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Glycolipids that Elicit IFN-γ-Biased Responses from Natural Killer T Cells

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SUMMARY

Natural killer T (NKT) cells recognize glycolipids presented by CD1d. The first antigen described, α-galactosyl ceramide (αGalCer), is a potential anticancer agent whose activity depends upon IFN-γ secretion. We report two analogs of αGalCer based on a naturally occurring glycosphingolipid, plakoside A. These compounds induce enhanced IFN-γ that correlates with detergent-resistant binding to CD1d and an increased stability of the lipid-CD1d complexes on antigen-presenting cells. Structural analysis on one of the analogs indicates that it is more deeply bound inside the CD1d groove, suggesting tighter lipid-CD1d interactions. To our knowledge, this is the first example in which structural information provides an explanation for the increased lipid-CD1d stability, likely responsible for the Th1 bias. We provide insights into the mechanism of IFN-γ-inducing compounds, and because our compounds activate human NKT cells, they could have therapeutic utility.

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 1) was identified as the lead anticancer 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 antitumor 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 (Oki et al., 2004; Yu et al., 2005). 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 anticancer immunity and host defense against infectious agents. Whereas 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). Whereas 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, whereas 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 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 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.

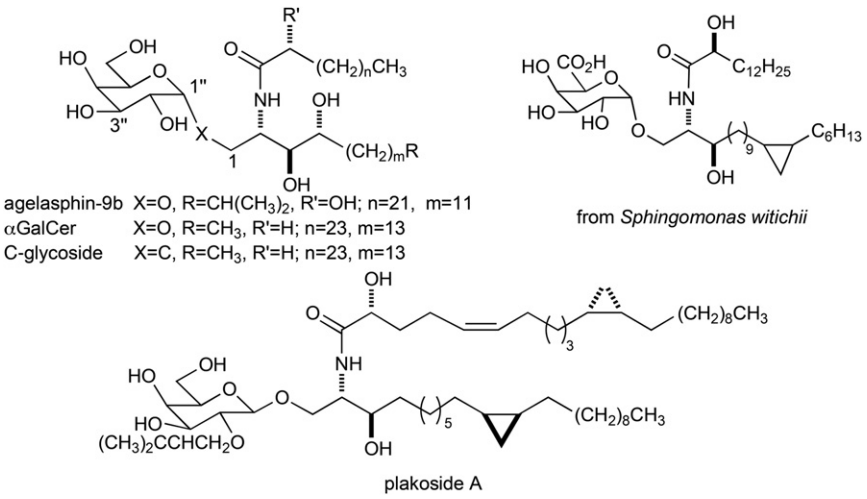

Figure 1. Structures of Immunostimulatory Natural Glycolipids, α-GalCer and C-glycoside

RESULTS

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 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 nonasymmetric, *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 longest linear sequence of more than 15 steps (Morita et al., 1995), with more steps required to make key intermediates. On the other hand, Gervay-Hague and colleagues 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 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, whereas the latter was employed for EF77 (Figure 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 if the

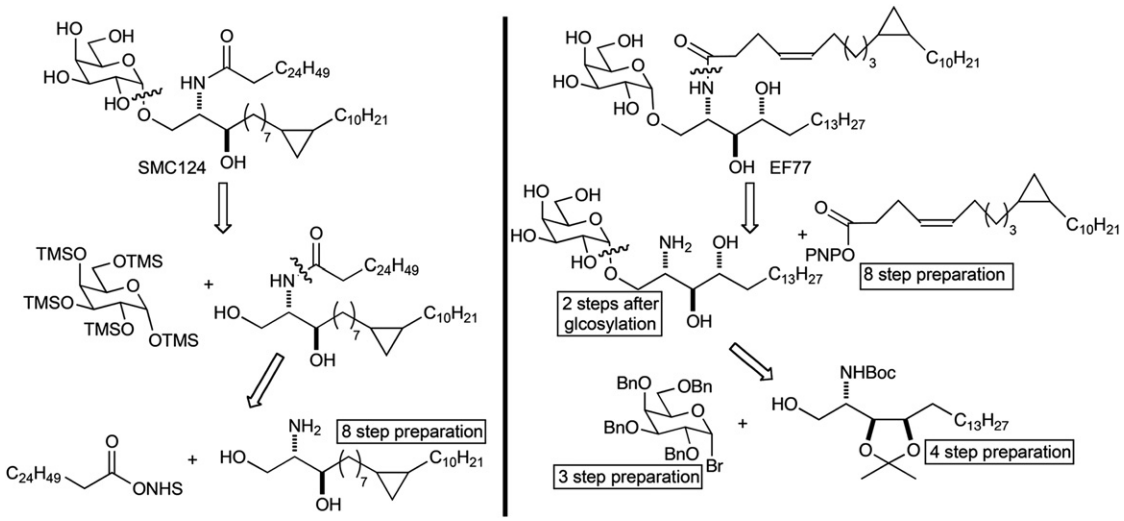

Figure 2. Strategies for the Syntheses of Plakoside A/αGalCer Hybrid Compounds SMC124 and EF77

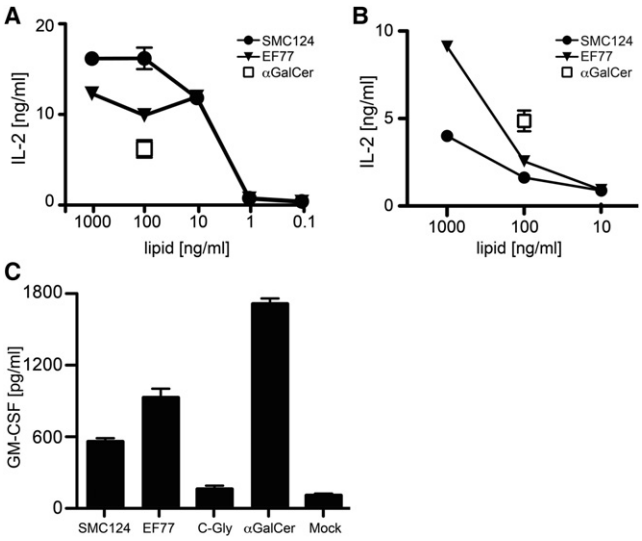

Figure 3. Immune Responses to Plakoside A Analogs

(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 hr of culture.

(B) Same as (A), except antigen presentation was carried out on CD1d-coated plates and incubated with the indicated glycolipids concentrations. Similar results were obtained in (A) and (B) with additional *i*NKT cell hybridomas.

(C) Peripheral blood mononuclear cells were pulsed with 100 ng/ml of the indicated glycolipids and cultured overnight. Cells were washed and cocultured with human *i*NKT cell lines for 24 hr. 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. See also Figure S1.

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, Figure S1). *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, whereas a 10-fold higher lipid concentration of SMC124 was required to elicit a similar response (Figure 3B). Similar results were observed using *i*NKT cell hybridoma 1.4 (Figure S1). 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 (PBMC) pulsed with lipids as APCs. Peripheral blood mononuclear cells were incubated with SMC124, EF77, C-glycoside, or αGalCer and then cocultured with human *i*NKT cell lines. GM-CSF secretion was analyzed because it has been previously demonstrated to be a sensitive measurement of TCR-mediated human *i*NKT cell activation. As previously reported, αGalCer stimulated human *i*NKT cells, whereas 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 3C, Figure S1).

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 (Figures 4A and 4B) and IL-4 (Figure S2) 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 hr that is sustained longer and up to 36 hr. The systemic IFN-γ in the sera at 6 hr 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 4A). Similar to C-glycoside, plakoside A analogs elicited lower IL-4 sera levels compared to αGalCer (Figure S2). Strikingly, SMC124- and EF77-injected mice consistently had seven- to eight-fold higher serum IFN-γ levels 22 hr post-lipid injection compared to either C-glycoside or αGalCer in six independent experiments (Figure 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 hr after lipid injection for IFN-γ production by natural killer cells. In agreement with the data from sera, natural killer cells from plakoside A analogs-injected mice 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 (Figures 4C and 4D). 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 CD40-mediated IL-12 production by DCs (Carnaud et al., 1999; Fujii et al., 2006; Kitamura et al., 1999). IL-12 in turn enhances natural killer-cell-mediated IFN-γ production. To confirm that the mechanism of *trans* activation by the plakoside A analogs is mediated through IL-12, we analyzed IL-12 serum levels from mice following lipid antigen injection. Correlating with the higher IFN-γ levels observed in the sera, IL-12 in the sera of mice that received SMC124 or EF77 was higher and sustained longer

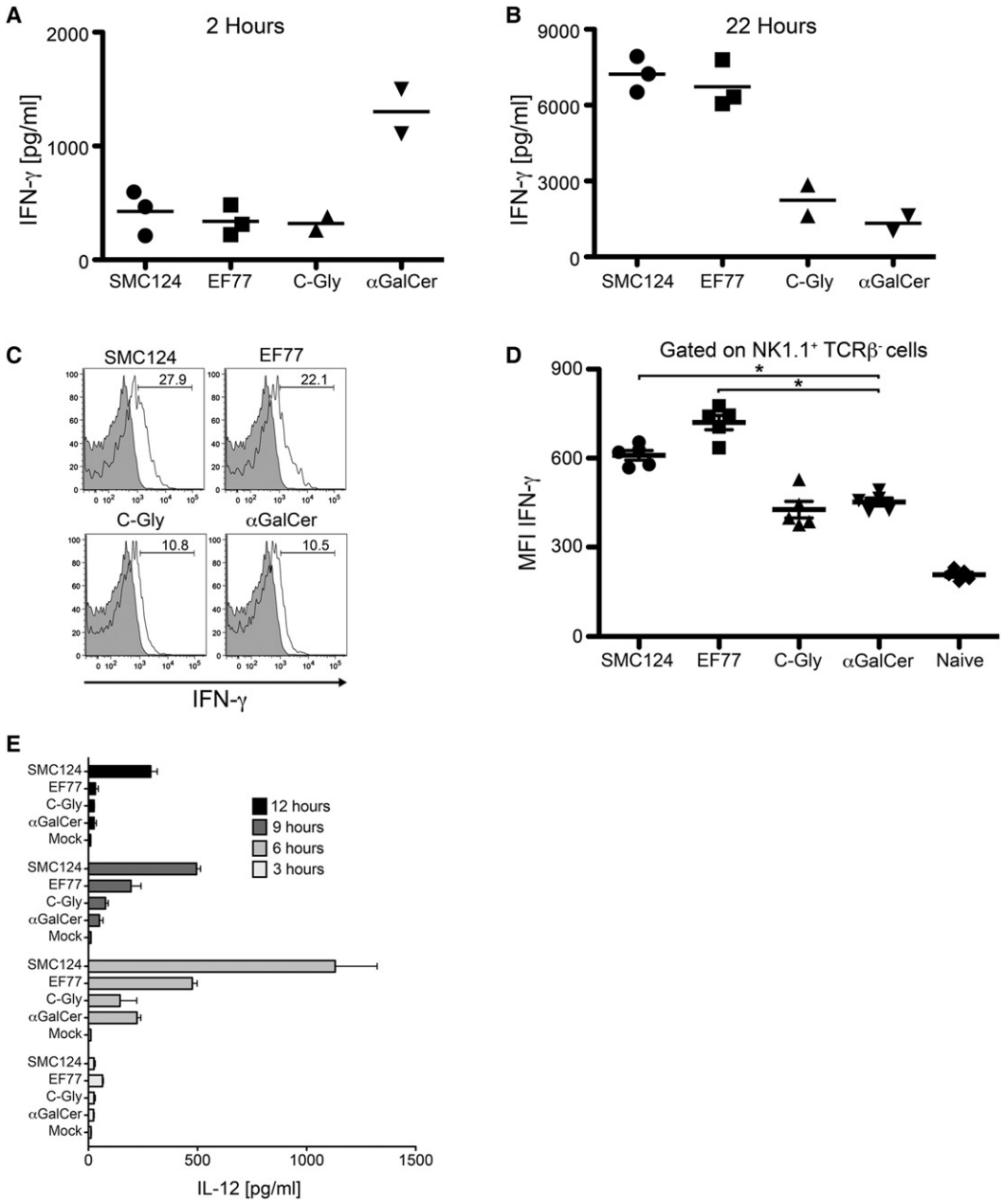

Figure 4. In Vivo Responses to Plakoside A Analogs

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 six independent experiments of two or three mice per group. Error bars represent ±SEM (C and D) Mice were sacrificed at 22 hr and $TCR\beta^{-}$, $NK1.1^{+}$ 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 hr postinjection of lipid (open histogram) compared with PBS injected control (shaded histogram). Histograms are representative plots of a minimum of three 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 four independent experiments.

(E) Serum samples were measured for IL-12p70 cytokine levels by ELISA. Data are representative of two independent experiments of three 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. See also Figure S2.

than in mice that received αGalCer (Figure 4E). Furthermore, in the absence of IL-12 receptor expression, IFN-γ was no longer observed at 22 hr postinjection (Figure S2).

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

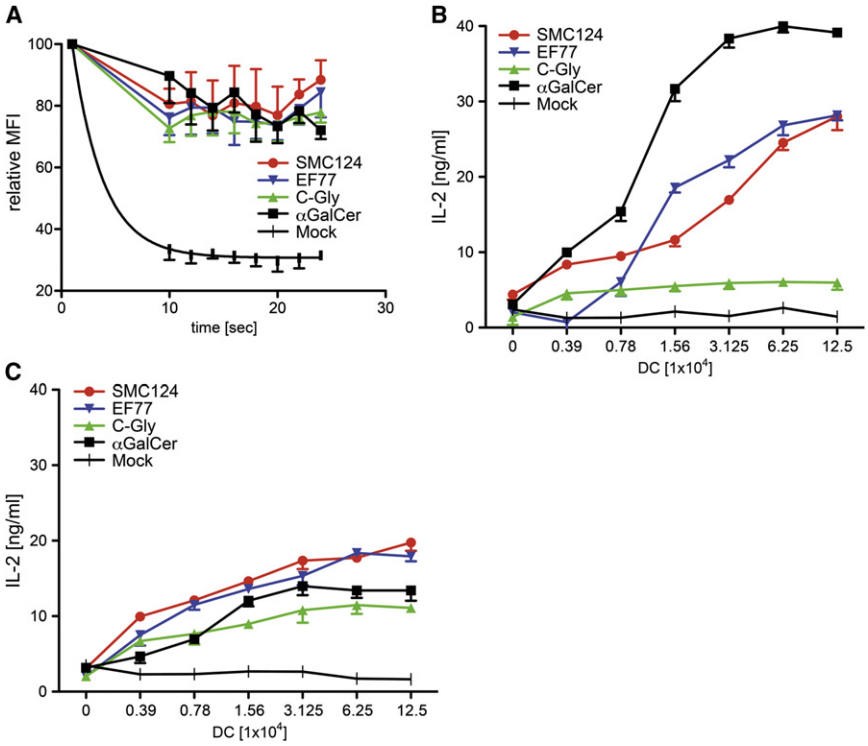

Figure 5. Stability of Lipid/CD1d Complexes

(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$^+$ DC were enriched using magnetic bead isolation at 2 (B) and 22 (C) hr postinjection. Varying number of enriched DC 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 two or three mice per condition.

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 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 (DC; >80% pure) isolated from lipid or DMSO-control-injected mice obtained at 2 and 22 hr postinjection were cocultured at increasing concentrations with a fixed number of *i*NKT cell hybridomas. Dendritic cells isolated from αGalCer-injected mice were able to activate the hybridoma at 2 and 22 hr postinjection, as measured by IL-2 secretion, although at 22 hr the stimulatory capacity of the DC declined by approximately 50% (Figures 5B and 5C). At 22 hr postinjection, EF77 and SMC124 retained a nearly undiminished ability to activate the *i*NKT cell hybridomas compared with 2 hr after injection (Figures 5B and 5C). A similar sustained capacity of the DC to activate *i*NKT cells ex vivo was observed with cells from C-glycoside-injected mice, although its antigenic potency was reduced.

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 (Figure 6A). This is a binding affinity similar to that observed with αGalCer (K_D = 24.6 nM (data not shown). 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}), whereas 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).

Crystal Structure of the TCR-SMC-CD1d Ternary Complex

In order to determine how the 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 noncrystallographic symmetry-related molecules in correspondence to the α3 domain of the CD1d molecule, the overall structure and the

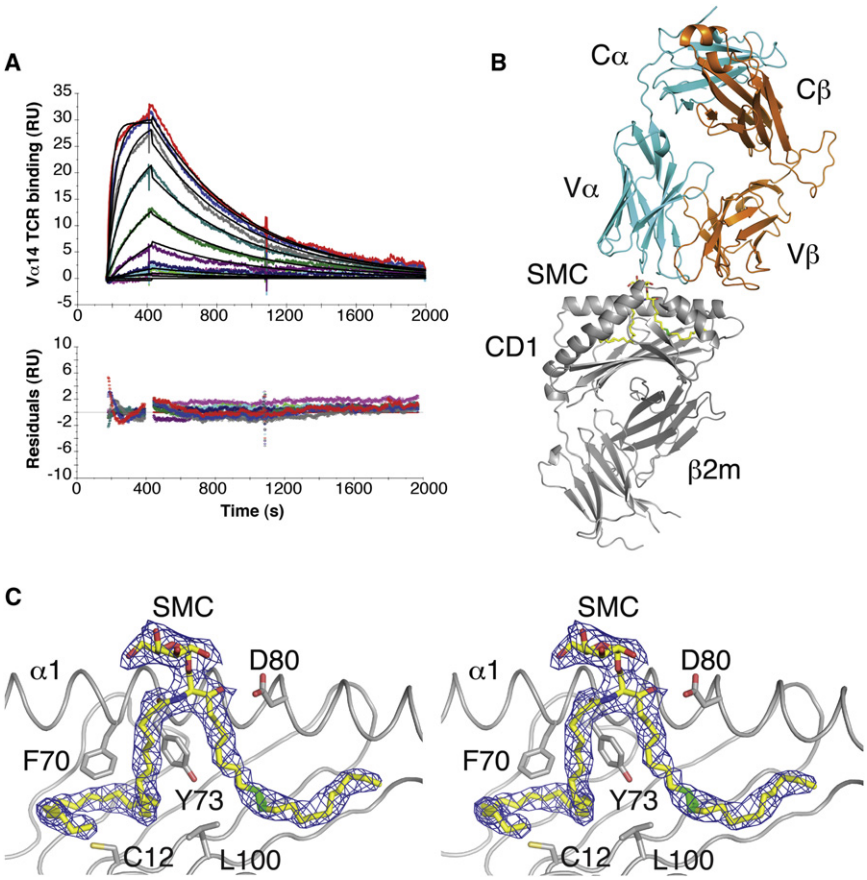

Figure 6. Biophysical and Structural Characterization of the CD1d-SMC124-*i*NKT TCR Interaction

(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 *2Fo−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. See also Figure S3.

conformation of the ligand and antigen-binding domain appear to be 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 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 to correspond to the SMC124 molecule (Figure 6C; Figure S3), 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 (Figures 7A and 7B), contribute to the stabilization of 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 (Figures 7C and 7D), as previously observed for αGalCer and other galactose-containing glycolipids (Aspeslagh et al., 2011; Li et al., 2010b; Wun et al., 2011).

Whereas 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 (Figures 7E and 7F). 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 (Figures 7E and 7F; Figure S4). Interestingly, whereas 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 (Figure S4). 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 and 34 for αGalCer; Table S1), 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 (Figures 7E and 7F). 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).

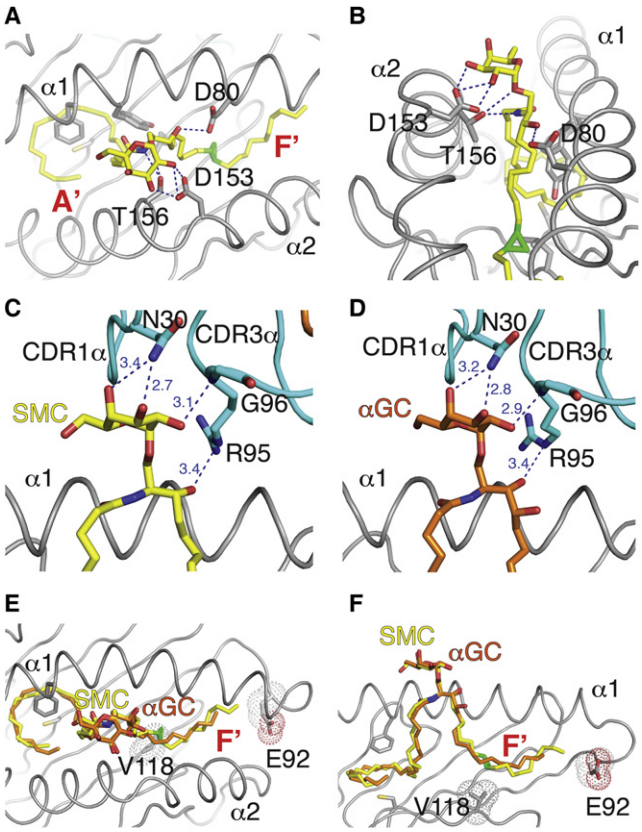

Figure 7. Binding of SMC124 to CD1d

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. See also Figure S4 and Tables S1 and S2.

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. Whereas 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 toward 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 that 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). Consistent with this mechanism, we observed increased induction of IL-12 in the sera 6–12 hr postinjection of the plakoside A analogs. Furthermore, the ability of natural killer cells to be *trans* activated by the plakoside A analogs, and therefore to skew a Th1 response, required the expression of the IL-12Rα. Additionally, we propose that the events leading to enhanced IFN-γ secretion at 6 hr and beyond also require continuing *i*NKT cell activation, which the ex vivo analysis suggests could be carried out by APC exposed to the plakoside A analogs in vivo hours earlier.

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 been clearly 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 located 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, whereas 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).

Whereas 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 antitumor agents or vaccine adjuvants.

SIGNIFICANCE

The number of glycolipids that bind CD1d and activate *i*NKT cells is continually growing. These lipids include both naturally occurring lipids and synthetic variants generated in an attempt to modulate the immune response to one that is beneficial to the host. Variations of the canonical *i*NKT cell Ag, αGalCer, have led to Th1- or IFN-γ-biasing conditions, but a number of these antigens have proven ineffective in activating human cells. Here, we have shown that structural analogs of a naturally occurring glycolipid isolated from the marine sponge *Plakortis simplex*, plakoside A, induce a potent Th1-polarized immune response and are effective in activating multiple human *i*NKT cell lines. These chemically synthesized lipids contain a ceramide structure similar to αGalCer but have unique lipid chains containing a cyclopropyl group on either the acyl chain or sphingoid base. The ternary crystal structure of one of the lipids bound to CD1d in complex with *i*NKT cell TCR suggests that CD1d can accommodate a long cyclopropyl containing sphingoid base and this may lead to increased stability. Furthermore, our biological assays indicate that these lipids have a prolonged biological stability, which likely accounts for the potent *trans* activation of natural killer cells and the copious amounts of IFN-γ detected in the serum after lipid injection. Additionally, our data suggests the plakoside A analogs are associated with lipid-raft microdomains. These data have revealed insights into the mechanisms that are necessary in polarizing a Th-1 biased *i*NKT cell response. Additionally, since our Plakoside A analogs efficiently activate human *i*NKT cell lines, these antigens have therapeutic potential where other Th1-biasing antigens have failed.

EXPERIMENTAL PROCEDURES

Chemical Synthesis

See the Supplementary Methods for synthetic procedures and characterization data.

Generation of Human *i*NKT Cell Lines

Human Vα24$^+$ *i*NKT cells were purified and expanded in accordance with published protocols (Rogers et al., 2004). Peripheral blood mononuclear cells were isolated by density-gradient centrifugation. Human donor PBMC (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.

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 hr. 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.

Cell-free Antigen Presentation Assay

Stimulation of *i*NKT cell hybridomas on microwell plates coated with soluble CD1d was carried out in accordance with published protocols (Naidenko et al., 1999; Sidobre et al., 2004; Tupin and Kronenberg, 2006). Indicated amounts of compounds or vehicle were incubated for 24 hr 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 hr, and IL-2 in the supernatant was measured by ELISA.

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 hr. GM-CSF release was evaluated in a sandwich ELISA following the manufacturer's instructions (R&D Systems).

Mice

C57BL/6, and *IL-12Rα*$^{-/-}$ 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.

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 1 mm pieces, digested using spleen dissociation media, and DCs were enriched by positive selection using a $CD11c^+$ isolation kit with RoboSep technology in accordance with the manufacturer's protocols (Stem Cell Technologies). Isolated DCs were cocultured at varying concentrations with hybridomas overnight.

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 were 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).

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 analogs or controls. After 16 hr of culture, cells were detached, washed three times, and resuspended in 50 μl of FACS buffer. After 10 min of incubation, 50 μl of staining solution containing 1 μg/ml of Alexa Flour 647 conjugated mAB L363 in FACS buffer was added and followed by 10 min 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 s. Triton X-100 was then added to a final concentration of 0.06% and followed by brief (~1 s) vortexing to mix the sample. Data collection was then resumed, and fluorescence intensities were monitored for another 30 s. Data were collected as flow cytometry standard files and analyzed using FlowJo software 7.5 (Treestar, Ashland, OR). Mean fluorescent intensity 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.

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).

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°C for 20 min. 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 min 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).

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. Three hundred 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.

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 = 96.3°). 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 (Protein Data Bank [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 $2Fo - Fc$ and $Fo - Fc$ difference electron density maps using the program COOT (Emsley et al., 2010). The lipid was built into $2Fo - Fc$ map and refined using REFMAC (Collaborative Computational Project, Number 4, 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, β2 m, 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). Data collection and refinement statistics are presented in Table S2.

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.

ACCESSION NUMBERS

Structure factors and coordinates have been deposited in the Protein Data Bank under accession code 3TVM.

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures and two tables and can be found with this article online at doi:10.1016/j.chembiol.2011.10.015.

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