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Mechanisms for Glycolipid Antigen-Driven Cytokine Polarization by Vα14i NKT Cells

Barbara A. Sullivan,*1 Niranjana A. Nagarajan,*2 Gerhard Wingender,* Jing Wang,† Iain Scott,*3 Moriya Tsuji,‡ Richard W. Franck,§ Steven A. Porcelli,¶ Dirk M. Zajonc,† and Mitchell Kronenberg*

Certain glycolipid Ags for Vα14i NKT cells can direct the overall cytokine balance of the immune response. Th2-biasing OCH has a lower TCR avidity than the most potent agonist known, α-galactosylceramide. Although the CD1d-exposed portions of OCH and α-galactosylceramide are identical, structural analysis indicates that there are subtle CD1d conformational differences due to differences in the buried lipid portion of these two Ags, likely accounting for the difference in antigenic potency. Th1-biasing C-glycoside/CD1d has even weaker TCR interactions than OCH/CD1d. Despite this, C-glycoside caused a greater downstream activation of NK cells to produce IFN-γ, accounting for its promotion of Th1 responses. We found that this difference correlated with the finding that C-glycoside/CD1d complexes survive much longer in vivo. Therefore, we suggest that the pharmacokinetic properties of glycolipids are a major determinant of cytokine skewing, suggesting a pathway for designing therapeutic glycolipids for modulating invariant NKT cell responses. The Journal of Immunology, 2010, 184: 141–153.

NKT cells with an invariant Vα14 TCR rearrangement (Vα14i NKT cells) are a distinct lineage of αβ T lymphocytes that have metaphorically been referred to as the “Swiss-Army knife” of the immune system (1) due to their remarkable ability to regulate many different aspects of immunity. They have characteristics that classify them as innate lymphocytes, functioning to give an immediate effector response. For example, Vα14i NKT cells constitutively express high levels of CD69 and CD44, and engagement of glycolipid/CD1d complexes stimulates the production of effector cytokines, including IL-4 and IFN-γ, within minutes (2). Vα14i NKT express a TCR comprised

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The sequence presented in this article (structure factor and atomic coordinates for the OCH/mouse CD1d complex) has been deposited in the Protein Data Bank under accession number 3G08.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: αGalCer, α-galactosylceramide; DC, dendritic cell; PFA, paraformaldehyde.

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therefore to enhance the clearance of parasites in a cerebral malaria model (15), to prevent melanoma metastases (17), and to ameliorate ocular autoimmunity in experimental autoimmune uveitis (18).

The current study was undertaken to determine the mechanisms for the altered cytokine bias induced by these glycolipid Ags. There have been a number of previous studies on the mechanism of Th2 cytokine polarization by OCH, and these suggest that the main cause is the reduced stability of binding of OCH to CD1d as a result of the decreased sphingosine base (10, 19) along with the reduced TCR affinity (19). According to this view, induction of IFN-γ secretion by Vα14i NKT cells requires a longer period of stimulation (10) and/or a higher Ag concentration (19). Vα14i NKT cells activated by OCH also had a reduced induction of CD40L expression (11). This in turn led to reduced production of IL-12 by APCs, which causes decreased synthesis of IFN-γ by NK cells, which are activated rapidly following Vα14i NKT cell stimulation. Therefore, the Th2 skewing effect of OCH was believed to be due to a combination of direct effects on Vα14i NKT cells and indirect effects on cells stimulated downstream of the activated Vα14i NKT cells, all attributable to the decreased duration of glycolipid Ag stimulation or decreased TCR affinity. All Th2 cytokine skewing by Vα14i NKT cells cannot be attributed to decreased antigenic potency, however, because the C20:2 glycolipid, with a fatty acid having two unsaturated bonds, causes Th2 cytokine skewing without decreasing antigenic potency (12), as do some other compounds with alterations in the fatty acid moiety (20). In this instance, evidence was provided that these compounds were much less dependent upon Ag internalization and excluded from CD1d molecules in lipid rafts (20), suggesting that the cellular site of Ag loading is important for function.

An implication of these studies on OCH is that the Th1 skewing Ag C-glycoside might have a higher TCR affinity or more stable binding to CD1d. There have been no previous comparisons, however, of OCH and C-glycoside, which are the paradigmatic Th2 and Th1 skewing glycolipid Ags. Therefore, here we carried out a comprehensive biochemical, structural, cellular, and in vivo comparison of these glycolipids. Our data are not consistent with the prevailing view that Th2 cytokine skewing relates in a simple, linear way to the stability of CD1d binding or to the affinity of the TCR interaction. Instead, our data show that the stability of in vivo presentation by CD1d is likely to be important, suggesting that the pharmacokinetic properties of the glycolipid Ag, allowing it to give a prolonged stimulus, may in some cases be the critical factor.

Materials and Methods

**Mice, reagents, and hybridomas**

C57BL/6d mice were purchased from the The Jackson Laboratory (Bar Harbor, ME). Mice genetically deficient for CD40 (CD40−/−) on the C57BL/6 background were the gift of Dr. Stephen Schoenberger (La Jolla Institute for Allergy & Immunology, La Jolla, CA). CD1d-deficient mice (CD1d−/−) were the kind gift of Dr. Luc Van Kaer (Vanderbilt University, Nashville TN). All mice were housed in accordance with the Institutional Animal Care Committee guidelines.

Mouse CD1d was produced in our laboratory as previously described (24). γGalCer and OCH were obtained from the Kirin Pharmaceutical Research (Gunma, Japan). C-glycoside was synthesized as previously described (17, 22). For experiments in which glycolipids were pulsed onto cells or injected into mice each glycolipid was dissolved in DMSO at a concentration of 100 μg/ml, then further diluted in PBS prior to incubation or injection. For experiments in which glycolipids were conjugated to CD1d to form tetramers, γGalCer and OCH were dissolved in vehicle (0.5% polysorbate 20 and 0.9% NaCl) at a concentration of 200 μg/ml and the mixture was dissolved in DMSO at a concentration of 1 mg/ml and then further diluted with vehicle to 200 μg/ml. In all of the experiments, glycolipids were heated to 56°C for 10 min and vortexed extensively prior to use. The DN3A4-1.2 (referred to as 1.2) and N38-2C12 (referred to as 2C12) Vα14i NKT hybridoma cells have been described previously (23, 24). A20-CD1d B lymphomas that stably overexpress CD1d have also been previously described (24).

**Tetrameric glycolipid/CD1d complexes**

Tetramers of CD1d bound to αGalCer, OCH, and C-glycoside were produced as described (21) with slight modifications. CD1d molecules were incubated at a 1:1 (CD1d-to-glycolipid) molar ratio with each glycolipid or vehicle. After 24 h, glycolipid/CD1d complexes were tetramerized by the addition of premium-grade streptavidin-labeled PE (Invitrogen, Carlsbad, CA). For equilibrium staining of hybridomas, 2 × 106 cells were stained in R10 medium (RPMI 1640 supplemented with 10% FBS, 100 μ/ml penicillin, 100 μg/ml streptomycin, and 0.292 mg/ml L-glutamine; Life Technologies/Invitrogen, Grand Island, NY) with azide (0.05%), to prevent internalization, for 3 h at room temperature in the dark with glycolipid/CD1d tetramers and 2.5 μg/ml FITC-labeled anti-TCRβ mAb. The cells were then washed twice in staining buffer and fixed in 1% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in PBS. The intensity of fluorescence of tetramer binding was determined by flow cytometry.

For tetramer dissociation experiments, Vα14i NKT hybridoma cells were incubated with a saturating dose of each glycolipid/CD1d tetramer for 1.5 h at room temperature. Excess tetramer was washed away, an anti-CD1d mAb (1B1; 100 μg/ml) was added to prevent the rebinding of tetramers, and samples were placed at 37°C for the decay period. After 15 min of equilibration, aliquots of the bulk tetramer-labeled population were removed and chilled in ice-cold R10 with azide. Following the decay period, hybridoma cells were incubated with anti-TCRβ on ice. Cells were washed again and fixed in 1% PFA in PBS. The intensity of fluorescence of tetramer binding was determined by flow cytometry. Mean tetramer fluorescence was calculated in hybridoma cells. Decay data are presented as time versus log(tetramer fluorescence for each point/initial tetramer fluorescence). After linear regression, tetramer half-life was determined by 1/2 = log 2/slope.

**Surface plasmam resonance**

All real-time binding experiments were performed at 25°C on a BIAcore 3000 biosensor system (GE Healthcare/Biacore, Piscataway, NJ). Kinetic parameters were obtained by fitting the specific sensorgrams with BIAEVAL 4 software.

**Decay of glycolipids from CD1d**

Analysis of the decay of glycolipids from soluble CD1d was performed with an αGalCer/CD1d-specific Ab L317.11.9 that has been described previously (25). Hexahistidine-tagged CD1d proteins were pulsed with the indicated glycolipids overnight. On the following day, glycolipid/CD1d complexes were bound to each pulsed channel of a Ni-NTA chip (GE Healthcare/Biacore). The glycolipid/CD1d complexes were allowed to decay for various times before addition of saturating doses of L317.11.9 and determination of the degree of binding.

**Measurement of ERK1/2 phosphorylation**

ERK1/2 phosphorylation was detected as previously described (26). Briefly, either 106 A20-CD1d cells per milliliter or 106 splenic B cells per milliliter positively selected with anti-CD19-conjugated MACS beads (Miltenyi Biotech, Auburn, CA) were pulsed with glycolipid Ags for 2 h or 106 BMDCs were pulsed with glycolipid Ags for 24 h at 37°C. The cells were washed once with cold medium, and Vα14i NKT hybridoma cells were mixed with pulsed APCs at a 4:1 ratio and plated on ice. At the indicated time points, cells were pelleted in a desktop centrifuge and placed at 37°C. Following the incubation, the supernatant was removed and the cell pellet was resuspended in ice-cold PBS with 2.5 mM EDTA followed immediately by the addition of PFA fixing buffer (4% PFA in PBS (pH7.4)). The cells were pelleted once more and Vα14i NKT cells were incubated with anti-Thy1.2 FITC for 30 min on ice. Following the incubation, cells were pelleted and resuspended in ice-cold permeabilization buffer (90% methanol and 10% water) and incubated on ice for 30 min. Cells were washed twice with room temperature staining buffer (4% PBS in PBS) and then incubated with anti-pp-ERK1/2 (E10 Alexa Fluor 647) for 30 min at room temperature. Cells were washed twice with staining buffer and resuspended in 1% PFA and analyzed by flow cytometry. Gates were set on Thy1.2 positive cells (Vα14i NKT cells) using t = 0 as a negative control. The data were plotted as time (minutes) versus (pp-ERK1/2% glycolipid – % pp-ERK1/2% vehicle).
Crystal structure determination

Soluble and fully glycosylated mouse CD1d/β2-microglobulin heterodimeric protein was expressed and purified as described (27). Synthetic OCH was loaded by incubating mouse CD1d with a fourfold molar excess of lipid. The CD1d/lipid mixture contained 100 mM Tris/HCl, 0.07% Tween 20, 10 mM NaCl (pH 8.5), and 0.07% OCH (pH 7.0) and was incubated for 16 h at room temperature under gentle agitation. The OCH/CD1d complexes were separated from unbound lipid by size exclusion chromatography on Superdex S200 resin (GE Healthcare) and concentrated to 8.5 mg/ml in 10 mM HEPES and 30 mM NaCl (pH 7.5) for crystallization. Crystals were grown over a period of 5 mo at 4°C in condition 35 of the commercial PEG/Ion screen, containing 16% polyethylene glycol 3350, 0.1 M Tris/HCl (pH 8.5), and 2% taurine (pH 8.0). Diffraction data for a single crystal were collected at beamline 11.1.1 of the Stanford Synchrotron Radiation Laboratory and processed to 1.6 Å with the Denzo–Scalepack suite (28) in space group P222 (unit cell dimensions: a = 42.2 Å; b = 107.1 Å; c = 106.7 Å). One OCH/CD1d complex occupies the asymmetric unit with an estimated solvent content of 50%, based on a Matthews’ coefficient (Vm) of 2.46 Å³/Da. Molecular replacement in Phaser (29) was carried out using the program MOLREP (30) and the Ig3/CD1d structure (PDB code 2QTY) as the search model, with the glycolipid ligand and carbohydrates removed, and resulted in a crystalllographic R factor (Rmerge) of 44.7% and a correlation coefficient of 0.53. Translation, libration, screw-rotation displacement parameterization in REFMAC (31) was essentially carried out as described for the pMCD1d/CD1d structure (32). The REFMAC library for OCH was created using the Dundee PRODRG2 server (33), after modification of the αGalCer coordinates from the αGalCer/CD1d human structure (10). The OCH/CD1d structure has a final Rmerge of 20.2% and Rfree of 23.2%, and the quality of the model (Supplemental Table II) was excellent as assessed with the program MolProbity (34). The software program PyMol (Delano Scientific, Palo Alto) was used to prepare Fig. 4. The PDB2PQR server (35) and the program APBS (36) were used to calculate the electrostatic surface potentials in Fig. 4. Structure factor and atomic coordinates for the OCH/mouse CD1d complex have been deposited in the Protein Data Bank (www.rcsb.org) under PDB ID code 3G08.

Cell preparation

Single-cell suspensions were prepared from the liver, spleen, thymus, and bone marrow. Prior to extraction, the liver was perfused with PBS via the portal vein until opaque and pressed through a 70-μm cell strainer (BD Biosciences, San Jose, CA). Total liver cells were then resuspended in 0.1% collagenase and 0.01% type II trypsin inhibitor in RPMI 1640 medium. Liver macrophages were isolated from the 40%/60% interface. The cells were washed once in R10 medium. Bone marrow cells were dissociated and filtered through a 70-μm cell strainer (BD Biosciences) and then washed once. Bone marrow cells from the spleen were enriched by incubation with anti-CD11c magnetic beads. Magnetic depletion of the CD8+ T cells was then carried out in the presence of 1 μg of aGalCer, OCH, C-glycoside, or vehicle and were bled at the indicated times after injection. Cytokine levels in the sera of mice immunized with glycolipids measured by a sandwich ELISA using paired anti-IL-4 and anti-IFN-γ mAbs (BD Pharmingen, San Diego, CA). Cytokine levels were determined by ELISA (Fig. 1B). As reported previously (9, 15), IL-4 peaked within a few hours after Ag presentation, whereas peak amounts of serum IFN-γ required a longer time. OCH induced a systemic Th2-biased response, with high levels of IL-4, equivalent to αGalCer, and relatively low levels of IFN-γ (Fig. 1B), in agreement with earlier studies (10, 14). Therefore, the overall Th2 bias resulting from OCH was due to reduced production of IFN-γ rather than increased IL-4. C-glycoside induced 3- to 4-fold less IL-4 in the serum than αGalCer, but it induced amounts of IFN-γ that were decreased less dramatically to ~50% of the amount induced by αGalCer (Fig. 1B), resulting in an overall Th1 profile of cytokine production. Therefore, for both OCH and C-glycoside, the cytokine bias is a relative one manifested most clearly as a difference in the proportion of the concentrations of IFN-γ produced relative to IL-4. This proportion is ~1:1 for OCH but nearly 10:1 for C-glycoside.

OCH and C-glycoside display reduced TCR avidity

As has been proposed for peptide recognition by conventional CD4+ T cells (37, 38), the altered pattern of cytokine production initiated by αGalCer/NKT cell activation could be due to differences in the binding kinetics or affinity of the glycolipid/CD1d complex for the Vα14i TCR. We therefore determined the affinity of αGalCer/NKT cell TCRs for CD1d bound to each Ag, using tetramer-based binding assays to measure the avidity and dissociation rates of TCRs expressed by αGalCer/NKT cell hybridomas and polyclonal Vα14i NKT cells. Tetramers of OCH/CD1d, C-glycoside/CD1d, and αGalCer/CD1d were used to determine the equilibrium dissociation constant (Kd), a measure of TCR avidity (Fig. 2A), and the dissociation rates to determine the half-life (t½) of the TCR interaction (Fig. 2B). Equilibrium tetramer binding measurements using the 2C12 αGalCer/CD1d cell hybridoma, with a Vα14i/Vβ8.2 TCR that has been analyzed extensively previously (39), showed that αGalCer/CD1d complexes bound with the highest avidity (Kd = 0.171 ± 0.024 nM). OCH and C-glycoside complexes bound to CD1d and the indications for this TCR was measured by a sandwich ELISA using paired anti-IL-4 and anti-IFN-γ mAbs (BD Pharmingen, San Diego, CA). Cytokine in the supernatant of hybridoma cultures were measured by a sandwich ELISA using paired anti-IL-2 mAbs (BD Pharmingen).

Results

Glycolipid Ags induce skewed systemic cytokine production

To analyze systemic cytokine production induced by different glycolipid Ags (Fig. 1A), C57BL/6J mice were injected i.v. with 1 μg of αGalCer, OCH, C-glycoside, or vehicle and were bled at the indicated times after injection. Cytokine levels in the sera were measured by ELISA (Fig. 1B). As reported previously (9, 15), IL-4 peaked within a few hours after Ag presentation, whereas peak amounts of serum IFN-γ required a longer time. OCH induced a systemic Th2-biased response, with high levels of IL-4, equivalent to αGalCer, and relatively low levels of IFN-γ (Fig. 1B), in agreement with earlier studies (10, 14). Therefore, the overall Th2 bias resulting from OCH was due to reduced production of IFN-γ rather than increased IL-4. C-glycoside induced 3- to 4-fold less IL-4 in the serum than αGalCer, but it induced amounts of IFN-γ that were decreased less dramatically to ~50% of the amount induced by αGalCer (Fig. 1B), resulting in an overall Th1 profile of cytokine production. Therefore, for both OCH and C-glycoside, the cytokine bias is a relative one manifested most clearly as a difference in the proportion of the concentrations of IFN-γ produced relative to IL-4. This proportion is ~1:1 for OCH but nearly 10:1 for C-glycoside.

Monoclonal Abs and flow cytometric analysis

The following mAbs were used in this study: CD1d (1B1), CD8α (53-6.72), CD8β (53-5.8), TCRβ (H57-597), Thy1.2 (30-H12 and 53-2.1), CD24 (M1/69), NK1.1 (PK136), TNF (M6P-XT22), IL-4 (1B11). IFN-γ (XMGL1.2), and p-ERK1/2 (E10). Abs were purchased from BD Biosciences or Cell Signaling Technology (Beverly, MA). Abs were biotinylated or conjugated to FITC, PE, PerCP-Cy5.5, Alexa Fluor 488, Alexa Fluor 647, or APC. Multicolor acquisition of fluorescently labeled cells was performed on a BD FACSCalibur or BD LSRII (BD Biosciences). Data analysis and production of figures was performed with FlowJo software (Tree Star, Ashland, OR).

Analysis of cytokine secretion

Cytokines in the sera of mice immunized with glycolipids measured by a sandwich ELISA using paired anti-IL-4 and anti-IFN-γ mAbs (BD Pharmingen, San Diego, CA). Cytokine in the supernatant of hybridoma cultures were measured by a sandwich ELISA using paired anti-IL-2 mAbs (BD Pharmingen).

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CD1d complexes to 1.2, another Vα14i NKT cell hybridoma with a Vα14i/Vβ8.2 TCR. Although these two hybridomas differ only in the CDR3 region of the TCR β-chain, OCH/CD1d complexes were barely able to bind the 1.2 hybridoma cells and the C-glycoside complexes did not achieve binding higher than that of the vehicle-loaded controls. Despite this, 1.2 binding αGalCer/CD1d complexes was similar to that of 2C12, although in agreement with previous results (39) the binding by 1.2 was slightly weaker (Fig. 2A, bottom; K_D = 0.305 ± 0.049 nM).

The decay of tetramer binding can be used as a measure of the stability of the interaction between tetramers and TCRs (40). For peptide-reactive T cells, the half-life of the interaction between peptide/MHC complexes and the TCR, as determined by surface plasmon resonance measurements, can be more indicative of antigenic potency than the equilibrium binding constant (41). The decay of glycolipid/CD1d tetramer binding to 2C12 hybridoma cells was more rapid for OCH/CD1d tetramers than that for αGalCer/CD1d tetramers (Fig. 2B), although C-glycoside/CD1d tetramers had an even shorter half-life of binding (Fig. 2B; t_1/2 = 385 ± 56, 114 ± 22, and 58 ± 5 min for αGalCer, OCH, and C-glycoside, respectively; αGalCer versus OCH p = 0.011, αGalCer versus C-glycoside p < 0.0001, OCH versus C-glycoside p = 0.016, Mann-Whitney U test). The decay of αGalCer/CD1d tetramer binding to 1.2 hybridoma cells also had a long half-life (Fig. 2B; t_1/2 = 197 min), although consistent with the equilibrium measurement, slightly shorter than that for 2C12. The low amount of staining of 1.2 hybridoma cells with tetramers loaded with the two other glycolipid Ags prevented this type of decay experiment.

**Glycolipid Ags display a hierarchy in intracellular signaling**

The reduced TCR half-life exhibited by OCH/CD1d and C-glycoside/CD1d in the tetramer decay experiments could be due to either a decreased TCR avidity or a decreased stability of binding of these glycolipids to CD1d. TCR engagement with CD1d/glycolipid complexes results in receptor proximal signaling. Therefore, we adapted an assay to measure the phosphorylation of ERK1/2 (26, 42), which requires only very brief periods to measure intracellular signaling events. As described by Altan-Bonnet et al. (42) for conventional T cells, we found that the Vα14i NKT cells exhibited an on–off or binary pp-ERK signal, with cells either ppERK+ or ppERK- (Fig. 2C). Thus, in Fig. 2D we display the data as percentage of ppERK+ cells over time. The percentage of Vα14i NKT cells that became pp-ERK1/2+ after incubation with glycolipid-pulsed APCs was dependent on the dose of the Ag, it could be blocked by an anti-CD1d mAb in a dose-dependent manner, and the MEK inhibitor UO126 ablated the signal (Supplemental Fig. 1). An unrelated peptide-reactive T cell hybridoma (B3Z) did not phosphorylate ERK1/2 after incubation with αGalCer-pulsed CD1d-transfected cells (Supplemental Fig. 1D). The rapidity, peaking at 1–5 min, and extent of ERK1/2 phosphorylation over the 30-min assay in these cells (Fig. 2D) was comparable to that of naive peptide-reactive T cells (26).

We measured the response to glycolipids presented by three APCs, including CD1d-transfected A20 B lymphoma cells, BMDCs, and splenic B cells (Fig. 2D). With fluorescent beads to quantify the number of CD1d molecules on each cell type, A20-CD1d cells expressed ~700,000 surface CD1d molecules,
BMDCs expressed ~30,000 molecules, and splenic CD19+ B cells expressed ~50,000 molecules (data not shown). The B cell population, however, is a mixture of more abundant follicular B cells, which expressed ~40,000 surface CD1d molecules, and marginal zone B cells, which expressed higher levels, ~130,000 (data not shown). When A20-CD1d cells were used as APCs, the extent of ERK1/2 phosphorylation induced by OCH and αGalCer were indistinguishable (Fig. 2D), despite the difference in equilibrium tetramer binding (Fig. 2A). This probably reflects the extraordinarily high expression of CD1d by the A20 cells, because when BMDCs or splenic B cells were used as APCs, OCH induced fewer 1.2 cells to phosphorylate ERK1/2 than αGalCer (Fig. 2D). These data are in keeping with the finding that OCH is a weaker Ag. C-glycoside induced very few 1.2 cells to phosphorylate ERK1/2 (Fig. 2D), in keeping with the finding that C-glycoside is a weaker Ag than either αGalCer or OCH. Therefore, the results from tetramer binding experiments and measurements of ERK phosphorylation are in agreement in showing that OCH and C-glycoside are both less potent Ags than αGalCer, with C-glycoside the weakest of the three Ags. Therefore, decreased TCR affinity for the Ag does not correlate with Th2 cytokine skewing.

Binding off-rate of glycolipid Ags to CD1d

We used the recently described monoclonal Ab L317, which recognizes αGalCer/CD1d complexes (25), to measure the off-rate of glycolipid Ag binding to CD1d. Using surface plasmon resonance we observed that L317 cross-reacts with CD1d in complex with either OCH or C-glycoside and that the affinity of L317 was the highest for αGalCer bound to CD1d, weakest for OCH, and significantly weaker for C-glycoside (Supplemental Table I). Because L317 was able to detect all three glycolipid Ags by surface plasmon resonance, we were able to measure the release of each of these glycolipids from CD1d, as described previously (13). After incubation for various times to induce decay of the glycolipid/CD1d complexes, the samples were analyzed by flowing L317 over the biosensor chip. Data were normalized to the maximum binding for each Ag. Note that the time needed for this Ab-based assay was too brief to be affected by the decay of Ab binding, even considering the shorter half-life of L317 binding to C-glycoside/CD1d complexes. The analysis indicated that αGalCer bound CD1d quite stably, with a half-life of 302.6 min (Table I and Supplemental Fig. 2). In keeping with previous reports (10, 19), OCH dissociated from CD1d more rapidly than αGalCer, with a half-life of 65.5 min. C-glycoside also dissociated more rapidly than αGalCer or OCH, with a half-life of 24.2 min. Thus, the off-rate for both OCH and C-glycoside for binding CD1d is faster than that for αGalCer, with C-glycoside showing a trend toward being even less stable in binding to CD1d than OCH (Table I). These results indicated that reduced stability of glycolipid ligand binding to CD1d was not likely to be sufficient for the induction of Th2 cytokine skewing.

OCH-CD1d crystal structure

OCH and αGalCer differ only in their alkyl chains, which are buried deep in the CD1d Ag binding groove. Therefore, the antigenic moieties appearing above the CD1d groove should appear similar. To analyze the underlying basis for the reduced TCR avidity for OCH/CD1d complexes and to determine if structural differences might account for the observed functional differences comparing OCH to αGalCer, we crystallized OCH bound to mouse CD1d and determined the structure to a resolution of 1.6 Å (Fig. 3 and Supplemental Table II). The general mode of glycolipid binding to mouse CD1d has been well documented (43, 44). More specifically, the OCH ligand binds with the C\textsubscript{24} fatty acid in the A’ pocket and the C\textsubscript{9} sphingosine moiety in the entrance to the F’ pocket, whereas the galactose is exposed at the CD1d surface for T cell recognition (Fig. 3B). Interestingly, because the sphingosine chain is truncated from the original C\textsubscript{18} of αGalCer to C\textsubscript{9}, the F’ pocket is not completely filled. Instead a spacer lipid is recruited to stabilize the remainder of the F’ pocket. Although not formally identified as such, the shape and length of the electron density for the spacer lipid suggests a short-chain fatty acid (C\textsubscript{8}, caprylic acid) rather than a more linear breakdown product of polyethylene glycol from the crystallization solution (Fig. 3B). However, both the weaker electron density and the higher b values for the spacer lipid suggest that not all of the CD1d molecules of the crystal are occupied by the spacer lipid. The galactose headgroup is stabilized by several hydrogen bond interactions with CD1d residues and also through water-mediated hydrogen bonds involving the 6’-OH of the galactose, the 4’-OH and the amide oxygen of the ceramide backbone of the ligand, and residues Thr\textsuperscript{156}, Ser\textsuperscript{76}, and Met\textsuperscript{69} of CD1d (Fig. 3C). These interactions have not been observed in any of the previous mouse or human αGalCer/CD1d structures (45, 46).

Although the overall binding of OCH is very similar to that of αGalCer, there is one major difference (Fig. 3D–H). The previously reported induced fit of mCD1d upon αGalCer binding (PDB 1Z5L) is not observed in the OCH structure, although it is observed in the recently determined structure of the trimolecular complex (PDB 3HE6). As a result, no F’ roof above the OCH ligand is formed, which in the αGalCer structure tightly tugs the sphingosine chain underneath by forming additional nonpolar van der Waals interactions. This structural difference explains the shorter half-life of the OCH/CD1d complexes as compared to that of the αGalCer/CD1d complexes. The induced fit is likely abrogated by the spacer lipid, which interferes with the reorientation of Leu\textsuperscript{84}. Reorientation of Leu\textsuperscript{84} and Leu\textsuperscript{150} are the key structural changes that lead to the induced fit (Fig. 3E), which in turn affects the position of amino acids such as Arg\textsuperscript{79} (Fig. 3E), which are known to be important for TCR recognition (23, 39, 47, 48). The binding of the galactose headgroup appears to be more dynamic. In the OCH as well as the αGalCer structure complexed with the TCR, the galactose is not as closely bound to the α2 helix, whereas the short-chain αGalCer from the unliganded mCD1d structure makes more intimate contacts (Fig. 3E). These differences between two highly similar ligands clearly illustrate that although the TCR docking orientation onto CD1d likely will be conserved in both ligand structures and likely will involve the same key residues of CD1d and the TCR, the interacting surfaces are not identical and, hence, likely to impact TCR avidity and signaling.

OCH and C-glycoside do not polarize Vα14i NKT cell cytokine production

As shown in Fig. 1, when the sera of immunized mice were analyzed, OCH induced a systemic Th2-biased response and C-glycoside induced a more Th1-biased response. We measured several features of Vα14i NKT cell activation to determine if these two Ags directly affect Vα14i NKT cells in different ways. Two hours after injection, αGalCer induced the highest percentage Vα14i NKT cells to produce IFN-γ, IL-4, and TNF (Fig. 4A and data not shown) and the highest level of CD69 and CD40L expression (Fig. 4C). By these criteria, OCH was less potent than αGalCer, and C-glycoside the least potent of the three. However, we did not observe an enhanced decline in the percentage of cells producing only one of the cytokines (Fig. 4B). We observed similar patterns of cytokine production both in the spleen at 2 h and in the spleen and liver 6 h after Ag injection (data not shown). Therefore, there was no immediate or early polarity in cytokine...
MECHANISMS FOR CYTOKINE POLARIZATION BY NKT CELLS

A

2C12

MFI of Tetramer Binding

12

Tetramer Concentration (nM)

0 5 10 15 20

αGalCer

△ OCH

▃ C-glycoside

■ Vehicle

B

2C12

Log (B/B0)

12

Time (Min.)

0 10 20 30 50 100

αGalCer 4°C

αGalCer

△ OCH

▃ C-glycoside

C

Time (min.): 0 1 5 10 20 30

αGalCer

0.84 83 89.4 48.3 64.1 66.9

C-glycoside

1.13 11.4 14.9 10.9 11.6 11.3

OCH

0.78 80.1 88.5 44.6 32.5 36.9

Vehicle

0.92 9.12 3.78 5 9.36 7.04

ppERK1/2

D

A20-CD1

BMDC

B cells

% ppERK1/2

0 10 20 30 40

Time (min.)

0 10 20 30

αGalCer

△ OCH

▃ C-glycoside

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production from Vα14i NKT cells activated with the different Ags, only a difference in the number of cells activated. These findings suggest that the systemic cytokine polarity of the immune responses induced by OCH and C-glycoside is likely mediated by events downstream, which could include the length of time of Vα14i NKT cell activation beyond 6 h or effects on other cells. Moreover, reduced early CD40L expression cannot by itself be responsible for Th2 cytokine polarization of the systemic immune response, as has been proposed (11), because OCH induced more CD40L than C-glycoside at 2 h after injection (Fig. 4C).

**Impaired NK cell trans activation by OCH**

Glycolipid Ag stimulation of Vα14i NKT cells in vivo causes the activation of several other cell types, a phenomenon sometimes called trans activation (49). Prominent among these events is the activation of NK cells to secrete IFN-γ (50), and in fact, most of the serum IFN-γ following αGalCer injection is due to NK cells (9). We examined IFN-γ production by NK cells 18 h after injection of Ags using intracellular cytokine staining (Fig. 5). αGalCer injection induced the highest percentage of IFN-γ–expressing NK cells in both the liver and the spleen, followed by C-glycoside and then OCH (Fig. 5). This corresponds to the levels of IFN-γ observed in the serum at 18 h (Fig. 1B), and it agrees with earlier observations comparing αGalCer to either C-glycoside (15) or OCH (11), although a direct comparison of OCH and C-glycoside has not been carried out previously. It is noteworthy, however, that the hierarchy of effectiveness for NK cell trans activation is different from those defined previously for TCR avidity and half-life, TCR proximal signaling events, and immediate cytokine production.

Because C-glycoside’s ability to trans-activate NK cells was discordant with its observed affinity for the TCR, we sought to determine if CD40/CD40L interactions were responsible, because they are required for αGalCer-mediated trans activation (9, 51). CD40L, expressed by Vα14i NKT cells after exposure to αGalCer, engages CD40 expressed by APCs. The APCs become activated and produce IL-12 (52), which contributes to NK cell production of IFN-γ. IFN-γ levels after either αGalCer or C-glycoside administration were strongly reduced in CD40-deficient mice (Supplemental Fig. 3). Therefore, the relatively strong activation of NK cells following C-glycoside, compared with OCH, requires a CD40-dependent mechanism, similar to trans activation following αGalCer, although as noted above, the amount of CD40L induced cannot explain the difference between OCH-induced and C-glycoside-induced responses.

**Enhanced stability of C-glycoside/CD1d complexes in vivo**

C-glycoside originally was synthesized because the replacement of the glycosidic oxygen with a CH₂ group likely would make this glycosphingolipid more resistant to catabolism in vivo. We tested the functional stability of the glycolipid Ags in vivo by immunizing mice and harvesting APCs from these mice at different times after immunization. These cells were then used in Ag presentation assays.

**Glycolipid Ags have a similar requirement for CD1d internalization**

A possible mechanism to explain the ability of the weak agonist C-glycoside to effectively trans-activate NK cells could derive from an enhanced requirement for this glycolipid Ag to be internalized for CD1d loading. This possibility is suggested by earlier work on the Th2 polarizing C20:2 glycolipid Ag, which exhibited a decreased requirement for CD1d internalization (12). A requirement for internalization could translate into preferential uptake in vivo by cell types, such as DCs, that might preferentially stimulate Th1 responses. Alternatively, as suggested previously (12), a requirement for internalization could be manifested at the single-cell level, by causing a preferential loading into CD1d molecules that locate to lipid rafts. Lipid raft localization of MHC class II molecules can affect the polarization of cytokine production by conventional CD4⁺ T cells (53), and some CD1d molecules are found in lipid rafts (54).

To address these possibilities with OCH and C-glycoside, we used mutant A20-CD1d cells, where CD1d was impaired in entry to endocytic compartments due to a Y → A point mutation in the four-amino acid motif, Tyr-Gln-Asp-Ile (YQDI), of the cytoplasmic tail. Three amino acids of this motif, including the tyrosine, are required for proper CD1d intracellular trafficking and presentation of glycolipid Ags dependent upon intracellular loading (55). In keeping with previously published reports (56), the disaccharide glycolipid Gal(α1-2)αGalCer (Fig. 1A) required the intact YQDI motif. This compound requires lysosomal carbohydrate processing to produce the monosaccharide αGalCer, which is loaded into CD1d molecules in lysosomes. The A20-CD1d cells with the Y → A point mutation (A20-CD1d YA) therefore could not present this compound. In this experiment, αGalCer stimulation had a partial requirement for normal CD1d internalization, as has been observed in some previous studies (12). By contrast, the presentation of OCH was not affected by the CD1d cytoplasmic tail point mutation (Fig. 6). C-glycoside was intermediate between OCH and αGalCer in terms of exhibiting sensitivity to the cytoplasmic tail mutation of CD1d. Our data therefore indicate that, unlike for the disaccharide, there is not an absolute requirement for normal endosomal localization of CD1d for Ag presentation. Furthermore, the data are not consistent with a strong requirement for normal CD1d internalization for Th1 cytokine production.

**Enhanced stability of C-glycoside/CD1d complexes in vivo**

C-glycoside originally was synthesized because the replacement of the glycosidic oxygen with a CH₂ group likely would make this glycosphingolipid more resistant to catabolism in vivo. We tested the functional stability of the glycolipid Ags in vivo by immunizing mice and harvesting APCs from these mice at different times after immunization. These cells were then used in Ag presentation assays.
We used anti-CD11c magnetic beads to enrich for DCs from the spleens of immunized mice, providing enrichment to ~30% CD11c⁺ MHC class II⁺ cells in the positive fraction, and a depleted fraction that was essentially devoid of this population (Supplemental Fig. 3A). We analyzed the DCs from these immunized mice to determine if DCs were altered differently following administration of particular compounds. Immunization with C-glycoside and OCH induced increased expression of CD80, CD86, and CD1d in splenic DCs to the same extent as αGalCer at 17.5 and 44 h (Supplemental Fig. 3B), suggesting that all three compounds induced a similar degree of DC maturation. To verify that the enriched DCs from the immunized mice remained capable of presenting glycolipids to Vα14i NKT cells, we incubated each enriched DC population with αGalCer in vitro, and the Ag-incubated cells were then re-isolated and cultured with the 1.2 hybridoma. DCs from mice immunized with each of the three Ags were capable of presenting αGalCer when isolated either 1.5 or 17.5 h after immunization (Supplemental Fig. 3C). When obtained from any of the glycolipid-immunized mice, however, the DCs induced less IL-2 from the 1.2 hybridoma than the DCs from vehicle-immunized mice. This suggests that Vα14i NKT cell activation reduced the ability of the DCs to present αGalCer to Vα14i NKT cells or that a highly stimulatory subpopulation of the DCs were eliminated in vivo.

A dose range of both the DC-enriched and the DC-depleted fractions from immunized mice were cultured with 1.2 hybridoma cells without exogenous Ag addition, and IL-2 release in the supernatant was used as a bioassay for the available glycolipid/CD1d complexes that remained. APCs from either the DC-enriched or the DC-depleted fractions stimulated IL-2 release when derived from mice immunized with αGalCer, OCH, or C-glycoside 1.5 h earlier (Fig. 2F). αGalCer gave the best response, and OCH led to a higher production of IL-2 than C-glycoside. At 17.5 and 44 h, however, the amount of activation induced following OCH immunization was considerably reduced compared with αGalCer and C-glycoside (Fig. 2F). This finding is particularly remarkable for C-glycoside, because it does not bind to CD1d in as stable a fashion as αGalCer. Furthermore, the number of available C-glycoside/CD1d complexes must be significantly higher than αGalCer/CD1d complexes to achieve the same IL-2 production, because the avidity of C-glycoside/CD1d for the invariant TCR is much lower. At 17.5 h, the DC-depleted fraction was capable of presenting αGalCer and C-glycoside to approximately one third of the amount as the enriched DCs (Fig. 2F).

**FIGURE 3.** Structural features of the mCD1d/OCH complex. A, The glycolipid OCH (yellow) is bound in the hydrophobic binding groove between the α1 and α2 helices of the CD1d heavy chain (gray) that noncovalently associates with β₂-microglobulin (β₂M, cream) to form a heterodimer. Three N-linked glycosylation sites (Asn²⁰ (N20), Asn⁴², and Asn¹⁶⁵) carry well-ordered carbohydrates (green sticks). A spacer lipid (cyan) is present in the binding groove to complement the short C₉ sphingosine chain of the synthetic ligand OCH. B, Electron density map (2₁Fo – F₂) for OCH and the spacer lipid bound in the A’ and F’ pockets of mCD1d is shown as a blue mesh and contoured at 1σ. The entire OCH ligand is very well ordered and gives rise to excellent electron density. Note that Leu¹⁰⁰ (orange) exists in two alternate conformations in the crystal structure, possibly because of the reduced length of the sphingosine chain. C, Hydrogen-bond interactions between CD1d residues (gray) and the polar moieties of OCH (yellow) are drawn as blue dotted lines with distance given in Angstroms for the direct CD1d/OCH hydrogen bonds. Water molecules (red balls with surrounding electron density) are bound to OCH and enhance the hydrogen-bond network between CD1d and OCH. D, Structure of CD1d with OCH bound (yellow) does not result in an induced fit, likely due to the presence of the short spacer lipid (cyan), which sterically interferes with reorientation of L84. The induced fit of mCD1d bound to short-chain αGalCer (PDB code 1Z5L in green) as well as full-length αGalCer (PDB code 3HE6 in gray) is shown superimposed. E, Direct binding comparison of OCH (yellow) and αGalCer (PDB code 1Z5L in gray) as well as αGalCer from the recent mCD1d/Vα14/β₂.TCR cocrystal structure (71) (PDB code 3HE6 in green). F–H, Surface representation with electrostatic potentials (red, electronegative; blue, electropositive; contoured from −30 to +30 kT/e). F, OCH (yellow) binding to mCD1d. G, αGalCer (green) binding to mCD1d (PDB 3HE6). H, αGalCer (gray) binding to mCD1d (PDB 1Z5L). Several residues that are involved in the shaping of the CD1d binding grooves or in polar interactions with OCH are depicted in the figure as one-letter code with residue number. Note the formation of the F’ roof in G and H, as a direct result of the induced fit, as illustrated in D.
44 h, only the enriched DCs induced detectable hybridoma IL-2 release, and this was decreased from the 17.5-h time point. Thus, from these data, we conclude that complexes of CD1d with αGalCer and C-glycoside are much more stable in vivo than those with OCH, with C-glycoside the most stable. This feature of C-glycoside allows for a sustained activation of Vα14i NKT cells that is correlated with its uniquely Th1-biasing properties.

Discussion

It is important to understand the rules by which Vα14i NKT cells can drive the overall immune response in either a Th1 or a Th2 direction, not only for achieving a basic understanding of the role of this T cell subset in immunity but also because clinical trials based on glycolipid Ag activation of the homologous population of human Vα24i NKT cells remain ongoing. It has been established that cytokines, such as the IL-12 elicited from APCs following engagement of TLRs, can stimulate IFN-γ secretion in the absence of IL-4 release by Vα14i NKT cells and, furthermore, that this does not require exogenous Ag (57–59). Similarly, IL-2 has been reported to cause the selective synthesis of IL-5 by Vα14i NKT cells (60). Functional subsets of Vα14i NKT cells that are precommitted to the synthesis of different cytokines (61, 62) may also be selectively engaged under some circumstances. The most practical means, however, of inducing preferentially Th1 or Th2 cytokines from the broad population of invariant NKT cells may be the use of particular glycolipid Ags. Here, therefore, we have focused on the mechanism whereby different glycolipid Ag variants that are closely related to αGalCer initiate different immune responses.

The finding that αGalCer stimulates Vα14i NKT cells to produce both Th1 and Th2 cytokines (63) triggered the synthesis of chemical variants that would cause a purely Th1- or Th2-biased immune response. Since that time, several variants have been produced (10–12, 14, 15, 64–70). Many of these variants bias toward a Th2 cytokine response, and of these, OCH, the first such Ag discovered, has been the most widely studied. It has been suggested that OCH acts to stimulate a more Th2 skewed response because of a reduced duration of TCR signaling, the result of a decreased half-life of binding to CD1d as well as a reduced TCR affinity (10, 19). Additional compounds have been reported to bias cytokines toward a Th1 response (65, 66). There have been only limited investigations, however, of the mechanism of action of Th1-biasing compounds. Furthermore, a side-by-side comparison of the mechanisms by which Th1- and Th2-biasing αGalCer variants skew the cytokine response had not been carried out previously.
We found that a shorter half-life of interaction with CD1d and a lower TCR affinity do not provide a simple explanation for Th2 cytokine skewing. OCH does indeed have a shorter half-life of binding to CD1d than αGalCer. This had been suggested previously using immune response assays and was established herein directly using a CD1d binding assay. C-glycoside binding to CD1d, however, tended to be even shorter lived than OCH binding. In agreement with earlier studies (10, 19), complexes of OCH/CD1d also had a lower TCR avidity than αGalCer/CD1d complexes. Regardless, with a variety of biochemical and functional assays, C-glycoside/CD1d complexes exhibited equal or even weaker binding to the Vα14i TCR. If there were a simple, linear relationship between cytokine polarization and the strength of the glycolipid/CD1d interaction or with the strength of the interaction of the glycolipid Ag/CD1d complex with the TCR, then C-glycoside should have stimulated a stronger Th2 response than OCH.

The decreased avidity of the invariant TCR for OCH complexes with mouse CD1d is surprising, because the chemical structures of the hydrophilic portions of OCH and αGalCer are identical. Previous surface plasmon resonance studies of the interaction of the human Vα24 invariant TCR with human CD1d, using different αGalCer variants, showed that the length of the sphingosine chain bound in the F′ pocket modulates TCR affinity (13). The authors proposed a model in which reduction of the sphingosine chain would result in partial collapse of the less than fully occupied F′ pocket, which in turn would change the CD1d T cell recognition surface. Although the mouse and human CD1d structures are very similar, the proposed F′ pocket collapse was not observed in the OCH/mouse CD1d structure, perhaps due to the recruitment of the spacer lipid. An additional difference comparing mouse and human is that binding of αGalCer does not lead to an induced fit in human CD1d, because there is no closing of the roof of the F′ pocket (13), although this occurs with mouse CD1d. It appears, however, that changes more subtle than the proposed F′ collapse are unique to αGalCer when bound to mouse CD1d. These changes, particularly those that close the roof over the F′ pocket, affect the CD1d surface and can account for the more stable CD1d binding exhibited by αGalCer. They also create a structure for TCR recognition of αGalCer that is different from that of OCH/mouse CD1d complexes, thereby explaining the TCR affinity difference. Interestingly, the recent crystal structure of the mouse CD1d/αGalCer/Vα14β8.2 TCR (71) also reveals the reported induced fit upon αGalCer binding. As a result, both α helices could be more dynamic in interacting with the various glycolipids than previously assumed.

Two factors could contribute to a polarization of the cytokine response induced following Ag activation of Vα14i NKT cells: the cytokine polarity of the Vα14i NKT cell response itself or the efficacy with which Vα14i NKT cells induce an indirect activation of other cell types. Our study examined both of these aspects. We have shown that although OCH induces amounts of serum IL-4 that are comparable to but not greater than αGalCer, it does induce less serum IFN-γ, whereas for C-glycoside the decrease in IL-4 synthesis is more profound. Therefore, for both OCH and C-glycoside, the cytokine bias is a relative one manifested most clearly as a difference in the proportion of the cytokines produced. Critically, although both glycolipids induced the predicted biased systemic cytokine responses, neither C-glycoside nor OCH caused a significant bias in the immediate cytokine production by Vα14i NKT cells, when measured by intracellular cytokine staining directly ex vivo. Instead, the immediate response to these compounds followed an affinity hierarchy, with αGalCer > OCH > C-glycoside, which related to the number of Vα14i NKT cells activated as opposed to a particular cytokine bias. This outcome agrees with results from some studies (15), but in other cases (11) a bias in the cytokines directly produced by Vα14i NKT cells was observed using different assays, such as cytokine production measured by ELISA. Although the ex vivo intracellular cytokine staining method that we used most closely reflects the in vivo activity of Vα14i NKT cells, because of the rapid TCR downregulation caused by some Ags, particularly αGalCer, it has the limitation of only detecting the early cytokine response. Therefore, it remains possible that at later times after activation the
different glycolipid Ags induced a more polarized cytokine response directly from Vα14i NKT cells, although this is unlikely to provide a complete explanation for their effects. In contrast to the TCR affinity hierarchy, C-glycoside induced a more effective trans-activation of NK cells to produce IFN-γ than OCH, although it was less effective than αGalCer. Therefore, our data are most consistent with the idea that the bias in cytokine production after OCH and C-glycoside immunizations occurs at least in part systemically rather than at the level of the initial response of the Vα14i NKT cell and that it is the result of differences in the trans-activation of other cell types, particularly NK cells, which are the major source of serum IFN-γ induced after stimulation with glycolipid Ags (9). Therefore, our data demonstrate that these Ags do not act as the glycolipid equivalents of altered peptide ligands, and rather than stimulating an altered pattern of cytokine production directly from Vα14i NKT cells, they act by altering the network of cellular interactions that occurs downstream following the initial stimulation event. This suggests that some other feature of the compounds, such as the length of time that they are able to stimulate Vα14i NKT cells, the preferential loading of the Ag in certain subcellular compartments, or the presentation by certain types of APCs, could explain the different responses that they elicit.

There is evidence that the Th2 bias elicited by the C20:2 analogue of αGalCer is related to its increased ability to be loaded into CD1d at the cell surface (12). While this paper was in preparation, we published a study showing that several Th2-biasing analogues of αGalCer, all with alterations in the fatty acid, are characterized by rapid and direct loading onto CD1d on the cell surface and exclusion from lipid rafts (20). Using cells that expressed a mutant version of CD1d with a reduced ability to enter endosomal compartments (55, 72), we observed that the responses to OCH and C-glycoside were not greatly different when compared with presentation by wild-type CD1d, although C-glycoside presentation was somewhat more affected. Of note, the glycolipids were pulsed onto the APCs for only 2 h prior to washing, and therefore in this time the cells expressing CD1d with a cytoplasmic tail mutation must have been able to take up for later binding, or bind to CD1d directly, similar or nearly similar amounts of glycolipid Ag as cells expressing wild-type CD1d. Our data suggest that factors other than exclusive or selective uptake of C-glycoside into deep intracellular compartments are likely responsible for some of the functional differences between OCH and C-glycoside and, furthermore, that there is unlikely to be a single explanation for the Th2-biasing effects of different compounds.

A striking finding from these studies is that C-glycoside forms long-lived, functional complexes on the surfaces of APCs in vivo, whereas OCH does not. APCs from mice injected with C-glycoside more than 17 h earlier stimulated Vα14i NKT cell hybridomas as effectively as APCs from mice injected with αGalCer. Considering that C-glycoside/CD1d complexes have a weaker TCR avidity, we conclude that within hours after Ag injection there must be more complexes of C-glycoside/CD1d than αGalCer/CD1d complexes. Our results are in agreement with those from a previous study that showed C-glycoside formed more stable complexes than αGalCer on bone marrow DCs loaded with compound in vitro and then tested for their ability to activate invariant NKT cells in vivo (73). While this paper was in preparation, it was reported that a Th2-biasing glycolipid with an acyl chain shortened to eight carbons can be internalized efficiently to lysosomes but that it is displaced from CD1d easily by other lipids in this acidic environment (74). As CD1d recycles from lysosomes to the cell surface, this internal displacement of the lipid Ag led to a shorter half-life of the glycolipid Ag/CD1d complexes on the cell surface (55, 72).

FIGURE 7. Stability of glycolipid/CD1d complexes in vivo. C57BL/6 mice were injected i.v. with 1 μg of the indicated glycolipids, and at the indicated times splenic CD11c+ DCs were enriched by collagenase digestion and MACS bead isolation. The enriched DCs or the DC-depleted cells were then cultured with the 1.2 Vα14i NKT hybridoma overnight, and IL-2 in the supernatant was assessed by ELISA. Results are plotted as the number of cells per well versus IL-2 (top, enriched DCs; bottom, DC-depleted cells). This experiment was performed twice with similar results.
such polarized cytokine responses. The Ag in cells will be a critical parameter for the induction of polarized cytokine response from human cells, and some success laboratories to synthesize glycosphingolipid Ags that induce a highly polarized cytokine response due to resistance to catabolism. Therefore, an important parameter is the functional stability of C-glycoside Ag presentation, which is likely due to a reduced stability of binding to CD1d. C-glycoside, by contrast, provides a weak TCR stimulus, but one that is prolonged and maintained over 17 h and increased relative to those of the other compounds, despite a reduced half-life of CD1 binding. This functional stability of C-glycoside Ag presentation is likely due to resistance to catabolism. Therefore, an important parameter for a glycolipid Ag to influence the cytokine response is the length of stimulation, with a longer stimulation in vivo promoting IFN-γ production due to more effective trans-activation of NK cells. The length of stimulation is likely influenced by the pharmacokinetics of the Ag. OCH and C-glycoside are not potent activators of human Vα24/γδ T cells, but efforts are underway in several laboratories to synthesize glycosphingolipid Ags that induce a highly polarized cytokine response from human cells, and some success is being achieved (75). Our findings suggest that the stability of the Ag in cells will be a critical parameter for the induction of such polarized cytokine responses.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary Figure Legends

Supplementary Figure 1. Detection of ERK1/2 phosphorylation is sensitive and specific. αGalCer induces a dose-dependent increase in ppERK. A20-CD1d cells were pulsed with the indicated dose of αGalCer for 2 h, washed and incubated with the 1.2 hybridoma for 5 min at 37°C. Phosphorylation of ERK1/2 was assessed by flow cytometry. Gates on the histograms indicate the percent positive cells. B. The phosphorylation of ERK1/2 can be inhibited by an anti-CD1d monoclonal antibody (1B1) in a dose-dependent manner. A20-CD1d cells were pulsed with 200 ng/mL αGalCer for 2 h, washed, incubated with 1B1 for 1 h, washed and incubated with the 1.2 hybridoma for 5 min at 37°C. C. The phosphorylation of ERK1/2 can be inhibited by the MEK inhibitor UO126. A20-CD1d cells were pulsed with 200 ng/mL αGalCer for 2 h, washed and incubated with the 1.2 hybridoma for 5 min at 37°C in the presence of the MEK inhibitor UO126 (20µM) or vehicle (Veh.). PMA was used as a positive control. “Vehicle” indicates A20-CD1d cells pulsed with the vehicle without αGalCer. “αGalCer + Veh” indicates αGalCer with the vehicle for UO126. D. A peptide-reactive hybridoma (B3Z) does not phosphorylate ERK1/2 in response to αGalCer. A20-CD1d cells were pulsed with 200 ng/mL αGalCer for 2 h, washed and incubated with the B3Z or 1.2 hybridomas for 5 min at 37°C. PMA was used as a positive control.

Supplementary Figure 2. Decay of CD1d/glycolipid complexes. Decay data are presented as time vs. normalized L317 mAb binding. After linear regression, glycolipid/CD1d complex half-life was determined by $t_{1/2} = \log_2$/slope. The calculated rates of decay for these assays are shown in Table I.
Supplementary Figure 3. CD40-CD40L interactions are essential for C-glycoside induced IFNγ production.

C57BL/6 mice (open symbols) and CD40-/− mice (closed symbols) were injected intravenously with 1 µg of the indicated glycolipid. After 6 h and 24 h, serum was isolated from the immunized mice and assessed for the quantity of IFNγ by ELISA. This experiment was performed twice with similar results.

Supplementary Figure 4. Enrichment of dendritic cells from the spleens of immunized mice.

1.5 h following glycolipid immunization, splenic CD11c⁺ dendritic cells were enriched by collagenase digestion and MACS bead isolation (see Materials and Methods for details) and analyzed by flow cytometry. For each glycolipid, or vehicle, immunized mouse, the enriched DCs (top panel) are compared with DC-depleted splenocytes (bottom panel), gating on IA^{b+} CD11c⁺ and IA^{b−} CD11c⁺ cells. Of these two populations, only the IA^{b+} CD11c⁺ cells carried antigenic complexes ex vivo and presented to the 1.2 hybridoma (data not shown). This analysis was carried twice out for each time point (1.5, 17.5 and 44h), with similar results.

Supplementary Figure 5. Phenotypic assessment of enriched DCs from glycolipid immunized mice.

After 1.5, 17.5 or 44h, splenic CD11c⁺ dendritic cells were enriched by collagenase digestion and MACS bead isolation and assessed for CD80, CD86 and CD1d expression by flow cytometry. Plots are shown gated on IA^{b+} CD11c⁺ cells. This experiment was performed twice with similar results.
Supplementary Figure 6. Presentation of αGalCer is reduced in glycolipid immunized mice.

After 1.5 or 17.5 h, splenic CD11c⁺ DC were enriched by collagenase digestion and MACS bead isolation (see Materials and Methods for details). Enriched DC from mice immunized with the indicated glycolipid were tested for the capacity to present αGalCer to the 1.2 Vα14i NKT hybridoma. Cells were cultured with 200 ng/mL αGalCer and the 1.2 Vα14i NKT hybridoma overnight. The level of IL-2 in the supernatant was assessed by ELISA. The 1.2 Vα14i NKT hybridomas alone did not produce IL-2 (data not shown). This experiment was performed twice with similar results.
Supplementary Figure 1  
Sullivan et al.
Supplementary Figure 3
Sullivan et al.

αGalCer C-glycoside

IFN, ng/ml

0 2 4 6 8 10 12

6 24

C-glycoside

0 0.5 1 1.5 2 2.5

6 24

Hours after injection
Supplementary Figure 4
Sullivan et al.

αGalCer | OCH | C-glycoside | Vehicle

Enriched DCs

DC-depleted splenocytes

CD11c | MHC II
time post immunization:

1.5 hrs

17.5 hrs

44 hrs

CD80

CD86

CD1d

αGalCer

OCH

C-glycoside

Vehicle

Figure 5

Supplementary

Sullivan et al.
Supplementary Figure 6
Sullivan et al.
### Supplementary Table I
Biophysical parameters of L317 binding to CD1d/glycolipid complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Analyte</th>
<th>$k_a$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
<th>$K_D$</th>
<th>interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1d/αGalCer</td>
<td>L317 monoclonal</td>
<td>$3.8 \times 10^6$</td>
<td>*</td>
<td>$5.1 \times 10^{-9}$</td>
<td>bivalent</td>
</tr>
<tr>
<td>CD1d/αGalCer</td>
<td>L317 Fab</td>
<td>$1.0 \pm 0.1 \times 10^6$</td>
<td>$5.3 \pm 0.1 \times 10^{-3}$</td>
<td>$5.1 \times 10^{-9}$</td>
<td>monovalent</td>
</tr>
<tr>
<td>CD1d/OCH</td>
<td>L317 monoclonal</td>
<td>$2.1 \times 10^6$</td>
<td>$1.4 \times 10^{-2}$</td>
<td>$6.7 \times 10^{-9}$</td>
<td>bivalent</td>
</tr>
<tr>
<td>CD1d/OCH</td>
<td>L317 Fab</td>
<td>$5.7 \pm 0.3 \times 10^6$</td>
<td>$1.7 \pm 0.6 \times 10^{-2}$</td>
<td>$3.0 \times 10^{-8}$</td>
<td>monovalent</td>
</tr>
<tr>
<td>CD1d/C-glycoside</td>
<td>L317 monoclonal</td>
<td>$2.7 \pm 0.7 \times 10^4$</td>
<td>$3.3 \pm 1.6 \times 10^{-2}$</td>
<td>$1.3 \times 10^{-7}$</td>
<td>bivalent</td>
</tr>
<tr>
<td>CD1d/C-glycoside</td>
<td>L317 Fab</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>monovalent</td>
</tr>
</tbody>
</table>

* no detectable dissociation
** no detectable association
Supplementary Table II
Data collection and refinement statistics for the mCD1d-OCH complex

<table>
<thead>
<tr>
<th>Data collection</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>30.0-1.6 (1.66-1.60)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.0 (97.7)</td>
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<tr>
<td>Number of unique reflections</td>
<td>63,903</td>
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<tr>
<td>Redundancy</td>
<td>5.8</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt;&lt;sup&gt;1,2&lt;/sup&gt; (%)</td>
<td>5.7 (63.2)</td>
</tr>
<tr>
<td>I/σ&lt;sup&gt;1&lt;/sup&gt;</td>
<td>29.7 (2.6)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Refinement statistics</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Number of reflections (f&gt;0)</td>
<td>63,900</td>
</tr>
<tr>
<td>Maximum resolution (Å)</td>
<td>1.6</td>
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<tr>
<td>R&lt;sub&gt;crys&lt;/sub&gt;&lt;sup&gt;3&lt;/sup&gt; (%)</td>
<td>19.5 (27.6)</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt; (%)</td>
<td>22.6 (27.7)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of atoms</th>
<th>3,524</th>
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<tbody>
<tr>
<td>Protein</td>
<td>2,932</td>
</tr>
<tr>
<td>Glycolipid ligand / spacer lipid</td>
<td>49 / 10</td>
</tr>
<tr>
<td>N-linked carbohydrate</td>
<td>56</td>
</tr>
<tr>
<td>Water</td>
<td>477</td>
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<table>
<thead>
<tr>
<th>Ramachandran statistics (%)</th>
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<tbody>
<tr>
<td>Favored</td>
<td>99.4</td>
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<tr>
<td>Disallowed</td>
<td>0</td>
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<table>
<thead>
<tr>
<th>R.m.s. deviation from ideal geometry</th>
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<tbody>
<tr>
<td>Bond length (Å)</td>
<td>0.015</td>
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<tr>
<td>Bond angles (°)</td>
<td>1.58</td>
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</table>

<table>
<thead>
<tr>
<th>Average B values (Å&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;5&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>22.0</td>
</tr>
<tr>
<td>Glycolipid / spacer lipid</td>
<td>32.1 / 44.4</td>
</tr>
<tr>
<td>Water molecules</td>
<td>36.7</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>37.0</td>
</tr>
</tbody>
</table>

| Disordered residues | A89-A91, A108-110, A198-202 |

<sup>1</sup>Number in parentheses refer to the highest resolution shell.
<sup>2</sup>R<sub>sym</sub>=(Σ<sub>i</sub>Σ<sub>j</sub> | I(i)-<I(h)>I| | Σ<sub>j</sub>I(h))x100, where <I(h)> is the average intensity of i symmetry-related observations for reflections with Bragg index h.
<sup>3</sup>R<sub>crys</sub>=Σ<sub>hkl</sub> | F_0-F_c | /Σ<sub>hkl</sub> | F_0 | x100, where F_0 and F_c are the observed and calculated structure factors, respectively, for all data.
<sup>4</sup>R<sub>free</sub> was calculated as for R<sub>crys</sub>, but on 3% of data excluded from refinement.
<sup>5</sup>B values were calculated with the CCP4 program TLSANL (Howlin, 1993 #70).