&D Systems).

2.2.4 Mice

C57BL/6 mice were purchased from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions and the experiments were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology. Mice were injected with 1 μg of lipids iv and the sera of immunized mice was subjected to sandwich ELISAs to measure total mouse IFN-γ levels.

2.2.5 Mouse and Human CD1d and TCR preparation

As reported previously (Zajonc et al., 2005), mouse CD1d-β2-microglobulin heterodimeric protein was expressed in a baculovirus expression system and human CD1d- β2-microglobulin was prepared analogous to the mouse protein. The recombinant soluble *i*NKT TCR was prepared by refolding α and β chains as previously reported (Wang et al., 2010).

2.2.6 CD1d loading

Each GSL was dissolved in DMSO at a concentration of 1 or 2 mg/mL. The individual GSLs were diluted in a vehicle solution (50 mM Tris-HCl pH 7.0, 4.8 mg/ml sucrose, 0.5 mg/ml sodium deoxycholate and 0.022% Tween 20) and were incubation at 80° for at least 20 minutes. The GSLs were then combined with CD1d in an approximate 3:1 molar ratio of lipid to protein overnight in the presence of 50 mM Tris-HCl pH 7.0.

2.2.7 SPR kinetic analysis

Biotinylated CD1d was processed and purified and studies were conducted using a Biacore 3000 (GE Healthcare) as reported previously (Wang et al., 2010) and lipid loading was done as reported above. Following overnight GSL loading, approximately 300 response units of CD1d-GSL complex were immobilized onto a streptavidin sensor

chip surface by injecting the CD1d-GSL mixture at 5 µl/min using HBS (10 mM HEPES, 150 mM NaCl, 3.0 mM EDTA, pH 7.4) running buffer. A reference channel was bound with unloaded CD1d and increasing concentrations of refolded TCR were passed over at a flow rate of 30 µl/min. Kinetic association and dissociation curves were calculated using BIAevaluation software version 4.1.

2.2.8 Crystallization and structure determination

Following overnight GSL loading, the CD1d-GSL complexes were incubated with refolded TCR at room temperature for at least 30 minutes, followed by isolating using Superdex S200 10/300 GL (GE Healthcare) equilibrated in buffer (50 mM Hepes pH 7.4, 150 mM NaCl). The isolated fractions containing the ternary complexes were concentrated and crystals were grown at 22.3°C by sitting drop vapor diffusion while mixing 0.5 ml protein with 0.5 ml precipitate. Precipitates were 0.2 M ammonium citrate dibasic pH 5.1 20% PEG 4000 for EF77, 17% polyethylene glycol, 8% tascimate pH 4 for 7DW8-5, 20% polyethylene glycol, 0.2 M dihydro ammonium citrate for NC-αGC, 17% polyethylene glycol, 0.2 M potassium acetate for GCK127 and 16% polyethylene glycol, 0.2 M di-ammonium hydrogen citrate for GCK152. Single crystals were harvested and flash cooled in a mother liquor containing 20% glycerol. Crystals were collected at the Stanford Synchrotron Radiation Lightsource and the Advanced Light Source and processed with the software Mosflm (Leslie, 2006). The structures were solved by molecular replacement in CCP4 (Collaborative Computational Project, Number 4) (Winn et al., 2011). First, the PDB coordinates for the protein component of PDB ID 2Q7Y were used, followed by the TCR coordinates of PDB ID 3QUZ. Models were rebuilt into σ_A-weighted $2Fo - Fc$ and $Fo - Fc$ difference electron density maps using

COOT (Emsley et al., 2010). The GSLs were built into 2*Fo –Fc* map and refined using REFMAC (Vagin et al., 2004). Refmac geometric libraries for the glycolipids were obtained using the PRODRG server (Schüttelkopf and van Aalten, 2004). Data collection and refinement statistics are summarized in Table 2.S1.

2.3 RESULTS

2.3.1 Modifications of the αGalCer structure

We compared the structure and antigenicity of αGalCer analogs that were modified in their three structural components, the galactose head group, fatty acid and sphingosine moiety (Figure 2.1). In addition, GCK127 and GCK152 have their O-glycosidic linkages replaced with an E-alkene linker between sugar and lipid moieties, similar to C-glycoside, while GCK152 also has a truncated fatty acid with a terminal phenyl group (C8-Ph) (Li et al., 2009). Naphtylcarba-αGalCer (NC-αGC) has an aromatic 6”-OH galactose modification, similar to the previously crystalized Naphtylurea (Nu)-αGalCer. However, unlike NU-αGC where this group is linked via a urea group, NC-αGC has a carbamate linker, which provides more flexibility to the napthyl ring (Aspeslagh et al., 2013). The rationale for introducing this flexible linker was to assess whether the rigidity of the urea linker or the aromatic nature and size of the napthyl group resulted in the reported structural change in the A´ roof of CD1d (Aspeslagh et al., 2011). EF77 is a plakoside A-like glycolipid, a GSL isolated from the marine sponge, *Plakortis simplex* (Costantino et al., 1997), and is the sister lipid to the previously crystallized SMC124 lipid. The sugar head group and O-glycosidic linkage are identical to αGalCer but the acyl chain has been modified to closer mimic plakoside A with a cyclopropyl group and an alkene bond (Tyznik et al., 2011). Like EF77, 7DW8-5 also has a

modification in the acyl group. The length of the chain has been shortened to 10 carbons and a parafluor-phenyl group has been added (C10-PH-F) (Li et al., 2010a; Padte et al., 2011). The rational behind those different modifications is that changes in the lipid moiety mostly influence antigen trafficking or the lipid interaction with CD1d, while changes in the galactose moiety and linkage can affect both resistance to endoglycosidases upon injection in mice and thus could increase the half-life in serum.

2.3.2 Real Time Binding Kinetics

First, we determined the TCR interactions of these lipids presented by both mouse and human using Surface Plasmon Resonance (SPR) (Table 2.1). According to the analysis with mouse CD1d and TCR (Table 2.1A), GCK127 and GCK152 give rise to the lowest *i*NKT cell Vα14Vβ8.2TCR binding kinetics of, K_D=247 ± 86 nM and K_D=197 ± 22 nM. This is similar to the affinity reported for the parent compound C-glycoside (K_D=247 nM), (Aspeslagh et al., 2011), but approximately 10-fold weaker than αGalCer, which in our hands ranges in affinity from 11-25 nM (Wang et al., 2010; Aspeslagh et al., 2013). Of the lipids tested, NC-αGC had the highest affinity of 37.1 ± 14.10 nM, similar to NU-αGC (Aspeslagh et al., 2011) followed by EF77 (44.7 ± 0.4 nM) and 7DW8-5 (94 ± 2.8 nM). This order was not maintained in the human biacore analysis using the Vα24Vβ11 TCR (Table 2.1B). GCK127 and EF77 appeared the weakest antigens at K_D=6.85 ± 2.6 μM and 3.4 ± 2.71 μM, respectively. The other lipids had similar affinity to αGalCer, which in our hands ranges from 1-3 μM. GCK127 (1.45 ± 0.05 μM) and NC-αGC (1.45 ± 0.35 μM) were very similar and 7DW8-5 had the highest affinity (1.13 ± 0.9 μM) of the group. We noted that in the mouse studies the off-rate for the *i*NKT cell TCR for both GCK127 and GCK152 (k_d =1.28 ± 0.0014 x 10^{-2} $s^{-1}$and 1.66 ± 0.0016 x10^{-2}

s^{-1}, respectively) is 10x faster than the other ligands including αGalCer (k_d= 2.2 ± 0.52x 10^{-3} s^{-1}) (data not shown) but similar to C-glycoside (Aspeslagh et al., 2011). Therefore, we assume that the TCR was not able to induce the F' roof closure of CD1d in the absence before TCR binding. As previously reported, some GSLs like αGalCer induce the formation of the F' roof prior to TCR docking through orienting CD1d side chains L84, V149, and L150 to optimal conformation for engagement by the TCR CDR3α residue L99. The ability to form this roof independently of the TCR has been correlated with an increased off-rate of the *i*NKT cell TCR (Li et al., 2010b). In the human SPR studies we noted that the off-rates for all the GSLs were similar, likely due to the inability of human CD1d to pre-form the F' roof as the L84 of mouse CD1d is altered to F84 in human CD1d and a fully closed F' roof has not been observed in the hCD1d-αGalCer structure (Koch et al., 2005).

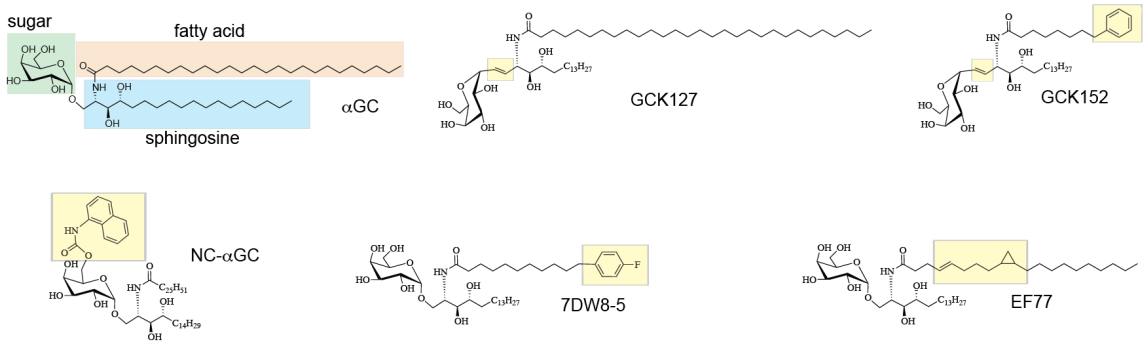


Figure 2.1. Chemical structures of glycosphingolipids. In green, the carbohydrate galactose sugar moiety, in red the fatty acid chain and in blue, the sphingosine base chain of αGalCer. In yellow are the deviations of each GSL from αGalCer.

2.3.3 Biological activity of the glycolipids

As all glycolipid antigens were bound by the TCR with moderate to high affinity, we investigated their activity *in vitro* and *in vivo*. Biological assays for each of the glycolipids had been reported previously, EF77 (Tyznik et al., 2011), GCK127 and 152 (Li et al., 2009), NC-αGC (Aspeslagh et al., 2013), 7DW8-5 (Li et al., 2010a; Padte et al., 2011; 2013; Venkataswamy et al., 2014; Xu et al., 2014), however, here we assessed their potency side-by-side. First, we performed a cell-mediated antigen presentation assay, in which a mouse B-cell lymphoma cell line (A20) stably expressing CD1d, was pulsed with various lipids and their ability to activate mouse Vα14 *i*NKT cell hybridoma cell lines was analyzed. *i*NKT cell hybridoma activation was measured by IL-2 secretion using an ELISA of cell culture supernatants. When the various lipids were assayed in this manner, all of the lipids stimulated the hybridomas over the mock control with 7DW8-5, EF77 and GCK152 being the most potent glycolipids at that concentration (Figure 2.2A). The synthesis of different lipids is done not only to understand mechanisms of *i*NKT cell activation and influence on the immune response, but also done to define possible therapeutic targets for humans. To address whether these lipids could activate human *i*NKT cells, we used a model whereby antigen-presenting cells in the form of PBMCs and *i*NKT cells were isolated from human donor blood samples. Various concentrations of lipids were added to PBMCs and activation of human *i*NKT cells was measured by GM-CSF cytokine secretion in the supernatant (one representative line is shown, Figure 2.2B). 7DW8-5 and NC-αGC were the most potent lipids and were capable of activating human *i*NKT cells in a dose-dependent manner. However, a high concentration of NC-αGC seemed to inhibit *i*NKT cell activation. Similarly, EF77 also gave a very strong response.

In comparison, both alkene versions of the parental C-glycoside compound, GCK127 and GCK152, failed to activate human *i*NKT cells, even at high concentrations.

We further tested the lipids *in vivo* by injecting 1 μg of each lipid into mice intravenously. 24 hours post injection, we analyzed serum IFN-γ cytokine levels using a sandwich ELISA. NC-αGC and EF77 were the two most potent lipids generating a Th1 skewing IFN-γ burst at this time point (Figure 2.2C). In summary, the pharmacokinetic properties of each lipid appear to affect the activation of *i*NKT cells. While NC-αGC is not able to activate *i*NKT cells when pulsed into A20 cells, it very strongly activates human *i*NKT cells using pulsed PBMCs and murine *i*NKT cells when injected intravenously into a mouse. In contrast, 7DW8-5 potently activated human *i*NKT cells and a murine *i*NKT cell hybridoma using pulsed A20 cells, but not when injected intravenously. EF77 was the only lipid that gave strong responses in all three assays, while the GCK series failed to give a robust response, even though the TCR binding kinetics were among the strongest for human *i*NKT cells (Table 2.1).

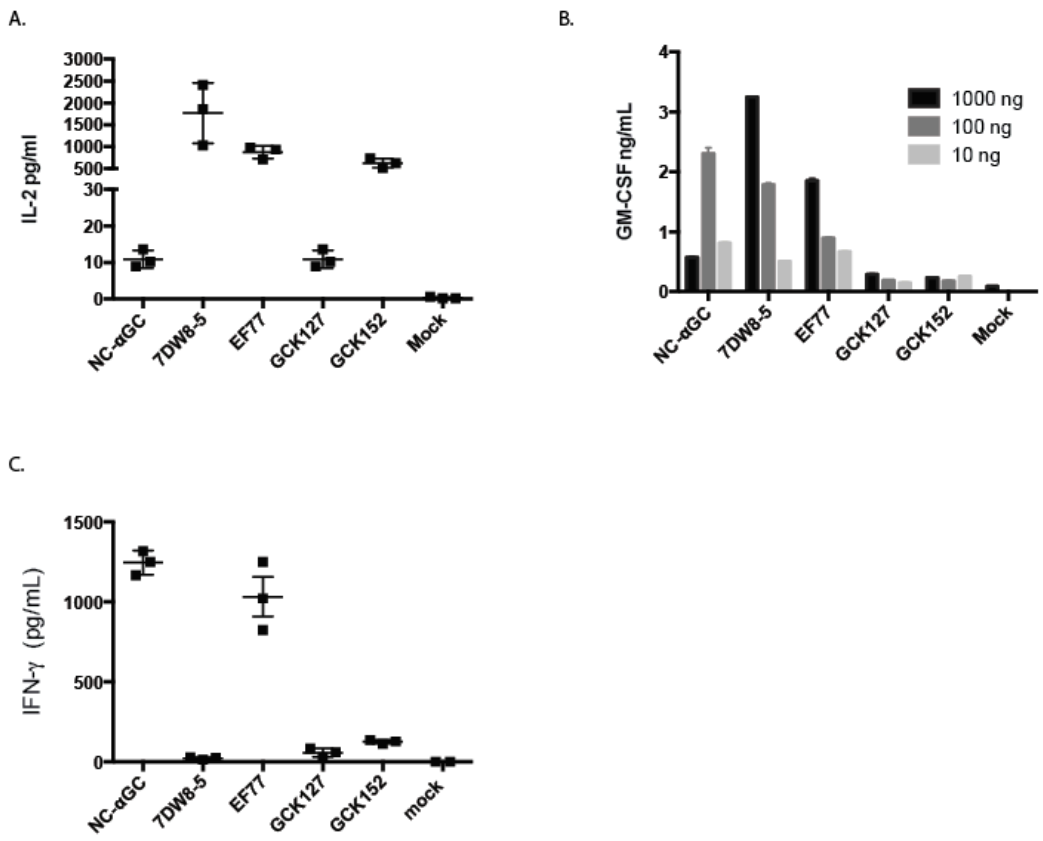


Figure 2.2. Biological assays with GSLs A. Cell based assay in which an A20 B cell lymphoma cell line transfected with CD1d was pulsed with 100 ng of the indicated GSL and co-cultured with an *i*NKT cell hybridoma (1.2). TCR engagement measured after 24 hours through IL-2 production in serum measured with a sandwich ELISA. Data represents samples in triplicate and are representative of three independent experiments. B. Human *i*NKT cells were activated by human PBMCs pulsed with the indicated glycolipid concentrations for 24 h. Levels of human GM-CSF were measured in the supernatant using a sandwich ELISA assay. Representative data from one of two experiments performed in triplicate wells using multiple human cell lines are shown. C. C57Bl/6 mice were injected with 1 μg of the indicated GSL, and sera were analyzed at 24 hours. Serum samples were measured for IFN-γ levels by ELISA. Data are representative of 2 independent experiments of 3 mice per group. Error bars represent ± SEM.

2.3.4 Crystal structures of the ternary complexes

In order to determine how each of these lipids is recognized by the TCR of *i*NKT cells, we crystalized each lipid in complex with mouse CD1d and TCR. All crystallography parameters are listed in Table 2.S1. The overall structure of all complexes was consistent with previously crystallized CD1d-glycolipid-TCR complexes with the TCR docked offset over the F′ pocket of CD1d in a parallel orientation (Figure 2.3), notably different from the diagonal footprint generated in MHC-peptide-TCR complexes. All GSLs bind with the fatty acid chain in the A′ pocket of CD1d, while the sphingosine chain nestles inside the F′ pocket and presents the galactose moiety at the CD1d binding groove portal for TCR recognition. Electron density for all of the lipids is well defined (Figure 2.4) except for the acyl chain of 7DW8-5. In this case, the electron density implies that the acyl chain can orient itself both clockwise and counterclockwise around cysteine 12 within the A' binding pocket. Thus, we have modeled both acyl chain orientations of 7DW8-5 with each 50% occupancy (occurrence). The altered acyl chain binding orientation may contribute to the biological properties of 7DW8-5 observed in mouse studies (Figure 2.4F).

2.3.5 TCR interactions

Previous studies established that the TCR of *i*NKT cells binds CD1d with a largely conserved binding footprint (Girardi and Zajonc, 2012; Mallevaey et al., 2011; Wun et al., 2011). Not surprisingly, the contact surfaces between CD1d and the TCR are very similar throughout the different ternary complexes (Table 2.2) All the TCR interactions with the antigen are formed with the conserved TCR α chain, while both TCR chains contact CD1d. TCR interactions with CD1d are overall dominated by the

TCRα chain (818-870Å^2 total buried surface area, (BSA)) but there is substantial involvement of the TCRβ chain (582-650Å^2 BSA) (Table 2.2). Contacts between TCRα and the ligand vary depending on the ligand modification that result in subtle differences in the galactose presentation to the TCR. Crystal structure analysis shows hydrogen bonding is maintained similar to αGalCer in the case of 7DW8-5, GCK127 and EF77 forming contacts between the galactose groups and residues N30, R95 and G96 of the *i*NKT TCR (Figure 2.5). GCK152 shows the most deviation from this binding. It fails to maintain the hydrogen bonds between the 4" hydroxyl group and N30, and the 3" hydroxyl group and R95 of the TCR. Furthermore, it also fails to make a hydrogen bond between the 3' hydroxyl group and D80 of the CD1d molecule. NC-αGC loses the hydrogen bond between the 4" hydroxyl group of the sugar and N30 of the TCR (Figure 2.5C) similarly to the previously crystallized NU-αGC, as the napthyl anchor binds within a newly formed pocket in the A' roof of CD1d, slightly pulling the galactose away from the TCR. Although NC-αGC was predicted to be more flexible than NU-αGC, when the two structures are overlayed (Figure 2.5F) there are no discernable differences between the two crystal structures at the resolution of this study, suggesting that the size and nature of the aromatic modification drives the induced fit observed in CD1d and not the flexibility of the linker region. We noted that the total buried surface area between the TCRα and GSL was highest in the ligands with the highest affinity measurements, NC-αGC, EF77 and 7DW8-5 (Table 2.2). However, the number of H-bonds between TCR and antigen did not always correlate with the affinity, suggesting that van der Waals interactions contribute greatly to both the TCR binding energy and affinity and can compensate for loss of individual H bond interactions (Figure 2.5). This is exemplified by

7DW8-5 that, despite having the maximum Hydrogen bond interactions, only shows the third highest affinity. Both GCK series antigens appear to be rather weak in both biological assays as well as TCR binding kinetics using the mouse TCR, suggesting that the exchange of the O-glycosidic bond with a carbon-carbon bond is less well tolerated as an αGalCer modification in mice. However, even though their antigenicity for human *i*NKT cells in our experiments is low as well, the TCR binding kinetics with the human molecules is rather high. The reason for which is currently unknown.

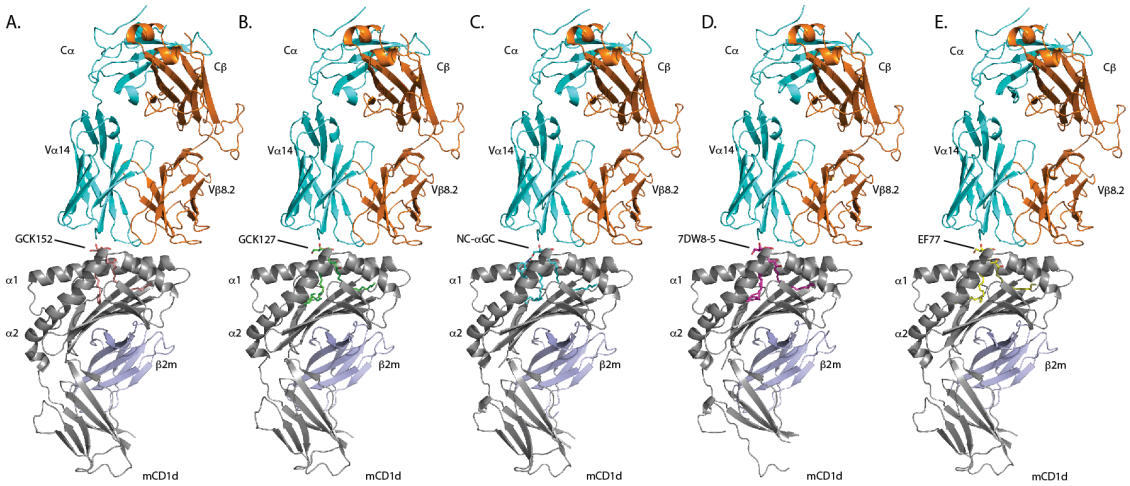


Figure 2.3. Ternary crystal structures of mouse CD1d-GSL-*i*NKT cell TCR complexes. A. GCK152 B.GCK127 C. NC-αGC D. 7DW8-5 E. EF77

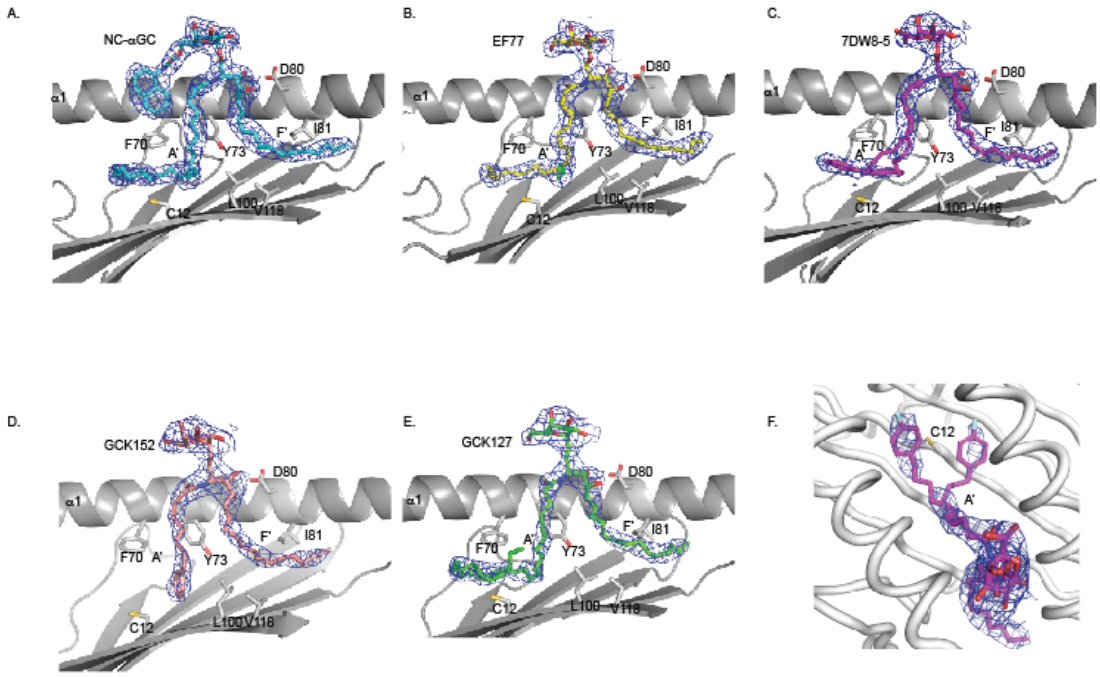


Figure 2.4. Electron density of GSLs shown with CD1d. Note: α2 helix removed for clarity A. NC-αGC B. EF77 C. 7DW8-5 D. GCK152 E. GCK127 F. Dual binding motif for acyl chain of 7DW8-5

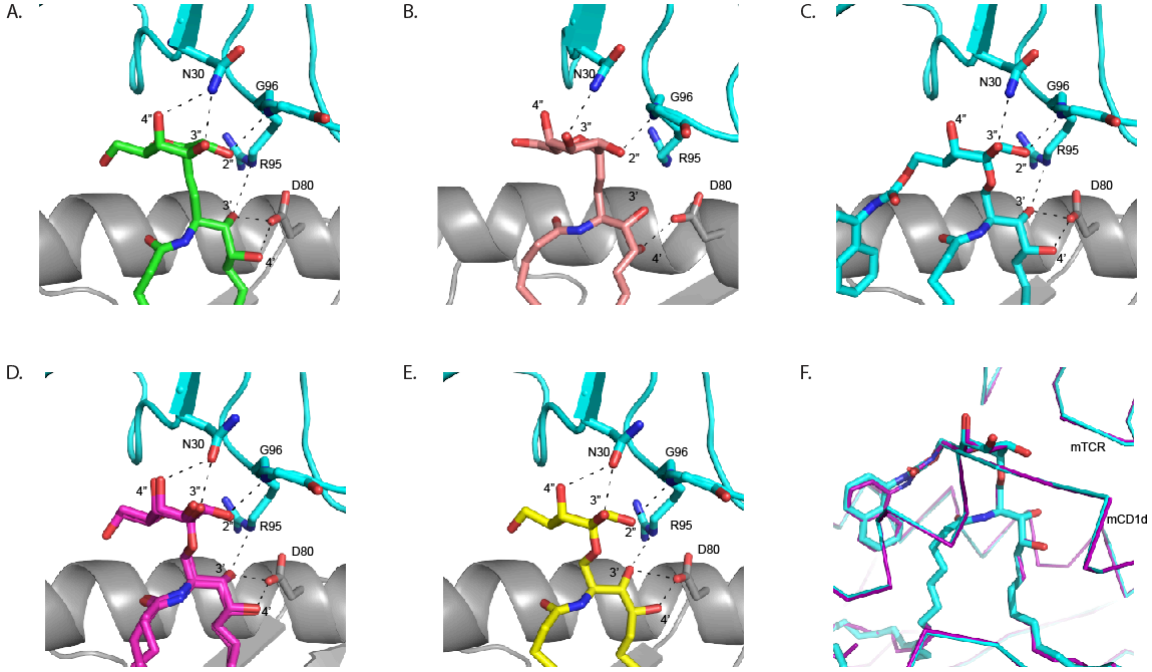


Figure 2.5. Mouse *i*NKT cell TCR hydrogen bond interactions with CD1d and GSLs A. GCK127 B. GCK152 C.NC-αGC D. 7DW8-5 E. EF77 F. Overlay of NC-αGC (cyan) with NU-αGC (purple) ribbon crystal structures

Table 2.1. Mouse and Human *i*NKT cell affinity measurements

A. Mouse CD1d and Mouse TCR SPR Analysis

Glycolipid	$K_{ass}(M^{-1}S^{-1})$	$K_{diss}(S^{-1})$	$K_D(K_{diss}/K_{ass})$
NC-αGC	3.8E+04 ± 5.30E+03	1.50E-03 ± 7.35E-04	37.1 ± 14.10 nM
EF77	4.5E+04 ± 0.6E+03	2.00E-03 ± 0.1E-03	44.7 ± 0.4 nM
7DW8-5	9.4E+04 ± 3.4E+03	8.86E-03 ± 9.31E-05	94 ± 2.8 nM
GCK152	8.5E+04 ± 1.5E+03	1.66E-02 ± 1.60E-03	197 ± 22 nM
GCK127	5.7E+04 ± 1.4E+04	1.28E-02 ± 1.4E-03	247 ± 86 nM

B. Human CD1d and Human TCR SPR Analysis

Glycolipid	$K_{ass}(M^{-1}S^{-1})$	$K_{diss}(S^{-1})$	$K_D(K_{diss}/K_{ass})$
7DW8-5	10.3E+04 ± 4.7E+04	0.163 ± 0.139	1.13 ± 0.9 μM
GCK127	4.4E+04 ± 2.9E+04	0.066 ± 0.045	1.45 ± 0.05 μM
NC-αGC	14E+04 ± 2E+04	0.205 ± 0.075	1.45 ± 0.35 μM
EF77	26.25E+04 ± 17.75E+04	0.295 ± 0.005	3.4 ± 2.71 μM
GCK152	1.4E+04 ± 0.4E+04	0.107 ± 0.063	6.85 ± 2.6 μM

Table 2.2. Total Buried surface areas between mouse TCR-CD1d and TCRα-glycolipid (in $Å^3$)

	CD1d-ligand-TCR complex				
Contact surfaces	GCK127	GCK152	NC-αGC	EF77	7DW8-5
TCRα-ligand	297.5	290	298.4	303.1	241
TCRα-CD1d	844.4	817.8	867.4	854.4	870.4
TCRβ-CD1d	610	646.9	633.1	636.9	582.2

2.4 DISCUSSION

Many *i*NKT cell GSLs have been assessed to predict how effective different lipids will be within the human and mouse immune systems (Anderson et al., 2013). Some studies see correlations, however the GSLs discussed in this paper clearly indicate that a variety of parameters are in play that cannot be addressed merely with one approach. Although we used different assays to look at these lipids in comparison to each other, we did not address the pharmacokinetic properties of each GSL and this is an aspect for future studies. It is interesting to note that, although the linker of NU-αGC has been altered in the NC-αGC lipid, allowing for more flexibility of the napthylurea group, the two crystal structure show no obvious differences and the overlay indicates the conserved binding orientation of the *i*NKT cell TCR trumps flexibly in these models. NC-αGC is very strong in the *in vivo* mouse studies and the human assay but fails to activate in the *in vitro* cell presentation assay. Conversely, EF77, the plakoside A GSL, activates in all the assays and 7DW8-5 activates the human *i*NKT cells and the cell based hybridomas. The ability of 7DW8-5 to activate human *i*NKT cells correlates with previously reported data that it is a very potent stimulus for human immune cells and that this GSL is potently able to serve as a vaccine adjuvant in primate models. Although this GSL does not by itself cause a robust IFN-γ cytokine profile in our mouse *in vivo* system, this could be due to the fact that this lipid may biodistribute differently than other GSLs. It has been noted that mouse intramuscular injection of this lipid causes it to localize to local lymph nodes whereas αGalCer is distributed systemically (Moriya Tsuji, personal communication). The EF77 crystal structure showed similar features to it's sister ligand SMC124 with the cyclopropyl group containing acyl chain binding in hydrophobic pocket of CD1d the A'

pocket instead of the F' pocket seen with the sphingoid chain-modified SMC124 (Tyznik et al., 2011). Similarly, the acyl chain modified 7DW8-5 ligand shows the phenyl ring localizing to the A' groove, however this GSL proved more difficult to model. Our crystal structure shows a duel binding motif of the acyl chain in the A' pocket indicating that the fatty acid chain may orient both clockwise and counterclockwise around C12, possibly altering it's binding association and disassociation parameters, although this was not seen in the SPR data. The GCK127 and GCK152 antigens are the weakest of these lipids, with rapid off-rates in the mouse SPR studies and this may be in part due to the inability of the CD1d F' roof to pre-form prior to TCR engagement (Joyce et al., 2011). This F' roof formation likely occurs upon binding of the other GSLs used in this study, accounting for the 10-fold difference in dissociation rates in the mouse SPR studies. Because this pre-formation does not occur in human CD1d, we do not see a disparity in the dissociation rates of the human *i*NKT cell TCR. The GCK152 GSL also fails to form some hydrogen bonds seen with lipids like αGalCer between the CD1d and TCR, also accounting for a faster off-rate of the TCR. Although the human TCR affinities would predict a human *i*NKT cell response, GCK127 and GCK152 do not strongly activate the human cell line in our hands, similar to what was seen for their parent lipid, C-glycoside. It is perhaps this alteration from an O-glycoside bond to a carbon linkage that is not prefered, or is perhaps degraded faster in a human system which would be an ironic twist as the carbon modification was predicted to stabilize the GSLs. Nonetheless, lipids of this nature do not seem to have the therapeutic potential that the other lipids in this study might possess. The buried surface area contacts between the TCRα loop and the CD1d moiety may shed some light on the variation in the biological and biochemical

parameters. In conclusion, these different GSLs have the capacity to form interactions between CD1d and *i*NKT cells, but have various responses in the mouse and human studies, indicating a lack of one-to-one correlation in these assays. Future studies that can accurately measure the pharmacokinetic properties of the GSLs, binding affinities for the GSL and CD1d molecules and biodistribution of different GSLs would be particularly interesting for this line of inquiry.

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Chapter 2, in part is currently being prepared for submission for publication of the material. Alysia Birkholz, Marek Nemčovič, Esther Dawen Yu, Enrico Girardi, Jing Wang, Archana Khurana, Nora Pauwels, Richard Franck, Moriya Tsuji, Amy Howell, Serge Van Calenbergh, Mitch Kronenberg, Dirk M. Zajonc. The dissertation author was the primary investigator and author of this paper.

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2.7 SUPPLEMENTARY DATA

Table 2.S1. Refinement statistics for the CD1d-GSL-TCR complexes

Data collection statistics	**CD1d-GCK127-TCR**	**CD1d-GCK152-TCR**	**CD1d-NC-αGC-TCR**	**CD1d-EF77-TCR**	**CD1d-7DW8-5-TCR**
PDB code	4Y4F	4Y4H	4Y16	4Y4K	4Y2D
Space group	$P2_1$	$P2_1$	$C222_1$	$C222_1$	$P2_1$
Cell dimension					
a, b, c, (Å)	78.6, 149.7, 101.4	79.4, 150.4, 102.5	79.6, 191.9, 151.9	79.1, 191.4, 151.3	79.4, 150.3, 100.8
a, b, g (°)	90, 96.5,90	90, 96.4, 90	90, 90, 90	90, 90, 90	90, 96.2, 90
Resolution range (Å) [outer shell]	40-3.2 [3.31-3.2]	40-3.1 [3.15-3.1]	66.21-2.60 [2.71-2.60]	95.7-2.9 [3.06-2.9]	500-3.05 [3.12-3.05]
No. reflections	38,286	42,694	34,681	25,092	44,418
R_{merge} (%)	13 [63.9]	11.3 [59.9]	10.3 [47.8]	17.2 [59.4]	9.3 [53.6]
R_{pim} (%)	n/a	n/a	7.0 [32.6]	11.1 [38.4]	n/a
Multiplicity	3.3 [3.3]	3.8 [4.0]	2.9 [2.9]	3.2 [3.4]	3.0 [3.0]
Average I/sI	13.7 [2.1]	21.5 [2.7]	6.8 [2.3]	5.9 [2.2]	13.2 [2.0]
Completeness (%)	98.2 [91.1]	99.4 [100]	96.2 [95.7]	97.4 [99.1]	99.3 [99.8]
Refinement statistics					
No. atoms	12,599	12,469	6,532	6,305	11,943
Protein	12,389	12,247	6,333	6,183	11,627
Ligand	126	96	73	56	208
Carbohydrate	84	126	80	66	108
Water	0	0	46	0	0
R/R_{free} (%)	21.8/25.7	24.2/28.3	21.1/24.4	21.9/26.5	21.7/25.7
Ramachandran plot (%)					
Favored	96.91	95.52	96.36	96.11	95.93
Allowed	99.75	99.68	100	99.75	99.87
R.m.s. deviations					
Bonds (Å)	0.006	0.01	0.01	0.005	0.005
Angles (°)	0.879	1.226	1.248	1.076	0.994
B-factors ($Å^2$)					
Protein	89.25	75.42	41.75	37.58	75.66
Ligand	67.87	58.88	33.77	28	50.79
Carbohydrate	83.86	87.85	54.56	44.54	96.54

Chapter 3:

Novel glycolipids that elicit IFN-γ-biased responses from natural killer T cells

3.1 INTRODUCTION

Natural killer T cells with an invariant T cell receptor α chain (*i*NKT cells) recognize microbial and synthetic glycolipids bound to and presented by CD1d. αGalCer (Figure 3.1) was identified as the lead anti-cancer candidate in structure activity relationship studies around Agelasphin 9b, a natural product isolated from the *Agelas* genus of marine sponges (Morita et al., 1995). αGalCer subsequently has been the key glycolipid used to elucidate the role of *i*NKT cells in the immune system (Bendelac et al., 2007). The anti-tumor activity of this compound depends on IFN-γ secretion, much of which is produced by natural killer cells that are activated downstream of antigen-specific *i*NKT cell stimulation (Yu and Porcelli, 2005). Interestingly, analogs closely related to αGalCer can elicit different cytokine profiles in the immune response. For example, in a number of cases enhanced IL-4 secretion is observed {Yu, 2005 #157}{Oki, 2004 #160}. Immunologists refer to outcomes of predominant IL-4 production as T helper type 2 (Th2) responses. Other analogs induce a more IFN-γ biased or Th1 response, which is important for anti-cancer immunity and host defense against infectious agents. While there has been a strong interest in developing Th1-biasing glycolipid antigens for *i*NKT cells, relatively few antigens fit into this category (Aspeslagh et al., 2011; Chang et al., 2007; Li et al., 2009; Lin et al., 2010; Lu et al., 2006; Tashiro et al., 2010). The prototypical Th1 biasing glycosphingolipid, known as C-glycoside, has the O-glycosidic

bond replaced with a carbon (Schmieg et al., 2003). While it effectively leads to IFN-γ synthesis *in vivo* in mice, C-glycoside does not stimulate human *i*NKT cells (Arora et al., 2011). Therefore, while a number of glycolipid antigens related to αGalCer with interesting properties have been characterized, there is a need for Th1-biasing compounds that strongly activate human *i*NKT cells.

In a search for additional glycolipids that induce a Th1 polarized *i*NKT cell response, we considered natural products similar to αGalCer, specifically, plakoside A (Figure 3.1), a glycosphingolipid isolated from the marine sponge, *Plakortis simplex* (Costantino et al., 1997). The ceramide of plakoside A shares structural features with αGalCer, as well as similarities to a cyclopropanated glycolipid isolated from *Sphingomonas witichii*. We synthesized two novel plakoside A analogs with either a cyclopropanated acyl chain or sphingoid base, and evaluated their ability to activate mouse and human *i*NKT cells. We characterized the glycolipids in terms of their antigenic responses, stability of CD1d-binding, T cell antigen receptor (TCR) binding kinetics, and the crystal structure of one glycolipid bound in the CD1d-TCR complex. Our data suggest that a more stable glycolipid-CD1d interaction *in vivo*, driven in part by a more stable binding of the antigen in the CD1d groove, is an important determinant of a Th1-skewed cytokine response. Therefore, this comprehensive analysis allows us to link the enhanced response elicited by these compounds to biochemical and structural features of their interactions.

3.2 MATERIALS AND METHODS

3.2.1 Generation of Human *i*NKT cell lines

Human Vα24^{+} *i*NKT cells were purified and expanded according to published protocols (Rogers et al., 2004). PBMCs were isolated by density-gradient centrifugation. Human donor PBMCs (1–1.5 ×10^{6}/ml) were cultured in RPMI 1640 complete medium containing 10% autologous heat inactivated serum. Human *i*NKT cell cultures were expanded by weekly re-stimulation with αGalCer-pulsed, irradiated PBMC and recombinant human IL-2.

3.2.2 Antigen presentation assays.

The antigen presentation assay has been described previously (Lawton et al., 2005). Briefly, A20-CD1d cells were pulsed with vehicle or the indicated glycosphingolipid overnight. APCs (1 × 10^{5} per well) were incubated with 5 × 10^{4} *i*NKT cell hybridomas for 20-24 h. The DN3A4-1.2 (1.2) and DN3A4-1.4 (1.4) Vα14 *i*NKT cell hybridomas have been described previously (Brossay et al., 1998). Cytokines in the supernatant of hybridoma cultures were measured by a sandwich ELISA using anti-IL-2 monoclonal antibodies.

3.2.3 Cell-free antigen presentation assay.

Stimulation of *i*NKT cell hybridomas on microwell plates coated with soluble CD1d was carried out according to published protocols (Naidenko et al., 1999; Sidobre et al., 2004; Tupin and Kronenberg, 2006). Indicated amounts of compounds or vehicle were incubated for 24 h in microwells that had been coated with 1.0 μg of CD1d. After washing, 5 × 10^{4} *i*NKT cell hybridomas were cultured in the plate for 20 h, and IL-2 in the supernatant was measured by ELISA.

3.2.4 GM-CSF Production by Human cells.

Plakoside A or αGalCer-pulsed APCs (1×10^5 per well) were seeded in 96 well plates and cultured in the presence of 5×10^4 Vα24$^+$ human *i*NKT cells for 20-24 h. GM-CSF release was evaluated in a sandwich ELISA following the manufacturer's instructions (R&D Systems).

3.2.5 Mice.

C57BL/6, and IL-12Ra$^{-/-}$ mice were purchased from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions and the experiments were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology. Mice were injected with 2 μg of lipids iv. As a positive control for *i*NKT cell responses, mice were injected with 2 μg of αGalCer or C-Glycoside (Sullivan et al., 2010). Standard sandwich ELISAs were performed to measure mouse IFN-γ, IL-12p70, and IL-4 in the sera of immunized mice.

3.2.6 Cell preparation.

Single cell suspensions of splenocytes and liver lymphocytes were isolated as described previously (Tyznik et al., 2008). For DC isolation from the spleen, the tissue was diced into 1mm pieces, digested using spleen dissociation media and DCs were enriched by positive selection using a CD11c$^+$ isolation kit with RoboSep technology according to the manufacturer's protocols (Stem Cell Technologies). Isolated DCs were co-cultured at varying concentrations with hybridomas overnight.

3.2.7 Flow cytometry and intracellular cytokine staining.

Lymphocytes were stained with αGalCer/CD1d tetramers labeled with streptavidin-allophycocyanin, anti-NK1.1-PerCp PE-cyanin (PECy)5, anti-CD8-PECy7,

anti-CD11b-PECy7, and anti-TCRβ-allophycocyanin-AF750. All antibodies and isotype controls were purchased from BD Biosciences, except anti-TCRβ-allophycocyanin-AF750 obtained from eBioscience. Cells were fixed and permeabilized using Cytofix/Cytoperm buffer and stained for intracellular IFN-γ with PE-labeled clone XMG1.2. The data were collected on a LSR II and FACsCanto flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

3.2.8 FACS-based detergent resistance assay

All screening experiments were performed as previously described (Arora et al., 2011). Briefly, JAWS II cells were seeded in U bottom 96 well plates. Cells were cultured with 100 nM concentration of each of the different glycolipid analogues or controls. After 16 hours of culture, cells were detached, washed 3 times, and resuspended in 50 µl of FACS buffer. After 10 minutes of incubation, 50 µl of staining solution containing 1 µg/ml of Alexa Flour 647 conjugated mAB L363 in FACS buffer was added followed by 10 minutes of incubation on ice. Cells were washed and analyzed on a FACS Calibur flow cytometer (BD Biosciences) with Cell Quest software. For estimation of lipid raft residency, the FACS analysis was performed in a kinetic mode. In the acquisition mode, the beginning fluorescence level was recorded for 10 seconds. Triton X-100 was then added to a final concentration of 0.06% followed by brief (~1 sec) vortexing to mix the sample. Data collection was then resumed, and fluorescence intensities were monitored for another 30 seconds. Data were collected as Flow Cytometry Standard (FCS) files and analyzed using FlowJo software 7.5 (Treestar, Ashland, OR). MFI values at time 0 (prior to addition of Tx-100) were normalized to

100, and relative decrease in MFI values for different agonists after addition of Tx-100 were compared.

3.2.9 Mouse CD1d expression, purification and Vα14-Vβ8.2 TCR refolding.

Mouse CD1d-β2-microglobulin heterodimeric protein was expressed in a baculovirus expression system as reported previously (Zajonc et al., 2005). The TCR construct design, refolding and purification processes were identical to the ones previously reported (Wang et al., 2010).

3.2.10 Glycolipid loading and SMC124-CD1d-TCR complex formation.

The SMC124 lipid was dissolved in DMSO at 1mg/ml. Before loading, 60 μl of the SMC124 solution was diluted to 0.25 mg/ml with 60 μl vehicle solution (50 mM Tris-HCl pH 7.0, 4.8 mg/ml sucrose, 0.5 mg/ml sodium deoxycholate and 0.022% Tween 20) and 120 μl 10 mg/ml Tween 20, followed by incubation at 80° for 20 minutes. SMC124 was loaded onto CD1d by incubating CD1d and the lipid (molar ratio of protein to lipid of 1:3) overnight in the presence of 50 mM Tris-HCl pH 7.0. For CD1d-TCR complex formation, the refolded TCR was incubated at room temperature for 30 minutes with lipid-loaded CD1d at a 1:2 molar ratio, as the SMC124 loading efficiency is approximately 50%, which was measured using the αGalCer specific antibody L317 (Yu et al., 2007) in a SPR binding experiment. The ternary CD1d-lipid-TCR complex was isolated from uncomplexed CD1d and TCR by size exclusion chromatography using Superdex S200 10/300 GL (GE Healthcare).

3.2.11 SPR binding analysis.

SPR binding studies were conducted using a Biacore 3000 (Biacore) as reported previously (Wang et al., 2010). Recombinant CD1d protein containing a birA-tag (LHHILDAQKMVWNHR) between the CD1d ectodomain and C-terminal hexahistidine tag was expressed and purified as reported above for the non birA-tagged CD1d. Pure birA-tagged CD1d was biotinylated using a commercial biotinylation kit (Avidity) and then isolated from free biotin on Superdex S200 10/300 GL. The lipid was loaded to biotinylated birA-tagged CD1d in the same way as non birA-tagged CD1d. 300 response units of CD1d-SMC124 were immobilized onto a streptavidin sensor chip (Biacore) surface by injecting the CD1d-SMC124 mixture at 5 μl/min (HBS running buffer without Tween 20 to prevent or slow down the washing of the glycolipid off CD1d: 10 mM HEPES, 150 mM NaCl, 3.0 mM EDTA, pH 7.4). A reference surface was generated in another flow channel with unloaded CD1d. During the association phase a series of increasing concentrations of TCR in duplicate were injected for 2 min, and the dissociation phase initiated by passage of running buffer alone, was continued over 45 min. Experiments were carried out at 25°C with a flow rate of 30 μl/min and were performed at least two times. Kinetic parameters were calculated after subtracting the response to CD1d molecules in the reference channel, using a simple Langmuir 1:1 model in the BIAevaluation software version 4.1. One representative sensorgram is shown.

3.2.12 Crystallization and Structure determination.

The CD1d-SMC124-TCR complex was isolated by Superdex S200 10/300 GL (GE Healthcare) equilibrated in buffer (50 mM Hepes pH 7.4, 150 mM NaCl). The fractions containing the complex were concentrated to 5 mg/ml. Crystals were grown at

22.3°C by sitting drop vapor diffusion while mixing 0.5 ml protein with 0.5 ml precipitate (100mM trisodium citrate pH 5.6, 10% PEG3350 and 2% tacsimate pH 5.0). Crystals were then flash-cooled at 100 K in mother liquor containing 20% glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Laboratory beamline 9.2 and processed with the software Mosflm (Leslie, 2006). The CD1d-SMC124-TCR crystallized in space group $P2_1$ (unit cell dimensions: a=79.0 Å; b=150.6 Å; c=102.0; Å b=96.3º). In the crystal, two CD1d-SMC124-TCR complexes occupy the asymmetric unit. The structure was solved by molecular replacement in CCP4 (Collaborative Computational Project, Number 4) (1994) using the protein coordinates from the CD1d-iGB3 structure [PDB code 2Q7Y] (Zajonc et al., 2008) as the search model followed by the *i*NKT TCR [PDB code 3HE6] (Pellicci et al., 2009). The model was rebuilt into σ_A-weighted 2*Fo –Fc* and *Fo – Fc* difference electron density maps using the program COOT (Emsley et al., 2010). The lipid was built into 2*Fo –Fc* map and refined using REFMAC (1994). The final refinement steps were performed using the TLS procedure in REFMAC with five domains (α1-α2 domain including carbohydrates and glycolipid, α3-domain, β2m, variable domain and constant domain of TCR). The CD1d-SMC124-TCR structure was refined to 2.8 Å to an Rcryst and Rfree of 22.6% and 27.3% respectively. The quality of the model was excellent as assessed with the program Molprobity (Lovell et al., 2003).

3.2.13 Statistical Analysis

An unpaired independent samples *t*-test was used for analysis of two separate sets of independent and identically distributed samples. Analysis was for equal variance. Normality was confirmed with a Kolmogorov-Smirnov test.

3.3 RESULTS

3.3.1 Synthetic strategies

Both the sphingoid base and the acyl chain of plakoside A have unique features in comparison to αGalCer and its analogs. With this in mind, we decided to evaluate separately the influence on *i*NKT cell stimulation of the sphingoid base and acyl chain moieties of plakoside A (Figure 3.1). Compound SMC124 contains a ceramide that couples the acyl chain of αGalCer with the sphingoid base of plakoside A. Compound EF77 has an acyl chain similar to that of plakoside A and links this to the phytosphingosine of αGalCer. We did not include the α-carbon OH group on the acyl chain of plakoside A in EF77 because it simplified access, and the studies with Agelasphin 9b had shown that this α-OH group was not essential for activity (Morita et al., 1995). A non-asymmetric, *syn*-selective cyclopropanation was done for SMC124 and EF77, because this, too, simplified the syntheses for initial evaluations. It is noteworthy that the sphingoid base of SMC124 is longer than any of the α-galactosyl ceramides that have been assessed to date and we speculated that the increased chain length could influence CD1d binding.

With the increased interest in glycolipid antigens for *i*NKT cells, aspects of their synthesis have become more streamlined. For example, the first published synthesis of αGalCer had a sequence of more than 15 steps (Morita et al., 1995), with several more steps required to make key intermediates. On the other hand, Gervay-Hague and colleagues have recently reported a three-step synthesis of αGalCer from commercially available starting materials (Du et al., 2007). In spite of the significant advances in

synthesis, glycolipids with variations in either the sugar, acyl chain or sphingoid base still represent challenging targets. The two most common strategies for glycolipid synthesis involve either glycosylation of a ceramide, or coupling of a donor sugar with some form of a sphingoid base, followed by acylation. The former approach was used to synthesize SMC124, while the latter was employed for EF77 (Figure 3.2).

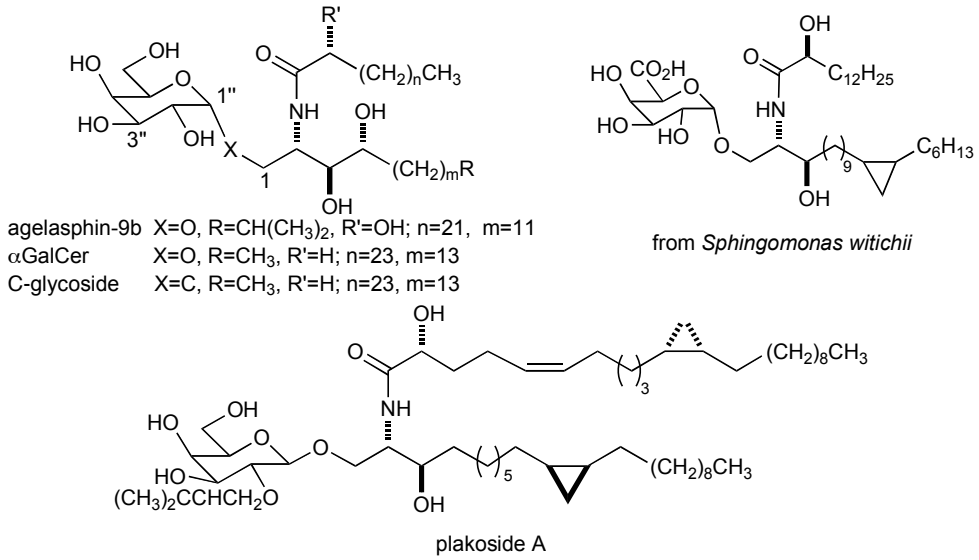


Figure 3.1. Structures of immunostimulatory natural glycolipids.

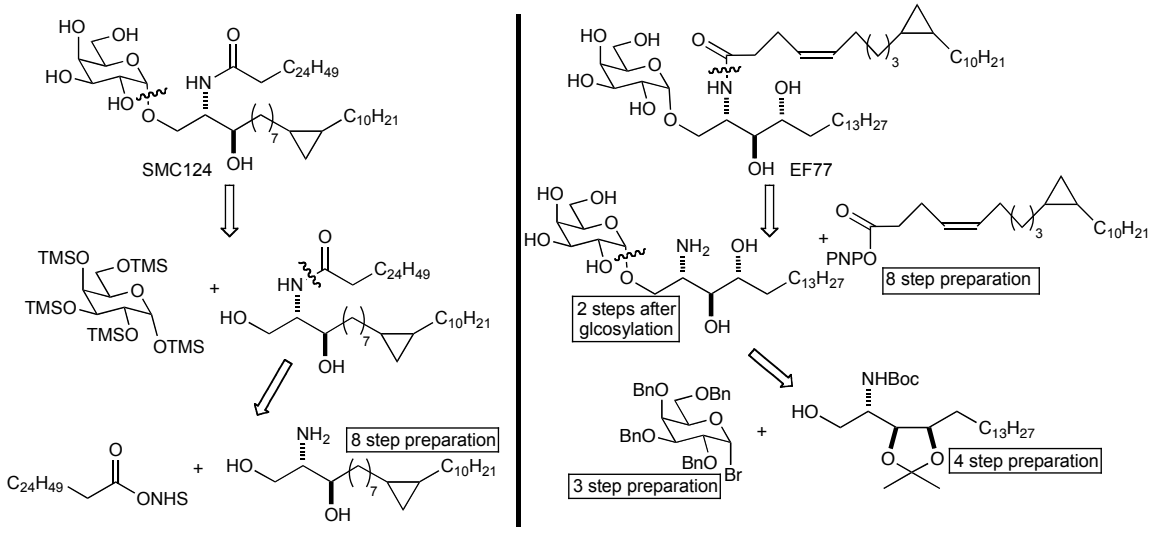


Figure 3.2. Strategies for the syntheses of plakoside A/αGalCer hybrid compounds SMC124 and EF77.

3.3.2 Plakosides are *i*NKT cell agonists

To analyze the biological activity of SMC124 and EF77 *in vitro*, we utilized mouse *i*NKT cell hybridomas and human *i*NKT cell lines. We first confirmed the ability of SMC124 and EF77 to stimulate immortalized *i*NKT cells (hybridomas) using the B lymphoma cell line A20 expressing surface mouse CD1d molecules as antigen presenting cells (APC). Regardless of whether the cyclopropane was located on the acyl chain or sphingoid base, both plakoside A analogs stimulated two *i*NKT cell hybridomas to similar levels (Figure 3.3). *i*NKT cell hybridoma 1.4, has an identical TCR α chain to hybridoma 1.2, but it utilizes Vβ10 instead of the Vβ8.2 segment that is much more common in *i*NKT cells (Brossay et al., 1998). To determine whether the stimulatory capacity of SMC124 and EF77 required internalization into cells, and perhaps lysosomal processing, we tested the ability of these glycosphingolipids to load onto immobilized CD1d molecules. These molecules were then tested to determine if they stimulate *i*NKT cell hybridomas in an APC-free assay measuring IL-2 release, a well-characterized bioassay for TCR engagement. At a concentration of 100 ng/ml, EF77 elicited a strong TCR-dependent response from the 1.2 hybridoma, while a 10 fold higher lipid concentration of SMC124 was required to elicit a similar response (Figure 3.3B). Similar results were observed using *i*NKT cell hybridoma 1.4. These data clearly demonstrate that the CD1d molecule can accommodate the cyclopropane group on either chain of the lipid and that the CD1d-bound antigens remain stimulatory for *i*NKT cell hybridomas.

We and others have observed that the first described Th1 skewing glycosphingolipid, C-glycoside, does not stimulate human *i*NKT cells (Li et al., 2009). To determine if human *i*NKT cells respond to SMC124 or EF77, we utilized expanded

human *i*NKT cell lines as responders and autologous peripheral blood mononuclear cells (PBMCs) pulsed with lipids as APCs. PBMCs were incubated with SMC124, EF77, C-glycoside, or αGalCer and then co-cultured with human *i*NKT cell lines. GM-CSF secretion was analyzed because it has been previously demonstrated that it is a sensitive measurement of TCR-mediated human *i*NKT cell activation. As previously reported, αGalCer stimulated human *i*NKT cells while the previously known Th1 skewing lipid in mice, C-glycoside, did not. Interestingly, SMC124 and EF77 induced significant amounts of GM-CSF secretion in all cell lines tested, although EF77 tended to be more potent for several of the cell lines (Figure 3.3C).

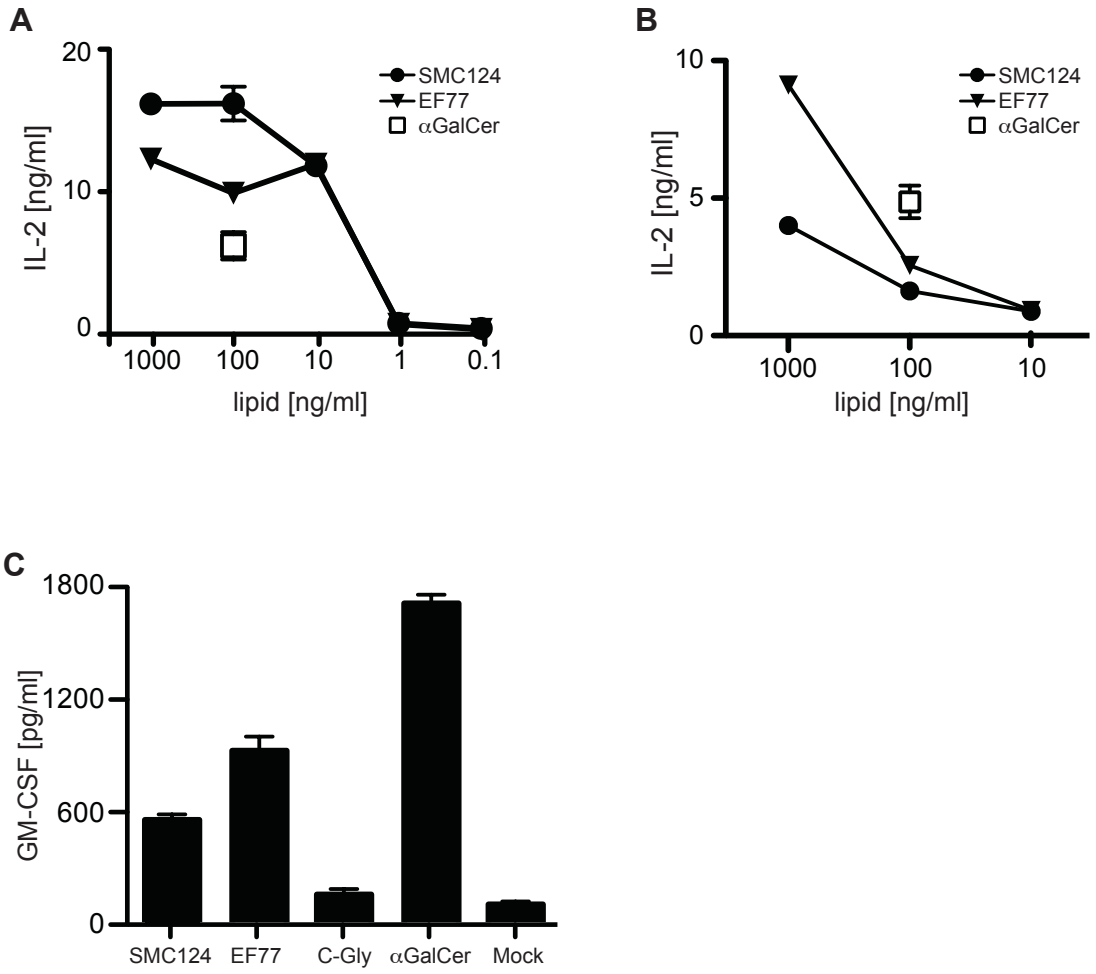


Figure 3.3. Immune responses to plakoside A analogs. (A) A20 cells transfected with CD1d and pulsed with the indicated glycolipid concentrations were cultured with *i*NKT cell hybridoma 1.2 and IL-2 cytokine levels in the supernatant were quantified by ELISA after 16 h of culture. (B) Same as (A) except antigen presentation was carried out on plates coated with CD1d and incubated with the indicated glycolipids concentrations. (A and B) Similar results were obtained with additional *i*NKT cell hybridomas. (C) PBMCs were pulsed with 100 ng/ml of the indicated glycolipids and cultured overnight. Cells were washed and co-cultured with human *i*NKT cell lines for 24 h. Cytokine levels for human GM-CSF were measured in the supernatant using ELISA. Representative data from one of 3-5 experiments performed in triplicate wells using multiple human cell lines are shown.

3.3.3 *In vivo* activation of *i*NKT cells

To determine if the plakoside A analogs activate *i*NKT cells *in vivo*, C57BL/6J mice were injected with lipid antigens, and sera were analyzed at various time points for IFN-γ (Figure 3.4A and B) and IL-4 by ELISA. It has been previously reported that IL-4 secretion peaks within two hours of αGalCer injection, with IFN-γ reaching a maximum level somewhat later (Matsuda et al., 2003; Schmieg et al., 2003). The Th1 biasing antigen C-glycoside causes a peak of IFN-γ by 6-12 h that is sustained longer, up to 36 h. The systemic IFN-γ in the sera at 6 h and later following either αGalCer or C-glycoside is primarily the result of secondary activation of natural killer cells downstream of *i*NKT cells (Matsuda et al., 2003; Yu and Porcelli, 2005). Two hours after injection of SMC124 or EF77, serum cytokine levels for IFN-γ were 3 fold lower than in αGalCer-injected mice (Figure 3.4A). Similar to C-glycoside, plakoside A analogs elicited lower IL-4 sera levels compared to αGalCer. Strikingly, SMC124 and EF77 injected mice consistently had 7-8 fold higher serum IFN-γ levels 22 h post lipid injection compared to either C-glycoside or αGalCer in six independent experiments (Figure 3.4B).

It has been previously reported that some glycolipids have the potential of inducing increased levels of *trans*-activation of several other cell types, including natural killer cells (Carnaud et al., 1999; Kawakami et al., 2001; Kitamura et al., 1999; Parekh et al., 2004). To address this, mice were analyzed by intracellular cytokine staining 22 h after lipid injection for IFN-γ production by natural killer cells. In agreement with the data from sera, natural killer cells from mice injected with the plakoside A analogs produced more IFN-γ, as measured on a per cell basis by the mean fluorescent intensity

(MFI) or when the percentage of IFN-γ^+ natural killer (NK1.1$^+$ TCRβ^-) cells in the spleen was assessed (Figure 3.4 C and D). Similar results were observed when cells from the liver were analyzed (data not shown). The mechanism of αGalCer and C-glycoside natural killer cell trans-activation is dependent on the lipid being presented on CD1d to the *i*NKT cell receptor leading to IL-12 production by DCs. IL-12 in turn enhances natural killer-cell-mediated IFN-γ production. To confirm the mechanism of trans-activation of our plakoside A analogs are mediated thru IL-12, we analyzed IL-12 serum levels from mice following lipid injection. It has been previously demonstrated that αGalCer and C-glycoside induces high levels of IL-12. Correlating to the high serum IFN-γ levels observed, IL-12 in the serum of SMC124 and EF77 was higher than αGalCer and sustained longer (Figure 3.4E). Furthermore, in the absence of IL-12 receptor, IFN-γ was no longer observed at 22 hours post injection. This was measured by both serum cytokine levels as well as single cell analysis of natural killer cells for intracellular IFN-γ.

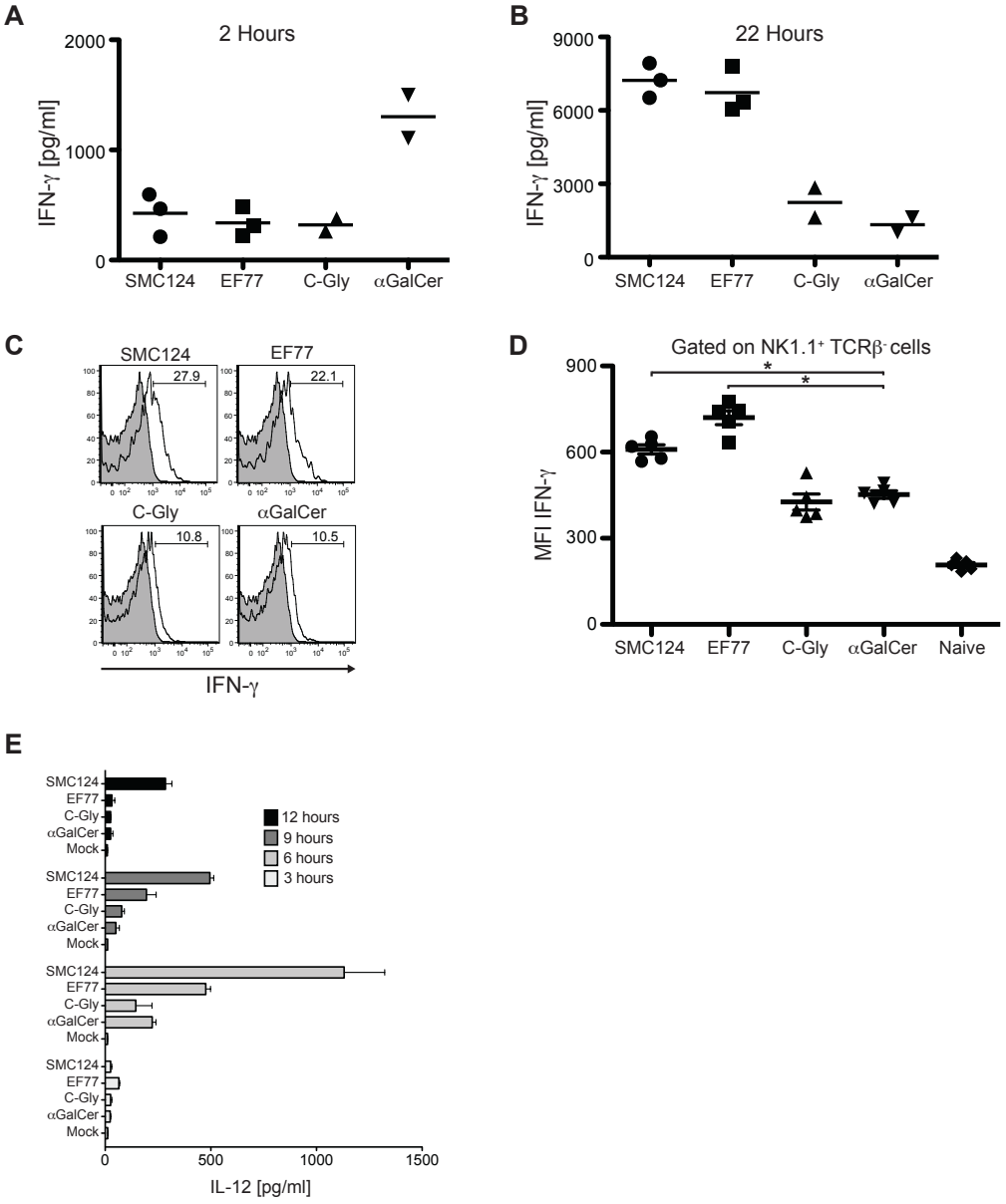


Figure 3.4. ***In vivo*** **responses to plakoside A analogs.** C57Bl/6 mice were injected with 2 μg of the indicated antigen, and sera were analyzed at the indicated time points. (A and B) Serum samples were measured for cytokine levels by ELISA. Data are representative of 6 independent experiments of 2 or 3 mice per group. Error bars represent +/- SEM. (C and D) Mice were sacrificed at 22 h and TCRβ$^-$, NK1.1$^+$ natural killer cells were analyzed for the production of IFN-γ by intracellular cytokine staining. (C) Percentage of IFN-γ$^+$ natural killer cells directly *ex vivo* 22 h post injection of lipid (open histogram) compared with PBS injected control (shaded histogram). Histograms are representative plots of a minimum of 3 independent experiments of 3-5 mice per group. Numbers represent percentage of IFN-γ$^+$ natural killer cells. (D) MFI scatter plot of IFN-γ intracellular fluorescence from 3-5 mice per group. Data are representative of 4 independent experiments. (E) Serum samples were measured for IL-12p70 cytokine levels by ELISA. Data are representative of 2 independent experiments of 3 mice per group. Error bars represent +/- SEM. Statistically significant differences using the equal variance unpaired Student *t* test are indicated with an asterisk (∗, $p \leq 0.001$) comparing αGalCer to either SMC124 or EF77.

3.3.4 Plakosides have prolonged *in vivo* activity

The ability of compounds to skew Th1 responses is correlated with loading into the groove of CD1d in endocytic compartments and subsequent appearance of the CD1d-glycolipid complexes selectively in lipid raft microdomains of the plasma membrane (Im et al., 2009). Recently a rapid, fluorescence-based assay for estimating the extent of lipid raft localization of CD1d/αGalCer complexes was developed for the efficient screening of compounds that skew Th1 cytokine responses (Arora et al., 2011). This assay uses fluorescently labeled antibodies that bind to CD1d only when it is loaded with αGalCer or closely related analogs, and it measures the kinetics of the loss of fluorescence associated with the cells after exposure to mild detergent concentrations. Since plasma membrane lipid rafts microdomains are detergent resistant, CD1d/glycolipid agonist complexes localized in lipid rafts cannot be extracted and a minimal decrease in fluorescence intensity is observed over time. In contrast, for the CD1d/glycolipid agonist complexes that are excluded from lipid rafts, the MFI decreases sharply following the Tx-100 addition. Utilizing this method, we observed that CD1d complexes loaded with either SMC124 or EF77 were stable to detergent to a similar extent as αGalCer or C-glycoside (Figure 3.5A). This is in sharp contrast to αGalCer C20:2, an analog with an unsaturated acyl chain that is Th2 biasing (Arora et al., 2011).

Another striking feature of the Th1-biasing antigen C-glycoside is that it has a prolonged biological stability *in vivo*, meaning that APC from mice injected hours earlier with C-glycoside have an enhanced ability to stimulate *i*NKT cells compared to αGalCer. To test if the plakoside A analogs also have a prolonged ability to stimulate *i*NKT cells *in vivo*, mice were injected with glycosphingolipids, and APCs were harvested at various

time points and tested directly *ex vivo* for their ability to stimulate *i*NKT cell hybridomas. Selected $CD11c^+$ dendritic cells (DCs) (> 80% pure) isolated from lipid or DMSO control injected mice obtained at 2 and 22 h post injection were co-cultured at increasing concentrations with a fixed number of *i*NKT cell hybridomas. DCs isolated from αGalCer-injected mice were able to activate the hybridoma at 2 and 22 h post injection, as measured by IL-2 secretion, although at 22 h the stimulatory capacity of the DC declined by approximately 50% (Figure 3.5 B and C). At 22 h post injection, EF77 and SMC124 retained a nearly undiminished ability to activate the *i*NKT cell hybridomas compared with 2 h after injection (Figure 3.5 B and C). A similar sustained capacity of the DC to activate *i*NKT cells *ex vivo* was observed with cells from mice injected with C-glycoside, although its antigenic potency was reduced.

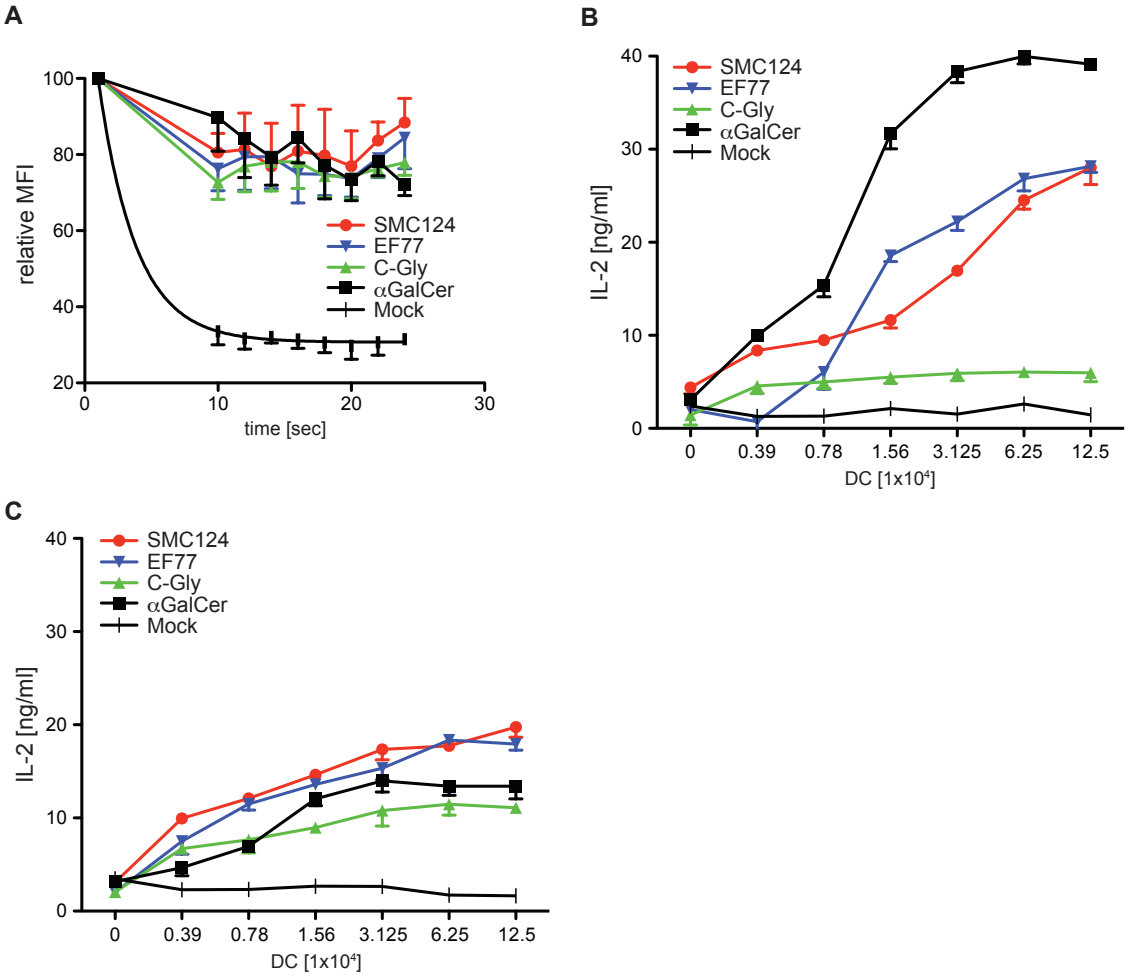


Figure 3.5. Stability of lipid/CD1d complexes. (A) Plot of detergent resistance of CD1d/glycolipid/antibody complexes formed with SMC124, EF77, and known Th1- and Th2-biasing αGalCer agonists on JAWS II DCs. (B and C) C57Bl/6 mice were injected with 2 μg of the indicated lipid antigens and $CD11c^+$ DCs were enriched using magnetic bead isolation at 2 (B) and 22 (C) h post injection. Varying number of enriched DCs were cultured with the 1.2 Vα14 *i*NKT cell hybridoma overnight and IL-2 in the supernatant quantified by ELISA. Data are plotted as the number of enriched DCs per well versus IL-2 (pg/ml). Data are representative of two independent experiments. Error bars represent +/- SEM of 2 or 3 mice per condition.

3.3.5 The CD1d-SMC124 complex affinity for the *i*NKT TCR

The potency of *i*NKT antigens is generally correlated to their affinity for the TCR (Wang et al., 2010; Wun et al., 2011). Equilibrium binding analysis using surface plasmon resonance (SPR) demonstrated a binding affinity (K_D) of the *i*NKT TCR for CD1d-SMC124 complexes of 51.9 ± 6.6 nM. This is a binding affinity similar to that observed with αGalCer (K_D = 24.6 nM, (Figure 3.6A), The kinetic parameters revealed that the TCR binds CD1d-SMC with an association-rate 2 fold weaker than αGalCer (k_a= 4.25 ± 0.46 × 10^4 M^{-1} s^{-1}) while the dissociation-rate (k_d= 2.2 ± 0.52 × 10^{-3} s^{-1}), is similar to that seen with αGalCer (k_a= 7.84 × 10^4 M^{-1} s^{-1} and k_d=1.61 × 10^{-3} s^{-1} respectively) (data not shown).

3.3.6 Crystal structure of the TCR-SMC-CD1d ternary complex

In order to determine how the novel structural features of the plakoside compound might affect binding to CD1d and recognition by the *i*NKT TCR, we determined the structure of the CD1d-SMC124-TCR complex by X-ray crystallography. The complex crystallized in space group $P2_1$ with two complexes in the asymmetric unit. Although differences are observed between the two non-crystallographic symmetry-related molecules in correspondence to the α3 domain of the CD1d molecule, the overall structure and the conformation of the ligand and antigen-binding domain appear very well conserved in both complexes. For this reason only one complex (chains A-D) will be described throughout the text.

The overall structure of the CD1d-SMC124-*i*NKT TCR complex is consistent with the structure of previously described CD1d-lipid-*i*NKT TCR complexes (Figure 3.6B) (Li et al., 2010b; Pellicci et al., 2009). The TCR docks in a parallel orientation on

top of the CD1d antigen binding groove, deviating significantly from the diagonal footprint generally observed for MHC-peptides-TCR complexes (Rudolph et al., 2006). The interaction of the TCR with CD1d is mediated by residues in the CDR3α and CDR2β loops and a CDR3β-dependent contact, as observed for other ternary complexes obtained using the same *i*NKT TCR construct (Aspeslagh et al., 2011; Li et al., 2010b). Well-defined density is observed corresponding to the SMC124 molecule (Figure 3.6C), suggesting an overall ordered conformation of the ligand in the binding groove, with its galactose moiety exposed for recognition by the TCR. Several polar interactions between the polar moieties of the ligand and CD1d, involving residues Glu80, Glu15, and Thr156 (Figure 3.7 A and B), contribute to stabilize the SMC124 conformation at the top of the binding groove. Similarly, conserved hydrogen bonds with CDR1α and CDR3α residues determine the modality of interaction of the SMC124 galactose moiety with the *i*NKT TCR (Figure 3.7 C and D), as previously observed for αGalCer and other galactose-containing glycolipids (Aspeslagh et al., 2011; Li et al., 2010b; Wun et al., 2011).

While the recognition constraints imposed by the *i*NKT TCR lock the galactose moiety of SMC124 in a conformation virtually identical to the one observed for αGalCer, differences are observed for the lipid moiety. SMC124 binds similarly to other sphingolipids, with its sphinganine in the F' pocket and the acyl chain in the A' pocket (Figure 3.7 E and F). Furthermore, when the structure of the complexed SMC124 and αGalCer or GalAGSL are superposed, in the A' pocket the acyl chain adopts a very similar conformation. However, the longer sphingoid base of SMC124 is forced to adopt a more compact conformation in the F' pocket, extending to the same depth in the pocket

as GalAGSL, and deeper than αGalCer (Figure 3.7 E and F). Interestingly, while the region defining the bottom of the F' pocket (residues 89-94) is not modeled in the CD1d-αGalCer-TCR complex, this loop is ordered in the CD1d-GalAGSL-TCR and CD1d-SMC124-TCR complexes, and it adopts the same conformation in both structures. Additionally, despite the comparable number of contacts mediated by the SMC124 and αGalCer sphingoid bases with the residues lining the F' pocket (32 van der Waals contacts for SMC124, 34 for αGalCer), differences are observed between the two compounds in terms of the residues involved. For example, there is a unique interaction of the SMC124 sphingoid base with the hydrophobic portion of the Glu92 side chain (Figure 3.7 E and F). There are also some differences in the number of contacts mediated by each interacting residue. This is seen in the increased number of interactions between Val118 and the cyclopropane moiety of SMC124. The compressed binding of SMC124 in the CD1d binding groove results in an increased buried surface area compared to αGalCer (1099$Å^2$ vs. 1027$Å^2$), as calculated by the PISA server, which could increase the stability of the CD1d-SMC124 complex (Krissinel and Henrick, 2007).

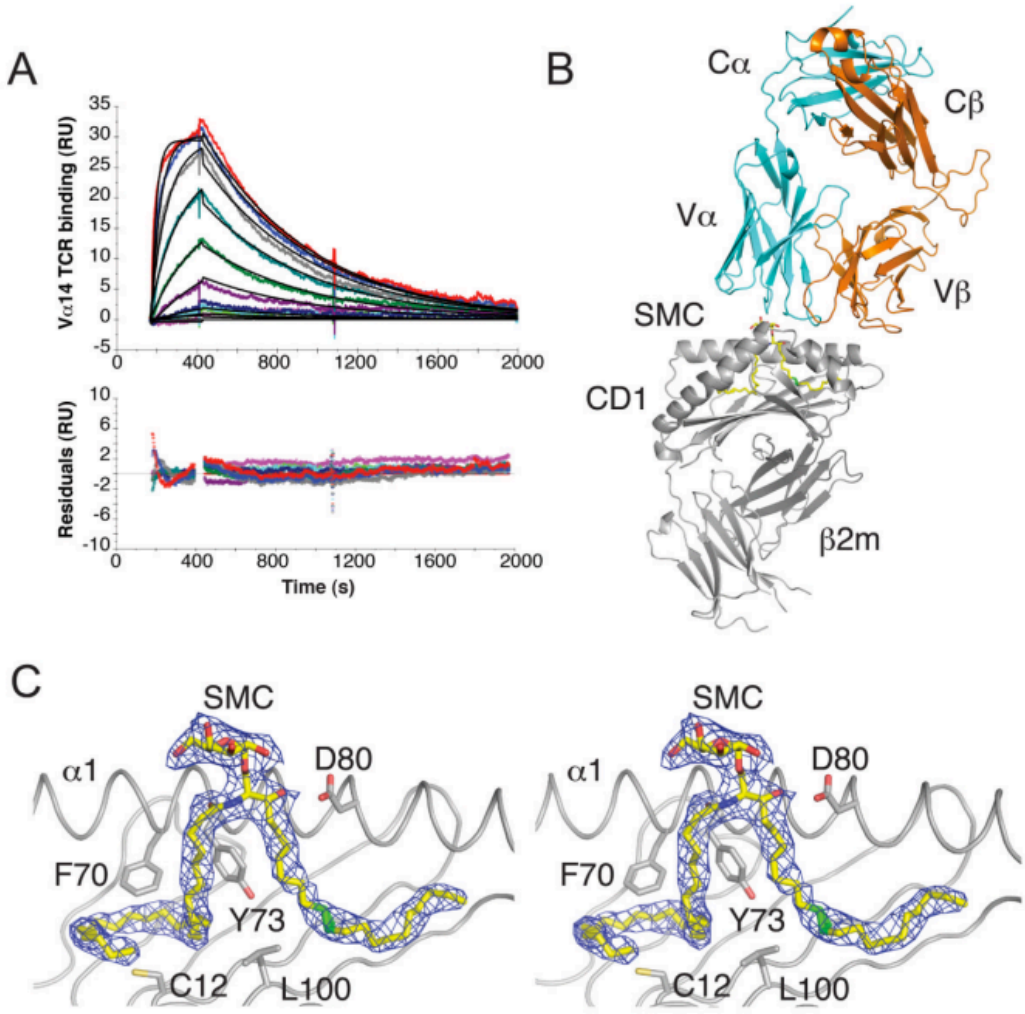


Figure 3.6. Biophysical and structural characterization of the CD1d-SMC124-*i*NKT TCR interaction
(A). Biacore sensorgram showing the binding of increasing concentrations of TCR (0.004 to 1 μM) to mouse CD1d-SMC124. (B). Overall structure of the ternary complex CD1d-SMC124-*i*NKT TCR. SMC124, yellow; CD1d heavy chain and β2-microglobulin chain, gray; TCR α chain, cyan; TCR β chain, orange. (C). Stereo image of the 2*Fo*−*Fc* electron density map of the ligand. The map is contoured at 1 σ and shown as a blue mesh around the ligand (in yellow) in side view with the α2 helix removed for the purpose of clarity. The cyclopropane modification on the sphingoid base is shown in green.

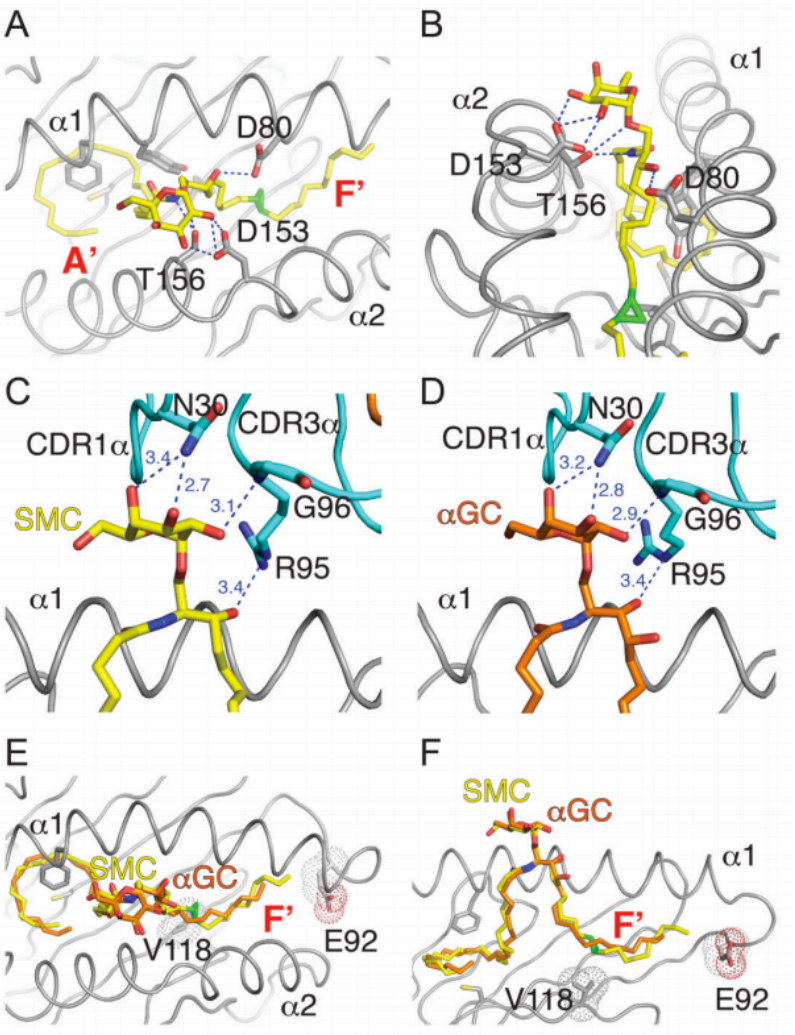


Figure 3.7. Binding of SMC124 to CD1d. Top (A) and side (B) view of the SMC124-CD1d interactions. SMC124 is shown in yellow. The cyclopropane modification on the SMC124 sphingoid base is shown in green. Hydrogen bond interactions between CD1d residues and the polar moieties of SMC124 are indicated with blue dashed lines. (C and D) Contacts between SMC124 (C) and αGalCer (PDB ID 3HE6) (D) with the *i*NKT TCR. The conserved hydrogen bonds with residues on the CDR1α and CDR3α are shown as dashed blue lines with the corresponding distances indicated in Å. SMC124, yellow; αGalCer, orange; CD1d heavy chain, gray; TCR α chain, cyan. (E and F) Conformation of the sphingoid bases of SMC124 and αGalCer in the F' pocket. (E) Top view of the CD1d binding groove. (F) Side view of the binding groove with the α2 helix removed for the purpose of clarity. SMC124 in yellow, αGalCer in orange. Note the more tightly packed conformation of the longer SMC124 sphingoid base in comparison to αGalCer.

3.4 DISCUSSION

The ability to modify or identify *i*NKT cell antigens that instruct either a Th1 or Th2 immune response is not only an area of active investigation, but the development of such selective compounds has potential therapeutic significance. While the prototypical *i*NKT cell ligand, αGalCer, is capable of strongly inducing both Th1 and Th2 cytokines, a few variants have been developed that skew towards either a Th1 or Th2 response (Arora et al., 2011; Aspeslagh et al., 2011; Bendelac et al., 2007; Chang et al., 2007; Im et al., 2009; Li et al., 2009; Li et al., 2010a; Tashiro et al., 2010). To identify unique *i*NKT cell antigens that are capable of providing both a Th1 response and activating human *i*NKT cells, we synthesized lipid variants of αGalCer based on the structure of plakoside A, a naturally occurring glycosphingolipid isolated from the marine sponge *Plakortis simplex.* Our studies show that compounds with either the acyl chain or the sphingoid base similar to plakoside A were capable of activating mouse *i*NKT cells, and although they are less potent than αGalCer, they cause a prolonged, systemic synthesis of IFN-γ *in vivo*, in part by enhanced *trans* activation of natural killer cells. Like the prototypical Th1 skewing antigen C-glycoside, these compounds predominantly are bound to CD1d molecules found in lipid raft domains of the plasma membrane, and they exhibit a prolonged ability to stimulate *i*NKT cells when tested *ex vivo*. The similar properties exhibited by these three compounds suggest there could be general rules for the properties required of Th1 cytokine inducing glycolipid compounds. Unlike C-glycoside, however, these plakoside-based antigens have the ability to stimulate human *i*NKT cells.

In our study, skewing to a Th1 response in vivo was dependent on an increased *trans*-activation of natural killer cells, despite decreased antigenic potency of the plakoside-like antigens early after lipid injection. This downstream activation of natural killer cells has been shown in other contexts to be dependent upon the expression of CD40L by activated *i*NKT cells (Carnaud et al., 1999; Yu and Porcelli, 2005). CD40L interacts with CD40 on DC and other APC, leading to the secretion of IL-12, a cytokine that directly stimulates natural killer cells (Fujii et al., 2007). Similar to these findings, we observe the induction of IL-12 in the serum 6-12 h post injection. The ability of natural killer cells to be trans-activated by Plakoside A analogs and skew a Th1 response required the expression of IL-12Ra on these cells. Additionally, we propose that the events leading to enhanced IFN-γ secretion at 6 h and beyond also require continuing *i*NKT cell activation, which the *ex vivo* analysis suggests would be carried out by APC exposed to the plakoside A analogs.

A prolonged activation of *i*NKT cells may depend on several factors, including differences in intracellular trafficking of the glycolipids, their ability to avoid degradation, and the stability of their binding to CD1d. It has clearly been established that lipid structure can influence the intracellular trafficking of lipids (Mukherjee et al., 1999). Therefore, glycosphingolipids with different ceramide moieties would be differentially exposed to degradative enzymes, which might affect their chemical stability. In fact, C-glycoside was originally designed in an attempt to identify a compound that would be resistant to degradation of the O-glycosidic bond (Schmieg et al., 2003). Furthermore, differential antigenic trafficking based on lipid structure also could lead to loading into CD1d in different compartments of the cell. CD1d molecules recycle between the plasma

membrane and various endosomal compartments, including lysosomes, and they can acquire antigens in intracellular compartments, as well as on the cell surface. This could influence the stability of the lipid/CD1d complex, because of exposure, for example in lysosomes, to lipid exchange and transfer proteins (Bendelac et al., 2007). Furthermore, the endosomal site of antigen loading could determine the ultimate localization of the CD1d/antigen complex to plasma membrane microdomains, and complexes there could be more effective at antigen presentation. Lipids that have shorter hydrophobic chains are not only less prone to localize to late endosomal compartments, but there is evidence that their binding to CD1d is destabilized in the more acidic environment in late endosomes[2]. Interestingly, some compounds that skew a Th1 response tend to have a longer acyl chain or sphingoid base, while truncation of these hydrophobic chains has been shown to skew toward a Th2 response (Chang et al., 2007; Fujio et al., 2006; Goff et al., 2004; Im et al., 2009; Li et al., 2010a; McCarthy et al., 2007; Miyamoto et al., 2001; Oki et al., 2004; Yu et al., 2005).

While lipid trafficking or the site of encounter with CD1d could be important determinants of Th1 cytokine skewing, our structural data suggest the hypothesis that enhanced stability of binding to CD1d also could be important, at least for SMC124, which has a larger area buried in the CD1d groove, is located deeper in the groove, and causes a more ordered conformation of the F' pocket compared to bound αGalCer. Recently, NU-αGalCer, a galactose modified αGalCer analog in which the 6"-OH group is replaced with a naphthylurea substituent, was shown to have additional contacts with the CD1d molecule, which might enhance the stability of binding (Aspeslagh et al., 2011). NU-αGalCer also causes a Th1 pattern of cytokine production, consistent with

the importance of the biochemical stability of the glycolipid CD1d interaction in determining the Th1 cytokine profile.

The synthesis of the plakoside A analogs, SMC124 and EF77, has added to the small number of Th1 biasing glycosphingolipids that are capable of stimulating both mouse and human *i*NKT cells. Furthermore, our mechanistic studies illuminate the importance of the prolonged stimulation required to achieve sustained IFN-γ production *in vivo* and some of the factors required to achieve it. It is remarkable that APC loaded with antigens such as SMC124, whose affinity of the TCR when bound to CD1d is similar compared to αGalCer, have an increased ability to stimulate *i*NKT cells approximately one day after injection. Given this, we consider it likely that multiple factors are contributing to the ability of the plakoside A analogs to accumulate as antigenic CD1d-complexes that can activate *i*NKT cells. Further experiments will be required to determine if these compounds behave similarly in human cells, and if they can, in fact, be developed as effective anti-tumor agents or vaccine adjuvants.

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Chapter 4:

A novel glycolipid antigen for NKT cells

that preferentially induces IFN-γ and IL-10 production

4.1 INTRODUCTION

Type 1 or invariant NKT (*i*NKT) cells are a lymphocyte population that is characterized by features of both the innate and adaptive immune responses. The multiple functionalities of these cells is remarkable in that they have been implicated in allergy, cancer, infection, autoimmunity, and a variety of other conditions (Berzins et al., 2011). Similar to other T lymphocytes, *i*NKT cells arise from a $CD4^+$, $CD8^+$ double positive thymocyte precursor, (Kronenberg and Gapin, 2002; MacDonald and Mycko, 2007; Porubsky et al., 2007; Watarai et al., 2012). Unlike classical T cells, which recognize peptide moieties presented by major histocompatibility complex (MHC)-encoded molecules, *i*NKT cells recognize lipid Ags that are often glycosphingolipids (GSLs). *i*NKT cells recognize these lipid Ags when they are presented by CD1d, a MHC class-I-like molecule (Barral and Brenner, 2007). The CD1d binding groove is composed of two hydrophobic pockets labeled A' and F' (Joyce et al., 2011). GSLs bind with the fatty acid chain localizing into the A' groove and the sphingoid base into the F' groove. This binding mode allows the carbohydrate head group to protrude out of the CD1d molecule such that it is exposed to be recognized by the *i*NKT cell TCR (Borg et al., 2007).

The *i*NKT cell TCR contains a highly restricted, invariant TCR α chain that is formed by a Vα14-Jα18 rearrangement in mice, and a homologous Vα24-Jα18 (TRAV10- TRAJ18) in humans (Lantz and Bendelac, 1994). Although the β chains of

these TCRs is not invariant, they are biased to Vβ8.2, Vβ7 or Vβ2 in mice, and Vβ11 (TRBV25-1) in humans, with diverse CDR3 regions. The prototypical GSL recognized by *i*NKT cells is alpha galactosylceramide (αGalCer) (Morita et al., 1995; Spada et al., 1998).

When stimulated by a strong agonist, such as the well-characterized αGalCer, *i*NKT cells secrete both Th1 and Th2 cytokines, such as IFN-γ and IL-4 (Bendelac et al., 2007). Stimulation with αGalCer causes long-term changes in the *i*NKT cell population that have been originally likened to anergy (Parekh et al., 2005; Sullivan and Kronenberg, 2005). However, we recently showed that stimulation with αGalCer leads to an expansion of an *i*NKT cell population capable of secreting IL-10, known as NKT10 cells (Sag et al., 2014). Interestingly, subtle chemical or structural alterations in αGalCer have been shown to alter the down-stream cytokine response, skewing it towards either a Th1 or a Th2 phenotype (Sullivan et al., 2010). Generation of an Ag capable of stimulating a strong Th1 cytokine profile has been an area of great interest, because this is beneficial for stimulating for anti-cancer responses and for use as a vaccine adjuvant. Based on prior work with αGalCer, the heightened IFN-γ response it causes is due, in large part, to the *trans*-activation of NK cells (Smyth et al., 2002; Yu and Porcelli, 2005; Brennan et al., 2013). Therefore, the Th1 response induced by this and some other GSL Ags represents not so much the tendency of an *i*NKT cell to produce IFN-γ with reduced IL-4, but represents the output of a cellular network that involves dendritic cells (DCs) expressing CD1d*, i*NKT cells to activate the response, and NK cells that are stimulated downstream of *i*NKT cells and that are crucial for continued IFN-γ release. Although αGalCer has been shown to suppress tumor metastases in mouse models (Schneiders et al., 2011), it

has not been overwhelmingly successful in human trials, possibly due in part to the mixed Th1 and Th2 response or the anergy it induces (Terabe and Berzofsky, 2008) or due to the potential induction of NKT10 cells (14). Because of this, many analogs of αGalCer have been generated in attempts to elicit a more pronounced Th1 skewed response. C-glycoside, which differs from αGalCer by replacing the carbon-oxygen glycosidic linkage with a carbon-carbon bond, was the first GSL Ag reported to have a Th1 polarizing potential (Patel et al., 2011). Although the initial findings were promising, C-glycoside cannot stimulate human *i*NKT cells (Sullivan et al., 2010) and can therefore not be used for therapeutic applications.

Here, we characterized in detail the biochemical properties, immune responses and mechanisms of action of a novel αGalCer analog, DB06-1. This compound is identical to αGalCer except for the replacement of the C2 carbonyl oxygen on the acyl chain replaced with a sulfur atom. DB06-1 was identified in a screen of lipids that associated with detergent resistant membrane domains, which is characteristic for other Th1 skewing compounds tested to date (Arora et al., 2011; Yu et al., 2005). Early after injection, DB06-1 promoted a Th1 skewed response that was more prominent than αGalCer. However, over the longer term DB06-1 induced more NKT10 cells than αGalCer.

4.2 MATERIALS AND METHODS

4.2.1 Statistical tests:

Unless otherwise noted, statistical comparisons were drawn with a 2-tailed Student T-tests were used. The symbols used in the figures are as follows: ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

4.2.2 Cell-free antigen presentation assay

Stimulation of *i*NKT cell hybridomas on microwell plates coated with soluble mouse CD1d was carried out according to published protocols (Naidenko et al., 1999; Sidobre et al., 2004; Tupin and Kronenberg, 2006). The indicated amounts of compounds or vehicle were incubated for 24 h in microwells that had been coated with 1.0 μg of CD1d. After washing, 5×10^4 *i*NKT hybridoma cells were cultured in the plate for 24 h, and IL-2 in the supernatant was measured by sandwich ELISA (R&D Systems) following the manufacturer's instructions. The DN3A4-1.2 (1.2) and DN3A4-1.4 (1.4) Vα14 *i*NKT cell hybridomas have been described previously (Brossay et al., 1998).

4.2.3 Antigen presentation assays

The GM-CSF bone-marrow derived DC culture has been described previously (Inaba et al., 2009). Briefly, cells were isolated from mouse femurs and were cultured in media containing GM-CSF for 7 days. The cells were then pulsed with GSL Ags overnight and were incubated with 5×10^4 *i*NKT hybridoma cells for 20–24 h. Similarly, the antigen presentation assay using A20 B lymphoma cells has been described (Lawton et al., 2005). Briefly, A20-CD1d transfectants expressing wild-type CD1d (WT) or tail deleted CD1d (TD) were pulsed with the indicated GSL Ags overnight. APCs (1×10^5 per well) were incubated with 5×10^4 *i*NKT hybridomas for 20–24 h. The DN3A4-1.2 (1.2)

Vα14 *i*NKT cell hybridoma used in both assays has been described previously (Brossay et al., 1998). IL-2 in the supernatant of hybridoma cultures was measured by sandwich ELISA (R&D Systems).

4.2.4 Generation and analysis of human *i*NKT cell lines

Human Vα24$^+$ *i*NKT cells were purified by magnetic enrichment and expanded according to a previously published protocol (Rogers et al., 2004). Briefly, PMBCs were isolated by Percoll (Sigma) density-gradient centrifugation. Human donor PBMCs (1–1.5 $\times 10^6$/ml) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) Pen-Strep-Glutamine (10,000 U/ml penicillin, 10,000 g/ml streptomycin, 29.2 mg/ml L-glutamine; Invitrogen). Human *i*NKT cell cultures were expanded by weekly re-stimulation with αGalCer -pulsed, irradiated PBMC and recombinant human IL-2. Ag-pulsed PBMCs (1×10^5/ well) were seeded in 96 well plates and cultured in the presence of 5×10^4 Vα24$^+$ human *i*NKT cells for 20–24 h. GM-CSF release, as a marker of *i*NKT cell activation, was measured by sandwich ELISA (R&D Systems).

4.2.5 Mice

C57BL/6 mice were purchased from The Jackson Laboratory. CD1-TD mice were generously provided by the laboratory of Dr. Albert Bendelac (Chiu et al., 2001). *Cd1d*$^{f/f}$ mice were generated in the laboratory using conventional strategies and were crossed with a CD11c$^+$ Cre transgenic line (Caton et al., 2007) obtained from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions and the experiments were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology. Mice were injected with 1-4 μg of lipids intravenously, αGalCer provided a positive control. Standard sandwich ELISAs (R&D

Systems) were performed to measure mouse IFN-γ, IL-12p70, and IL-4 in the sera.

4.2.6 Cell preparation

Single cell suspensions of splenocytes were generated as described previously (Tyznik et al., 2008). For DCs isolation, the tissue was diced into 1 mm pieces, digested using spleen dissociation media (Stem Cell Technologies) and DCs were enriched by positive selection using a $CD11c^+$ isolation kit with RoboSep technology (Stem Cell Technologies) or MACS Technology according to the manufacturer's protocols (Miltenyi Biotec). Isolated DCs were co-cultured at varying concentrations with 1.2 *i*NKT cell hybridomas overnight and activation was measured by sandwich ELISA of culture supernatants for IL-2.

4.2.7 Flow cytometry and intracellular cytokine staining

Lymphocytes were isolated from mouse spleens and cultured in media containing consisting of RPMI 1640 (Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) Pen-Strep-Glutamine (10,000 U/ml penicillin, 10,000 g/ml streptomycin, 29.2 mg/ml L-glutamine; Invitrogen) in the presence of GolgiPlug and GolgiStop (BD Biosciences) at 37°C for 4 hours. The cells were then washed and stained with Live/dead aqua, αGalCer/CD1d tetramers labeled with the fluorochrome BV421 (BD Biosciences) (generated in our laboratory) (Matsuda et al., 2000), CD45R/B220 (BD Biosciences), NK1.1 (eBiosciences), CD8 (BD Biosciences), TCRβ (Biolegend), and CD3ε (Biolegend) antibodies. For intracellular cytokine staining, the cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and stained for intracellular IFN-γ (eBioscience) to measure NK cell activation. To measure *i*NKT cell IL-10 (BD Biosciences), splenocytes were purified by use of Lymphoprep (Axis-Shield,

Oslo, Norway) density gradient centrifugation before the cells were re-stimulated with PMA and ionomycin (both from Sigma-Aldrich) for 4 hours at 37°C in the presence of GolgiPlug and GolgiStop. The data were collected on a LSR II or Fortessa flow cytometers (BD Biosciences) and analyzed using FlowJo software (Tree Star).

4.2.8 NK Cell depletion

C57BL/6 mice were depleted of NK cells by injecting 50 μl anti-asialo GM1 rabbit polyclonal Ab (Wako) 24 h prior to GSL challenge. NK cell depletion ($NK1.1^+$ $TCR\beta^-$ cells) was verified by flow cytometry.

4.2.9 Mouse CD1d expression, purification and Vα14-Vβ8.2 TCR refolding

Mouse CD1d-β2-microglobulin heterodimeric protein was expressed in a baculovirus expression system as reported previously (Zajonc et al., 2005). Human CD1d- β2-microglobulin was prepared analogous to the mouse protein. The TCR construct design, refolding and purification processes were identical to the ones previously reported (Wang et al., 2010), while the human TCR 4C1369 (Matulis et al., 2010) was generously provided by Dr. Rossjohn and prepared as reported (Gadola, 2006).

4.2.10 Glycolipid loading and DB06-1-CD1d-TCR complex formation

The DB06-1 lipid synthesized in the laboratory of Gurdyal S. Besra, was dissolved in DMSO at 1mg/ml. Before loading, 25 μl was diluted to 0.25 mg/ml with 25 μl vehicle solution (50 mM Tris-HCl pH 7.0, 4.8 mg/ml sucrose, 0.5 mg/ml sodium deoxycholate and 0.022% Tween 20) and 50 μl 1% Tween 20 and incubated at 80°C for 20 min. DB06-1 was loaded onto CD1d overnight (molar ratio of protein to lipid of 1:3) in the presence of 10 mM Tris-HCl pH 7.0. Refolded TCR was incubated at room

temperature for 1 h with lipid-loaded CD1d at a 1:2 molar ratio and the ternary CD1d-lipid-TCR complex was isolated from uncomplexed CD1d and TCR by size exclusion chromatography using Superdex S200 10/300 GL (GE Healthcare).

4.2.11 Surface plasmon resonance binding analysis

Surface plasmon resonance (SPR) binding studies were conducted using a Biacore 3000 as reported previously (Wang et al., 2010). Briefly, approximately 300 response units of biotinylated CD1d (either human or mouse) loaded with DB06-1 were immobilized onto a streptavidin sensor chip (GE Healthcare) surface by injecting the CD1d-DB06-1 mixture at 3 μl/min in Hepes buffered saline (HBS) running buffer. A reference surface was generated in another flow channel with unloaded CD1d. Experiments were carried out at 25°C with a flow rate of 30 μl/min and were performed at least twice. Kinetic parameters for the mouse molecule interactions were calculated after subtracting the response to CD1d molecules in the reference channel, using a simple Langmuir 1:1 model in the BIAevaluation software version 4.1. One representative sensorgram is shown. Human kinetic parameters were obtained using steady state solution graphs plotting T_{eq} vs concentration and were fitted with binding response at equilibrium BIAevaluation software version 4.1.

4.2.12 Crystallization and Structure determination

The mouse CD1d-DB06-1-TCR complex was isolated by Superdex S200 10/300 GL (GE Healthcare) column chromatography in 50 mM Hepes pH 7.4, 150 mM NaCl and concentrated to 0.86 mg/mL. Crystals were grown at 22.3°C by sitting drop vapor diffusion while mixing 2 μl protein with 2 μl precipitate (0.2M ammonium citrate dibasic pH 4.98 20% PEG 4000). Crystals were then flash-cooled at 100°K in mother liquor

containing 20% glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Laboratory beamline 9.2 and processed with the software Mosflm (Leslie, 2006) . The CD1d-DB06-1-TCR crystallized in space group $C222_1$.The structure was solved by molecular replacement in CCP4 (Collaborative Computational Project, Number 4) (1994) using the protein coordinates from the CD1d-iGb3 structure as the search model [PDB code 2Q7Y] (Zajonc et al., 2008) as the search model followed by the *i*NKT cell TCR [PDB code 3QUZ] (Aspeslagh et al., 2011). The model was rebuilt into σA-weighted 2*Fo – Fc* and *Fo – Fc* difference electron density maps using the program COOT (Emsley et al., 2010). The lipid was built into 2*Fo –Fc* map and refined using REFMAC (1994). The final refinement steps were performed using the TLS procedure in REFMAC with five domains (α1-α2 domain including carbohydrates and glycolipid, α3-domain, β2m, variable domain and constant domain of TCR). The CD1d-DB06-1-TCR structure was refined to 2.83 Å to an Rcryst and Rfree of 20.9% and 25.6% respectively. The quality of the model was excellent as assessed with the program Molprobity (Lovell et al., 2003). Crystal statistics are listed in Table 4.S1.

4.3 RESULTS

4.3.1 DB06-1 is a Th1 skewing *i*NKT cell agonist

The chemical structure of DB06-1 compared to αGalCer, is shown in Figure 4.1A. DB06-1 is identical to αGalCer except the replacement of the C2 carbonyl oxygen on the acyl chain for a sulfur atom. We used several assays to measure the antigenic potency of this compound. Initially, we tested DB06-1 in a cell-free antigen presentation assay, whereby a soluble CD1d molecule was coated on a plate, GSL Ags were added, and then the IL-2 release from an *i*NKT cell hybridoma was used to determine if the lipid could

activate the *i*NKT cell TCR. DB06-1 only weakly stimulated the *i*NKT cell hybridoma compared to αGalCer (Figure 4.1B). We also used a cell-based antigen presentation assay, with bone marrow derived DCs as the APC. This a more physiologically relevant experimental setup as it allows for the endolysosomal loading of GSL Ags into CD1d. In this experimental set-up, DB06-1 was a more effective Ag, although it remained weaker in comparison to αGalCer (Figure 4.1C).

As noted in the Introduction, some lipids that activate mouse *i*NKT cells do not stimulate their human counterparts, and therefore such compounds in this category are not relevant for the development of immune therapies. However, DB06-1 efficiently activated two distinct human *i*NKT cell lines cultured with CD1d expressing APC (Figure 4.1D), however, demonstrating its effectiveness in both species.

Th1 cytokine skewing following GSL stimulation is believed to be the product of a cellular network and responses that occur within the first 24 h (Brennan et al., 2013). We therefore analyzed the *in vivo* response to DB06-1 by measuring the concentration of cytokines in the sera of mice 2 and 22 h after injection. Previous results (Arora et al., 2011), showed that DB06-1 can induce a robust serum IFN-γ *in vivo*. The initial IFN-γ response induced by DB06-1 after 2h was higher than that induced by αGalCer (Figure 4.2A). The production of IFN-γ at 22 h after GSL injection has been attributed to the *trans*-activation of NK cells, due in part to IL-12 production from APCs (Carnaud et al., 1999; Kawakami et al., 2001; Kitamura et al., 1999; Parekh et al., 2004). We noted that serum IL-12 levels from mice injected with DB06-1 at 6 h post injection were higher than mice injected with αGalCer (Figure 4.2B). To measure the *trans*-activation directly, we analyzed IFN-γ production of splenic NK cells ($NK1.1^+$ $TCR\beta^-$) cells from mice injected

with DB06-1 24 h prior. After a 4 h stimulation, splenic NK cells were identified by flow cytometry and intracellular IFN-γ was measured (Figure 4.2C). NK cells of mice injected with DB06-1 produced more IFN-γ than NK cells from αGalCer injected mice (Figure 4.2C). To determine that the sera IFN-γ production at this 24h time point was indeed due to NK cell *trans*-activation, we repeated the experiment in mice depleted specifically of NK cells with anti-asialo-GM1 antibodies (Tyznik et al., 2014) (Figure 4.S2). As expected, the serum IFN-γ levels at 24 h after DB06-1 injection were significantly lower in these NK cell depleted mice than in control mice (Figure 4.2D). Therefore we conclude that DB06-1 acts like other previously described Th1 skewing GSL Ags, by stimulating *i*NKT cells to elicit increased secretion of IL-12 from APCs that leads to increased IFN-γ secretion from NK cells.

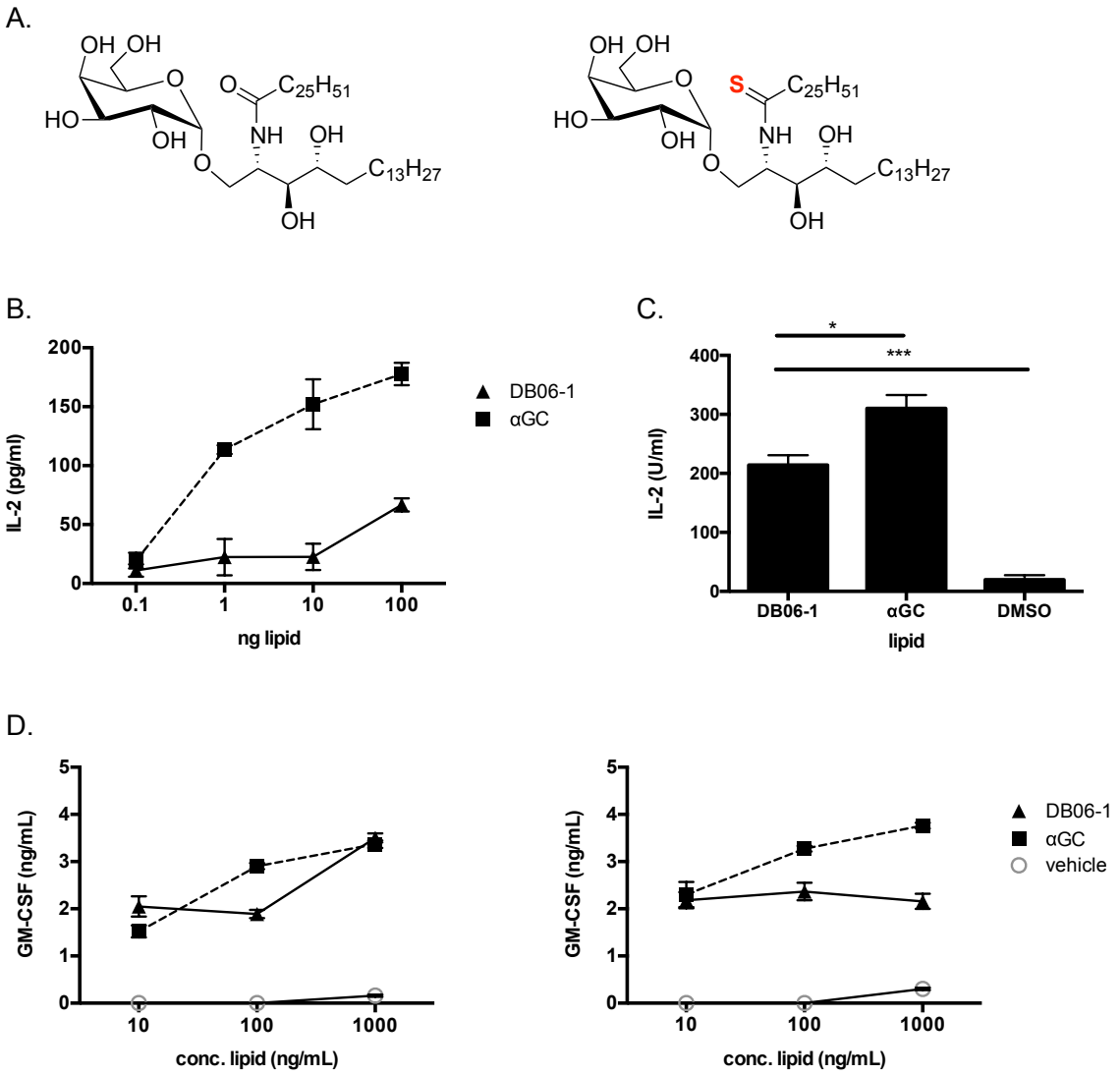


Figure 4.1. DB06-1 is recognized by *i*NKT cells. A.) Chemical structures of αGalCer (αGC) and DB06-1 B.) Cell-free, CD1d coated plate assay. Data are representative of two independent experiments. Error bars represent ± SEM of three wells per condition. C.) Bone marrow DC presentation assay using 100 ng/mL lipid. Data are representative of two independent experiments. Error bars represent ± SEM of three wells per condition. D.) Two human *i*NKT cell lines, stimulated with the indicated Ag concentrations. Data are representative of two independent experiments. Error bars represent ± SEM of three wells per condition.

4.3.2 DB06-1 has prolonged biological stability and is presented by DCs

A previous study has indicated that $CD8\alpha^+$ $CD11c^+$ DCs are the dominant antigen presenting cell type essential for activation of *i*NKT cells by injected GSL Ags (Arora et al., 2014), although, in some circumstances, macrophages have been shown to be important too, especially for Ags that are in particulate form (Barral et al., 2010). To determine if DCs were essential for the presentation of DB06-1, we generated a mouse strain with floxed CD1d alleles (*$Cd1d^{f/f}$ mice*) and crossed this line with a *CD11c-Cre* transgenic mouse strain ($Cd1d^{f/f}$ Cre^+ mice), thereby deleting CD1d expression on $CD11c^+$ cells, including most DCs (Figure 4.3A). When such $Cd1d^{f/f}$ Cre^+ mice were with DB06-1 we observed a significant decrease in the amount of IFN-γ levels in the serum at 24 h (Figure 4.3B). However, as IFN-γ production was not completely absent, these data suggest that $CD11c^+$ DCs may not be the sole population capable of presenting DB06-1 to *i*NKT cells *in vivo*. IL-4 levels at 2 h were also determined and were found to be decreased in $Cd1d^{f/f}$ Cre^+ mice as well (Figure 4.S1). Therefore, we conclude that $CD11c^+$ DCs likely are important for DB06-1 presentation *in vivo*, although the participation of other cell types is possible.

We previously found that a common feature of several Th1 cytokine skewing αGalCer analogs is that they persist longer as complexes with CD1d on the surface of APCs *in vivo* (Tyznik et al., 2011). This was analyzed by injecting mice with GSL Ags, isolating APCs from the mice at various time points and using these APCs cells to stimulate *i*NKT cell hybridomas *in vitro* (Sullivan et al., 2010; Tyznik et al., 2011). The Th1 skewing lipids that have been analyzed in this way showed an increased ability to activate *i*NKT cell hybridomas at 24 h compared to αGalCer. In accordance with these

studies, we observed that APCs purified 24 h after Ag injection were indeed better able to activate *i*NKT cell hybridomas *in vitro* when they had been exposed *in vivo* to DB06-1 than to αGalCer (Figure 4.3D). Unlike the previous studies, however, even at 2 h after Ag injection the presentation of DB06-1 by APC induced clearly stronger *i*NKT cell responses *in vitro* than αGalCer (Figure 4.3C). This suggests that DB06-1 is capable of loading faster *in vivo* compared to αGalCer, in addition to the greater stability it manifested over 24h when DCs were analyzed *ex vivo*.

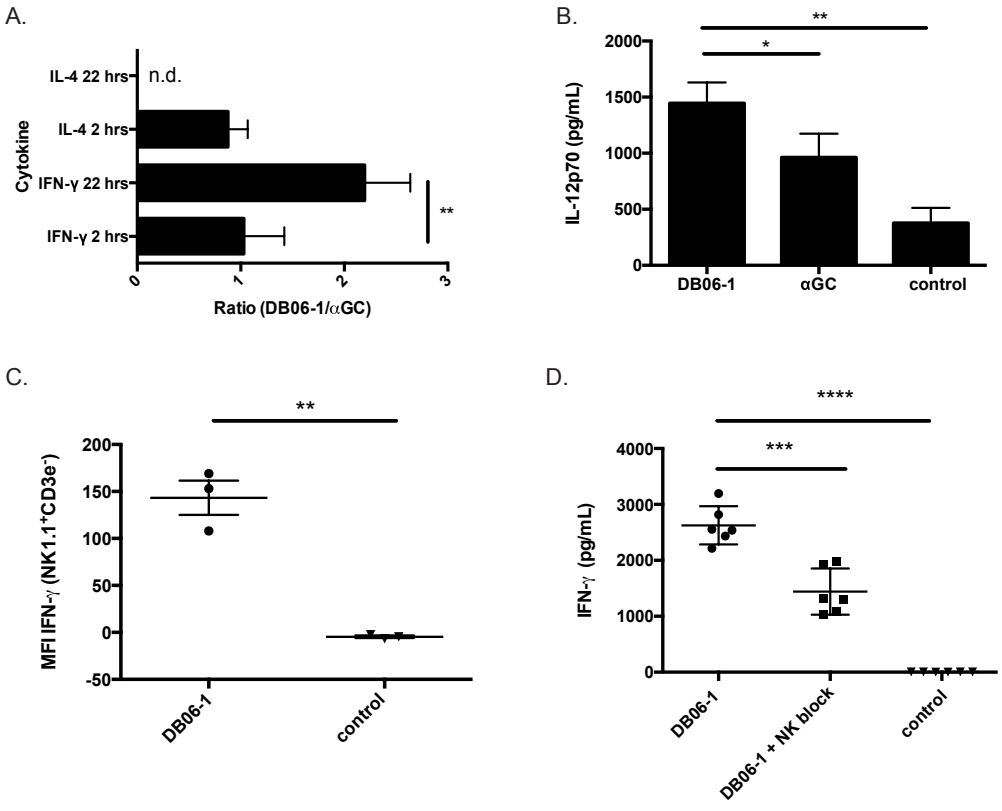


Figure 4.2. DB06-1 is a Th1 cytokine skewing lipid. A.) Ratio of serum cytokine concentrations (DB06-1/ αGalCer) from C57BL/6 mice after i.v. injection of 1 μg lipid. Data are representative of four independent experiments. Error bars represent ± SEM of at least three mice per condition, n.d. indicates not detectable. B.) IL-12p70 measured by ELISA in sera from C57BL/6 mice injected i.v. with 1 μg of indicated GSL Ag 6 h earlier. Data are representative of two independent experiments. Error bars represent ± SEM of at least three mice per condition. C.) NK cell IFN-γ production in C57BL/6 mice injected iv with 1 μg DB06-1. Splenocytes from DB06-1 injected mice and uninjected controls were isolated and cultured in Brefeldin A for 4 h. and gated NK cells ($NK1.1^+ TCR\beta^-$) were analyzed by ICCS. The total IFN-γ MFI of NK cells is plotted. Data are representative of two independent experiments. Error bars represent ± SEM of at least two mice per condition. D.) C57BL/6 mice depleted of NK cells with α-asiolo-GM1-Ab (NK block) were compared to controls for IFN-γ in the sera at 24 h post injection of DB06-1. Data are representative of two independent experiments. Error bars represent ± SEM of at least three mice per condition.

4.3.3 CD1d recycling is required for DB06-1 presentation

Previous data indicated that CD1d-presentation of Th1 skewing lipid Ags is augmented by trafficking of the CD1d molecules through endosomal compartments. Furthermore, CD1d molecules presenting Th1 skewing Ags are preferentially associated with lipid rafts (Arora et al., 2011). CD1d contains a tyrosine motif in its cytoplasmic tail that allows trafficking to endosomal compartments. To study the role of endosome trafficking in the presentation of DB06-1, we used transfected B cell lines that either expressed (CD1d-WT A20) or lacked (CD1d-TD A20) this tyrosine motif in CD1d. As reference ligand we used galactosyl (α1-2) galactosyl ceramide (GGC) that is known to require lysosomal processing to cleave the terminal sugar to yield active αGalCer for presentation to *i*NKT cells (Sagiv et al., 2007). Like GGC, DB06-1 presentation to an *i*NKT cell hybridoma was greatly reduced when CD1d-TD A20 cells were used compared to the CD1d-WT A20 cells (Figure 4.4A). In order to confirm that this recycling was also important *in vivo*, we used a mouse strain that lacked the tyrosine motif of the *cd1d* gene (CD1-TD). Although surface expression of CD1d is higher on APCs from CD1-TD mice compared to control mice, *i*NKT cells do not develop in CD1d-TD mice (Chiu et al., 2001). To study the antigen-presenting capability in these mice, we injected CD1-TD and control mice with DB06-1, isolated splenic $CD11c^+$ cells 24 h post injection, and used the DCs to stimulate an *i*NKT cell hybridoma *in vitro*. Weaker stimulation of *i*NKT cells was observed after interaction with DB06-1 exposed DCs derived from CD1d-TD mice compared to control DCs (Figure 4.4B). Together, these data demonstrate that DB06-1 is best presented when CD1d traffics normally through late endosomal compartments.

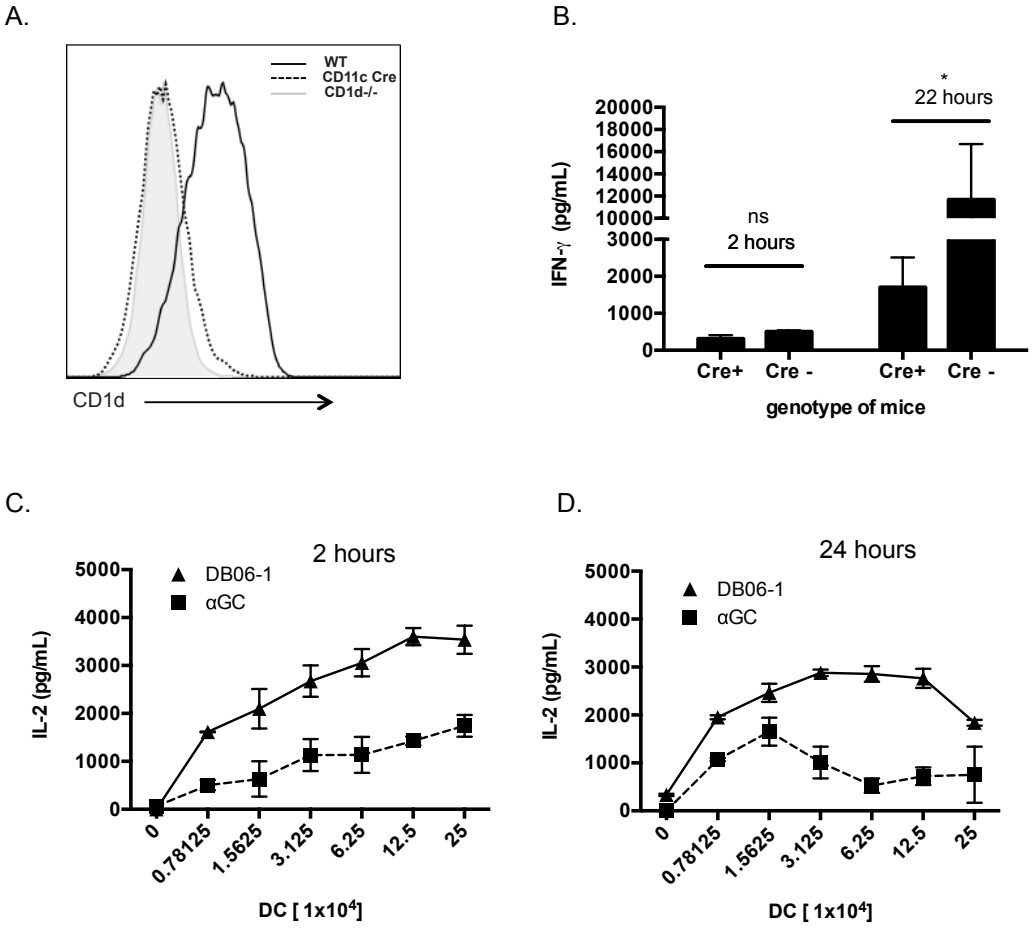


Figure 4.3. DC presentation of DB06-1. A.) CD1d deletion in $CD11c^+$ cells in $Cd1d^{f/f}$ CD11c Cre mice. Analysis of gated live cells that were $B220^-$, $TCR\beta^-$, $CD11c^+$ cells is shown. B.) $Cd1d^{f/f}$ mice ± CD11c Cre were injected with 1 μg DB06-1 and were bled at 2 and 22 h. Serum IFN-γ was measured by ELISA. Data are representative of two independent experiments. Error bars represent ± SEM of at least two mice per condition. C, D.) C57BL/6 mice were injected with 1 μg of the indicated glycolipid and $CD11c^+$ splenic DCs were enriched using magnetic bead isolation at 2 h (C.) and 24 h (D.) post injection. Indicated numbers of enriched DCs were cultured with the 1.2 *i*NKT cell hybridoma overnight and IL-2 in the supernatant was measured by ELISA. Data are plotted as the number of enriched DCs per well versus IL-2 (pg/ml). Data are representative of three independent experiments. Error bars represent ± SEM of three mice per condition.

4.3.4 The *i*NKT cell TCR has a high affinity for the CD1d-DB06-1 complex

One hypothesis for the ability of Ags to induce a Th1-skewed response *in vivo* that has been put forward is that those Ags may have an increased affinity for the *i*NKT TCR when bound to CD1d (Oki et al., 2004). Equilibrium binding analysis using SPR demonstrated a binding affinity (K_D) of the mouse Vα14Vβ8.2 *i*NKT cell TCR for mouse CD1d-DB06-1 complexes of 56 ± 6 nM (Figure 4.5A), approximately 2-fold weaker than the binding affinity observed with αGalCer loaded CD1d (24 nM) (data not shown). The association rate (k_a =5.2 ± 0.7 $10^4 M^{-1}$ s^{-1}) and dissociation rate (k_d=2.9 ± 0.7 10^{-3} M^{-1} s^{-1}) were comparable to the rates for αGalCer (k_a = 7.84 10^4 M^{-1} s^{-1} and k_d = 1.61 10^{-3} s^{-1}, respectively; data not shown).

We also analyzed the affinity of a human *i*NKT cell TCR for the human CD1d-DB06-1 complex (Figure 4.5B). Using steady state kinetic modeling, whereby we plotted the residual units of the sensorgram against the concentration of the TCR and calculated a line of best fit (Figure 4.5C), we determined that the affinity of the human TCR for the CD1d-DB06-1 complex (0.485 μM ± 0.265) was again approximately 2-fold weaker than the CD1d-αGalCer complex (0.22 μM ± 0.11). Therefore, we conclude that although the *i*NKT cell TCR has a high affinity for the CD1d-DB06-1 complex, the interaction with the CD1d- αGalCer complexes is even stronger. These results are consistent with other data demonstrating that increased TCR affinity cannot explain increased Th1 cytokine release resulting from *i*NKT cell stimulation.

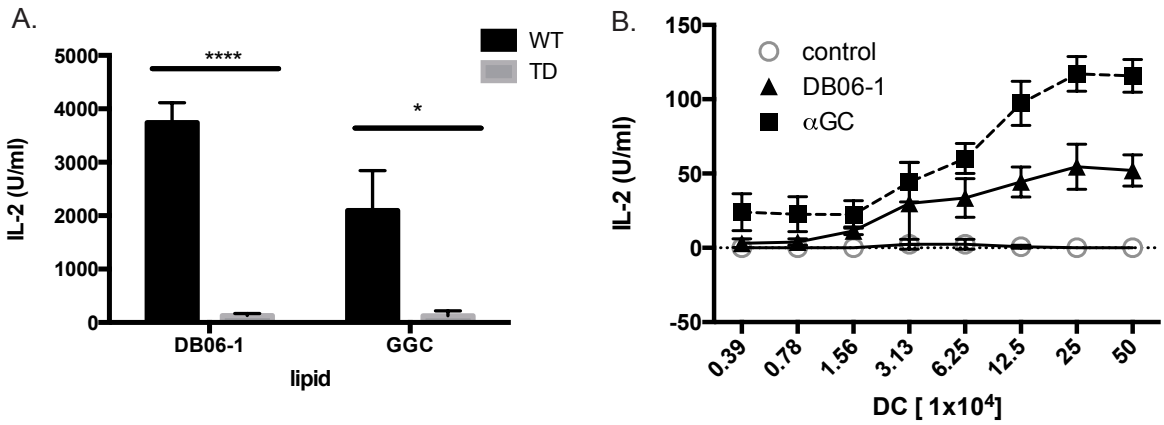


Figure 4.4. Endolysosomal localization of CD1d is required for presentation of DB06-1. A.) A20 cells expressing wild-type (WT) or tail-deleted (TD) CD1d were pulsed with 0.1 ng DB06-1 or 100 ng GGC overnight. Cells were then cultured with the 1.2 *i*NKT cell hybridoma overnight and IL-2 in the supernatant was quantified by ELISA. Data are representative of two independent experiments. Error bars represent ± SEM of three wells per condition. B.) Mice with the WT CD1d gene replaced with a tail-deleted CD1d (TD) gene were injected i.v. with 1 μg of the indicated lipid antigens and at 22 h splenic $CD11c^+$ DCs were enriched by magnetic bead isolation and varying concentrations were co-cultured with 1.2 *i*NKT cell hybridoma. Data plotted as the number of enriched DCs per well versus IL-2 (U/mL). Data are representative of two independent experiments. Error bars represent ± SEM of three mice per condition.

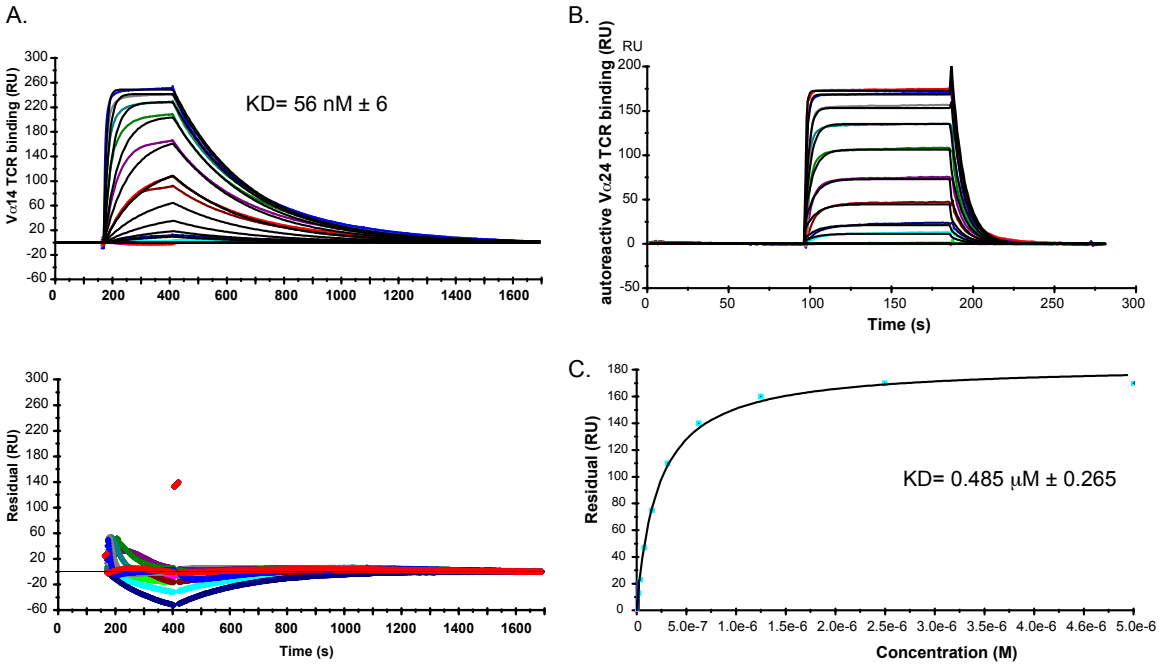


Figure 4.5. TCR binding to CD1d-DB06-1 complexes. A.) Biacore SPR sensorgram showing the binding of increasing concentrations of a mouse *i*NKT cell TCR (0.004 to 2 μM) to complexes of mouse CD1d-DB06-1. Data are representative of three independent experiments. B.) Binding of a human Vα24 *i*NKT cell TCR (0.2 to 5 μM) to human CD1d-DB06-1 complexes as assessed by SPR. Data are representative of three independent experiments. C.) Saturation plot demonstrating equilibrium binding of the human *i*NKT cell TCR to immobilized CD1d-DB06-1.

4.3.5 Structure of the mouse TCR-DB06-1-CD1d ternary complex

In order to characterize the biochemical features of the binding of the DB06-1 GSL to CD1d and the *i*NKT TCR, we determined the structure of the ternary complex by X-ray crystallography (Figure 4.6A). The complex crystallized in the space group $C222_1$, with one complex in the asymmetric unit. The binding orientation of the *i*NKT cell TCR is consistent with the conserved parallel docking mode previously described (Rossjohn et al., 2012). The TCR docks over the CD1d antigen binding groove in an orientation that is markedly different than the diagonal binding footprint seen in MHC-peptide-TCR complexes. As previously shown with models using the same TCR construct (Tyznik et al., 2011), the TCR interacts with CD1d using amino acids in the TCR CDR3α and CDR2β loops as well as a CDR3β-dependent contact (Figure 4.6B). The same previously seen polar contacts are also formed between the α chain (N30, R95, G96) of the *i*NKT TCR and the DB06-1 Ag (Figure 4.6B). There is well-defined density for the DB06-1 ligand (Figure 4.6C), with the galactose head group exposed to recognition by the *i*NKT cell TCR. Polar contacts between the GSL Ag and amino acids E80, E153, and T156 of CD1d are virtually identical to the interactions of CD1d with αGalCer. The binding of orientation of DB06-1 within the hydrophobic A' and F' grooves of CD1d is also conserved, with the acyl chain localizing to the A' groove and the sphingoid base to the F' groove, with minimal to no differences when superimposed on the CD1d/αGalCer structure (data not shown). Because the two molecules are chemically similar in these regions, this similarity was expected. DB06-1 differs from αGalCer by the replacement of the carbonyl group with a thioamide. The sulfur atom, which is 50% bigger than the oxygen atom present in αGalCer (Figure 4.6D) took up more space in CD1d (Figure

4.6E), possibly forming more intimate contacts with the surrounding CD1d residues.

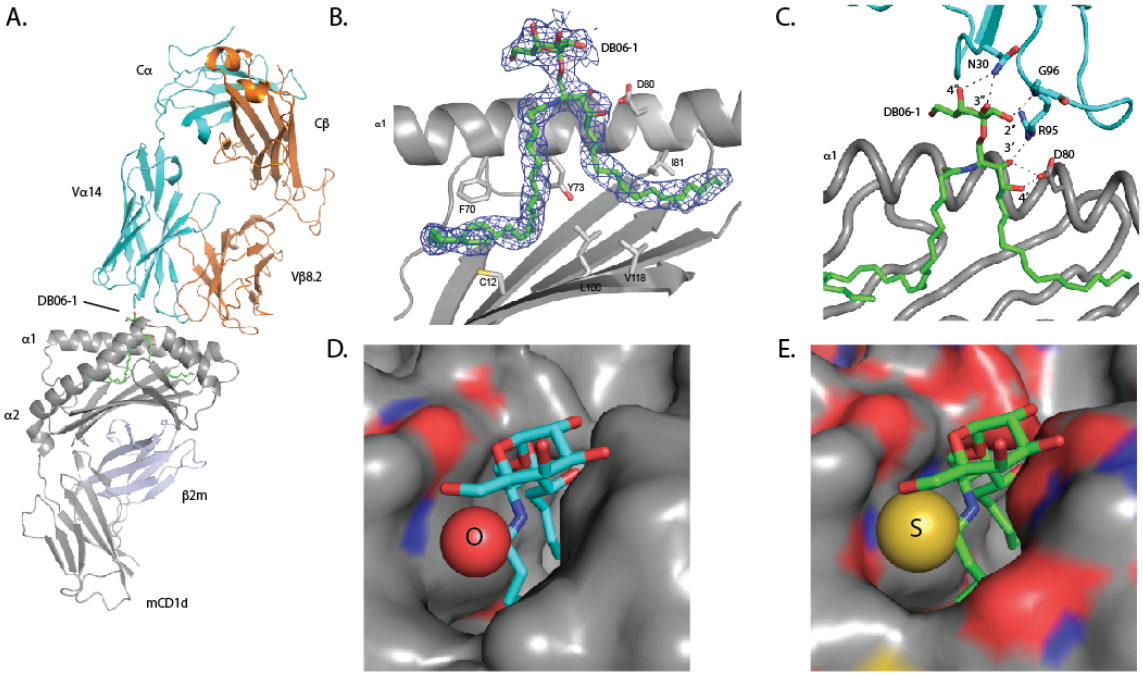


Figure 4.6. Crystal structure of the mouse CD1d-DB06-1-Vα14Vβ8.2 TCR ternary complex. A.) Overview of the ternary complex. B.) TCR/glycolipid Ag contacts. C.) Final $2F_0$-F_c electron density map as shown as blue mesh, contoured at 1.0 σ level for the DB06-1 glycolipid. Note that the α2-helix is removed for clarity. D, E.) CD1d surface showing fit of αGalCer with oxygen highlighted (D) and DB06-1 with the sulfur and local amino acids highlighted (E).

4.3.6 DB06-1 induces IL-10

Recent studies have identified an *i*NKT cell subset, which we called NKT10 cells, that produces IL-10 and which may have regulatory function (Sag et al., 2014; Lynch et al., 2015) Previous experiments demonstrated that *i*NKT cells exposed to αGalCer *in vivo* were more capable of producing IL-10 when re-stimulated even weeks to months later. In order to compare a strongly Th-1 biasing GSL Ag to αGalCer for the induction NKT10 cells, we injected mice with DB06-1 or αGalCer, and four weeks later measured the capacity for splenic *i*NKT cells to produce IL-10 following a brief stimulation *in vitro* with PMA and ionomycin followed by intracellular cytokine staining. Remarkably, the frequency of IL-10+ *i*NKT cells one month after DB06-1 immunization was significantly larger than after αGalCer immunization (Figure 4.7). A similar enhancement of IL-10 production was observed after immunization with DB06-1 when the *i*NKT cells were re-stimulated with Ag one month later (manuscript in publication). These data demonstrate that DB06-1 is superior to αGalCer at inducing/expanding NKT10 cells *in vivo*.

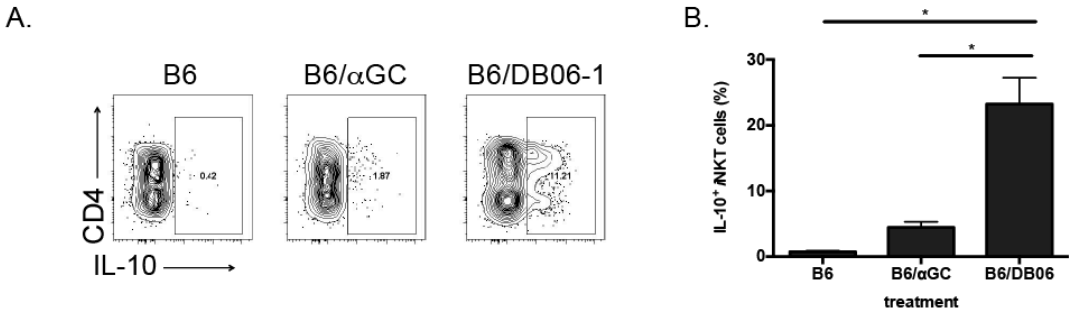


Figure 4.7. Increased IL-10 induced by DB06-1. A, B.) IL-10 production as measured by intracellular staining of gated splenic *i*NKT cells from C57BL/6 mice injected with 4 μg of indicated Ag one month prior. Cells were re-stimulated with PMA and ionomycin *in vitro* as described in Materials and Methods. A) Representative flow cytometry plots, B.) Summary graph. Data are representative of two independent experiments. Error bars represent ± SEM of at least three mice per condition.

4.4 DISCUSSION

Understanding how structural changes in GSL Ags can differentially modulate the immune response is important, not only for understanding how *i*NKT cells influence immunity, but also for developing therapeutic GSLs. For example, Ags that preferentially skew the immune response towards Th1 cytokine production could be useful as vaccine adjuvants and anti-cancer therapeutics (Padte et al., 2013; Uchida et al., 2008). Here we characterized DB06-1, a GSL Ag that differs from the well-studied αGalCer by only a single atom. Despite this subtle change, we show that DB06-1 leads to significant changes in the immune system of mice that are more pronounced than those induced by αGalCer. Within one day after immunization, DB06-1 caused an increased Th1-cytokine response, and on the long-term it induced/expanded more NKT10 cells when compared to αGalCer. Moreover, because DB06-1 can activate human *i*NKT cells it, or related antigens with thioamide groups, could be therapeutically relevant.

A number of theories have been proposed for Th1 cytokine skewing following GSL Ag immunization, but despite much effort and investigation, this process remains incompletely understood. Selective uptake and presentation by an APC, for example DC versus B lymphocytes could be important (Bai et al., 2012), although recent evidence indicates that $CD8\alpha^+$ DC are the most essential APCs for the presentation of injected lipid Ags, regardless if they are Th1 or Th2 skewing (Arora et al., 2014). The effect of a GSL Ag on the APC could be critical, perhaps a result of its trafficking in the cell and the site where it is loaded into CD1d. Th1 cytokine skewing has been associated with a requirement for Ag internalization for loading into CD1d, as opposed to loading into CD1d on the cell surface (Lalazar et al., 2008). This type of immune response is also

correlated with appearance of CD1d-GSL complexes on the cell surface in detergent resistant domains (Arora et al., 2011). Consistent with this, DB06-1 was originally identified in a screen of lipid Ags that were associated with detergent resistant domains when bound to CD1d. Furthermore, this Ag was previously shown to stimulate robust IFN-γ production *in vivo*, but cytokine production was not compared to αGalCer, which should be classified as a Th0 Ag considering the ratio of IL-4 to IFN-γ that it induces. For unknown reasons, the different trafficking of GSLs in APCs are linked to changes in the DC, such as increased expression of CD86, that help to stimulate Th1 responses.

Another theory for Th1 cytokine skewing proposes that IFN-γ production is a result of prolonged stimulation by GSL-CD1d complexes, possibly due to increased TCR affinity, increased stability of GSL-CD1d complexes, or pharmacokinetic properties. For example, a decreased rate of compound degradation, as originally proposed for C-glycoside, could contribute to prolonged *i*NKT cell stimulation. In fact, our data indicate that several Th1 skewing GSL Ags have an increased half-life *in vivo* as CD1d-Ag complexes on the surface of APCs (Sullivan et al., 2010; Tyznik et al., 2011). Furthermore, the results from structural studies indicate that some Th1 skewing glycolipids may have increased contacts with CD1d that may promote prolonged antigenic stimulation (Aspeslagh et al., 2013; Tyznik et al., 2011). Although these different theories have merit, one class of theory alone probably does not account for all compounds that cause Th1 biasing cytokine release, and these mechanisms are not mutually exclusive.

The novel compound characterized here fits with several of the theories and mechanisms described above for the activity of Ags that cause Th1 biased cytokine

responses. For example, DB06-1 is preferentially presented by $CD11c^+$ DCs to *i*NKT cells. As previously mentioned, it was known that DB06-1 bound to CD1d localized to detergent resistant domains (Arora et al., 2011). Moreover, DB06-1 also shows a strong requirement for internalization by APC for effective presentation. Although DB06-1 can be loaded into CD1d *in vitro* in a cell free assay, optimal presentation of this lipid is achieved by recycling through endosomal compartments. While DB06-1 has a slightly weaker TCR affinity when presented by CD1d than αGalCer, this is not surprising as it has been observed with other Th1 biasing GSL Ags, including the most well-studied Ag, C-glycoside (Sullivan et al., 2010). Similar to other Th1 biasing *i*NKT cell GSL Ags (Tyznik et al., 2011), DB06-1 does persist as GSL-CD1d complexes on DCs *in vivo.* Moreover, the larger sulfur atom may make CD1d loading more difficult in an *in vitro* cell free assay, but this may be overcome in the presence of lipid transfer proteins in the lysosome. Conversely, the sulfur atom also may allow for better locking within the CD1d groove, inhibiting the GSL Ag from replacement. Furthermore, sulfur is also less electronegative than oxygen, and this may allow DB06-1 to be maintained in the CD1d hydrophobic pocket longer. Therefore we speculate that as for other Th1 biasing *i*NKT cells Ags, the increased stability of the molecular interaction of DB06-1 with CD1d will permit a prolonged *i*NKT cell stimulation that leads to increased IFN-γ production by *trans*-activated NK cells.

Despite properties that are consistent with other Th1 biasing *i*NKT cell GSL Ags, DB06-1 has some novel features that emerged from this study. Not only does DB06-1 persist on DCs *in vivo*, as shown by the *ex vivo* analysis of DC at 24h, but this Ag also loads more rapidly as shown at the 2h time point. This property has not been observed

with other GSLs, such as C-glycoside and the plakoside-like *i*NKT cell GSL Ags (Sullivan et al., 2010; Tyznik et al., 2011). Although the mechanism for this rapid loading is not known, the sulfur atom could influence the ability of the GSL Ag to interact with proteins involved in Ag transport or uptake.

A population of *i*NKT cells that produce IL-10, called NKT10 cells, has recently been described (Lynch et al., 2015; Sag et al., 2014). These NKT10 cells are induced or expand greatly after a strong antigenic stimulation, for example αGalCer immunization, are long-lived and they preferentially localize to adipose tissue. A second intriguing property of DB06-1 described here is the robust IL-10 production by *i*NKT cells from mice injected with this compound one month earlier when compared to those injected with αGalCer. We have made a similar observations of increased IL-10 production when *i*NKT cells were re-stimulated weeks after injection with several other Th1-biasing GSL Ags, suggesting this property could be a more general one. This effect extending far beyond the initial IFN-γ burst could have profound implications regarding the development of *i*NKT cell GSL Ags as therapeutics. Although humans have NKT10 cells, it is unknown if the profound response seen in mice will be found in humans.

In summary, DB06-1 is a powerful activating GSL Ag capable of impacting the mouse immune system days and weeks after immunization. Its chemical properties allow for stable formation of complexes with CD1d when it can be internalized in DC *in vivo*. The characterization of this GSL, along with previous *i*NKT cell GSL Ags, contributes to our understanding of the mechanisms for diverse *i*NKT cell influences on the immune response and will aid in the logical design of potential future *i*NKT cell GSL Ag therapeutics.

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4.7 SUPPLEMENTARY DATA

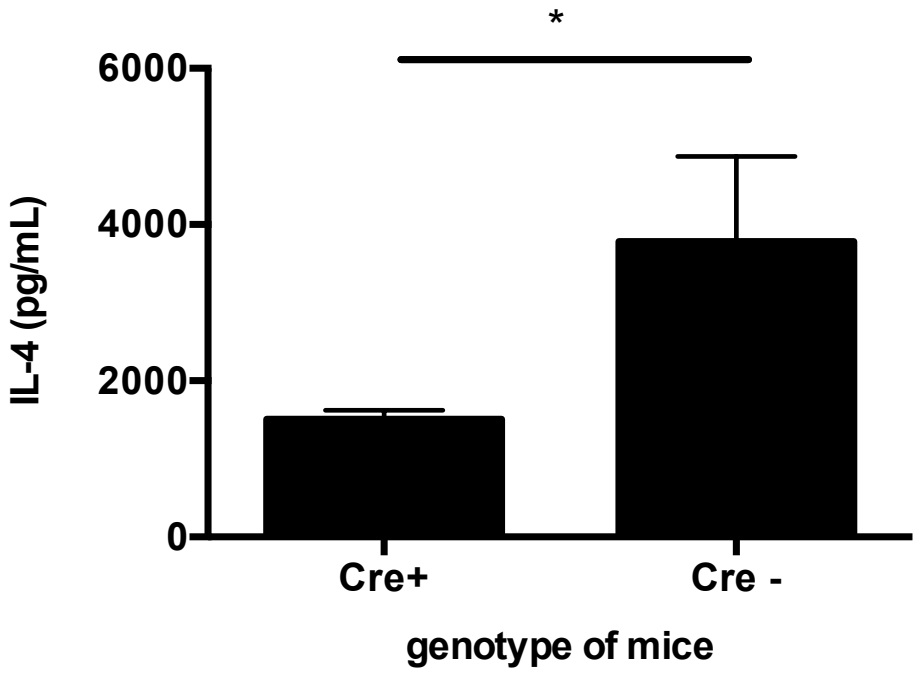


Figure 4.S1. DCs are important for DB06-1 Ag presentation. IL-4 concentration at 2 h in the sera of *cd1d*$^{f/f}$ mice, with or without the *CD11c-Cre* transgene, injected i.v. with 1 μg of DB06-1.

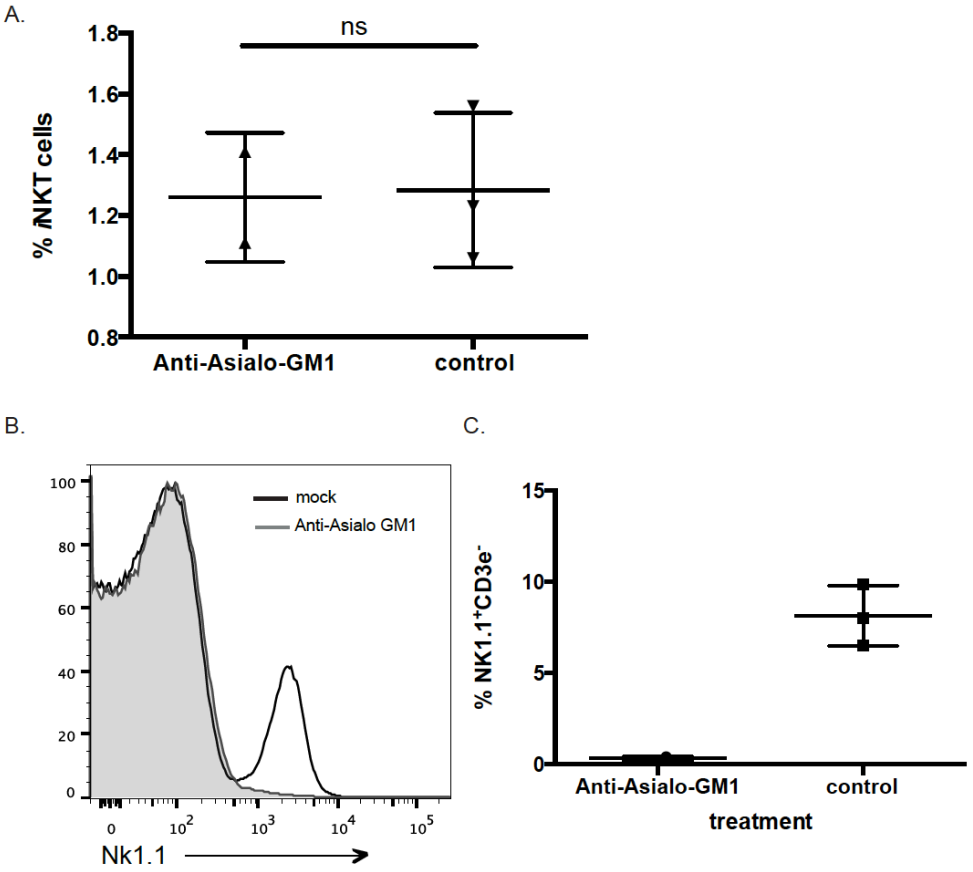


Figure 4.S2. Effective depletion of NK cells by anti-asialo-GM1 treatment. A.) NK cell depletion had no impact on *i*NKT cell numbers. Splenic *i*NKT cells (gated on live, $B220^-$, $CD3\varepsilon^+$, CD1d-Tet^+) from mice receiving anti-asialo-GM1 antisera or controls were assessed by flow cytometry. B. C.) Depletion of splenic NK cells, gated as live, $B220^-$, $CD3\varepsilon^-$, $NK1.1^+$ cells comparing control mice to those that received anti-asialo-GM1 treatment.

Table 4.S1. Data collection and refinement statistics from CD1d-DB06-1-TCR mouse ternary crystal structure.

Data collection statistics	**CD1d-DB06-1-TCR**
Space group	$C222_1$
Cell dimension	
a, b, c, (Å)	78.9, 190.5, 150.8
a, b, g (°)	90.0, 90.0, 90.0
Resolution range (Å) [outer shell]	50-2.83 (2.93-2.83)
No. reflections	27210 (2706)
R_{merge} (%)	12.2 (57.3)
R_{pim} (%)	6.1 (28.2)
Multiplicity	3.9 (3.8)
Average I/sI	10.4 (1.9)
Completeness (%)	98.1 (98.6)
Refinement statistics	
No. atoms	6,341
Protein	6,225
Ligand	60
Carbohydrate	56
R/R_{free} (%)	20.9/25.6
Ramachandran plot (%)	
Favored	97
Allowed	100
R.m.s. deviations	
Bonds (Å)	0.005
Angles (°)	0.978
B-factors ($Å^2$)	
Protein	50.2
Ligand	40.4
Carbohydrate	58.9

Chapter 5:

Discussion: Impact of synthetic GSLs on *i*NKT cells and the immune system

5.1 CONCLUSIONS

Studying *i*NKT cells has proven challenging. Since their initial characterization approximately 25 years ago (Bendelac et al., 1995; Yankelevich et al., 1989), the extraordinary capacity of these cells to change the immune system has been evaluated and explored. The rational as to why certain GSLs can bias the immune system toward a Th1 or a Th2 response has been under debate for some time, and has clinical relevance as the alteration of the immune response by GSLs has therapeutic potential. The desire for a Th1-biasing GSL is logical in that this response is necessary to fight intracellular viral infections (Lalazar et al., 2006) and may have relevance in cancer and adjuvant studies. In accordance with this, one ligand discussed in Chapter 2, 7DW8-5, is about to be used in clinical trials for use as a vaccine adjuvant (Padte et al., 2013). The series of studies in this dissertation explores theories regarding the mechanism of action of Th1 biasing ligands and their effects on the mammalian immune system using a wide variety of αGalCer analogs. This manuscript shows that *in vitro* model systems and biochemical kinetic assays cannot accurately predict the mouse *in vivo* response. In accordance with this, mouse models are excellent, however, testing in a human cell line is necessary to determine if the mouse model is recapitulating likely activation mechanism in a human system.

One of the characteristics of a Th1 skewing GSL is their differential ability to *trans*-activate NK cells. The exact reason behind this ability is likely due to prolonged *i*NKT cell stimulation *in vivo*. This hypothesis provides a clue as to why *in vitro*

experiments do not necessarily predict the proclivity of a GSL to bias the immune response, as was shown in Chapter 2. The *in vivo* system milieu likely alters the pharmacokinetic properties of the lipid, due to the presence of various lipid transport proteins, and therefore this complexity cannot be addressed in typical *i*NKT cell studies carried out *in vitro*. The degradation, biodistribution and intracellular trafficking of GSLs may change the likelihood of presentation to *i*NKT cells. As was discussed in Chapters 3 and 4, once the GSL localizes to the DCs, the stability of the CD1d-GSL complex, as measured by an increased half-life in the experiment in which the stimulation capacity of the APC was measured *ex vivo*, may override a threshold whereby downstream NK cell activation can occur.

The crystallographic structures presented in this work provide a sublime representation of the impact that the *i*NKT cell TCR has on shaping the CD1d-GSL-*i*NKT cell TCR ternary structure. The parallel docking motif with the exact same orientation over the CD1d-GSL complex in every structure is validated by similar ternary complexes in the Protein Database, including the trimolecular structure of CD1d-αGalCer-*i*NKT cell TCR. The structures reveal possible mechanisms for increased and/or prolonged interactions of the lipid antigen with CD1d, which could contribute to the eventual NK cell *trans* activation.

In the future, more work should be done to understand the CD1d/GSL interaction. Many of the recent studies have focused on the *i*NKT cell side of the equation. While that has led to the discovery of *i*NKT cell subsets, including our recently described NKT10 subset (Sag et al., 2014), it has not fully allowed us to elucidate all of the components necessary to generate a preferential Th1 or Th2 biased response. In recent work, there has

been an indication that the $CD8^+$ DCs are the sole presenters of GSLs to *i*NKT cells and it is the regulation of cell surface markers on this APC that may play a role in matriculating the response of the *i*NKT cell. If this theory holds true, certain cell surface markers such as PD-L1 and PD-L2 could serve as predictors of the *i*NKT cell-mediated immune response (Arora et al., 2014). In line with this, it is very difficult to measure the binding stability of a GSL antigen with CD1d; however, thermocalorimetry experiments may be able to measure these interactions. The measurement of the stability of the independent GSL without any bound protein is also challenging. The assault that the GSL faces upon entry *in vivo* is likely to be distinct to the individual chemical formations.

Many more studies are necessary to divulge the secrets of *i*NKT cells, their GSL stimulators and the antigen presenting cells. With this knowledge, the therapeutic potential of this unique lymphoid population could have highly significant clinical implications.

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