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*J Immunol* 2011; 187:4705-4713; Prepublished online 30 September 2011;
doi: 10.4049/jimmunol.1100794
http://www.jimmunol.org/content/187/9/4705

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/09/30/jimmunol.1100794.DC1

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NKT TCR Recognition of CD1d-α-C-Galactosylceramide

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NKT cells respond to a variety of CD1d-restricted glycolipid Ags that are structurally related to the prototypic Ag α-galactosylceramide (α-GalCer). A modified analog of α-GalCer with a carbon-based glycosidic linkage (α-C-GalCer) has generated great interest because of its apparent ability to promote prolonged, Th1-biased immune responses. In this study, we report the activation of spleen NKT cells to α-C-GalCer, and related C-glycoside ligands, is weaker than that of α-GalCer. Furthermore, the Vβ8.2 and Vβ7 NKT TCR affinity for CD1d-α-C-GalCer, and some related analogs, is ~10-fold lower than that for the NKT TCR-CD1d-α-GalCer interaction. Nevertheless, the crystal structure of the Vβ8.2 NKT TCR-CD1d-α-C-GalCer complex is similar to that of the corresponding NKT TCR-CD1d-α-GalCer complex, although subtle differences at the interface provide a basis for understanding the lower affinity of the NKT TCR-CD1d-α-C-GalCer interaction. Our findings support the concept that for CD1d-restricted NKT cells, altered glycolipid ligands can promote markedly different responses while adopting similar TCR-docking topologies. The Journal of Immunology, 2011, 187: 4705–4713.

The online version of this article contains supplemental material.
profound effects on NKT cell function (26, 27). Whereas some AGLs have the ability to promote Th2-biased responses (IL-4 > IFN-γ) downstream of NKT cell activation (28, 29), others promote Th1-biased responses (IFN-γ > IL-4) downstream of NKT cell activation. The AGL α-C-GalCer is considered the prototypical Th1-biased Ag, also known as C-glycoside, which possesses a CH2-based glycosidic linkage rather than the oxygen-based glycosidic linkage of α-Galectin and other natural glycosphingolipids (30). Further, α-1C-GalCer, a nonisosteric α-GalCer analog in which the anomeric carbon of galactose is bonded directly to the sphingolipid backbone, was found to induce a higher IFN-γ/IL-13 ratio in human NKT cells than α-GalCer and α-C-GalCer (31). This C-glycoside modification is intended to provide resistance to enzymatic degradation at the glycosidic linkage by glycosidases, thus increasing the stability of the glycosphingolipid in vivo (30). NKT cell stimulation with α-C-glycoside resulted in a Th1-biased response in vivo, and this is thought to be attributable to more stable presentation by APCs, more efficient activation of NKT cells, and prolonged downstream activation of IFN-γ production by NK cells (32). Consequently, α-C-GalCer provides superior protection against melanoma metastasis and malaria infection in mice (30, 33). It has also been suggested that the altered glycosidic linkage may change the way that this glycolipid sits in the CD1d Ag-binding cleft, resulting in structural differences and changes in NKT TCR interactions and affinity (30). This was supported by a recent study (34) that used a CD1d-α-C-GalCer tetramer dissociation assay, which indicated that CD1d-α-C-GalCer had significantly lower affinity for the NKT TCR than CD1d-α-GalCer.

Thus, studies using glycolipid AGLs demonstrate the feasibility of using these to manipulate the NKT cell response, which can translate to more tailored NKT cell-based therapies (reviewed in Ref. 5). However, to achieve more targeted NKT-based therapeutics requires a greater understanding of the molecular basis of antigenic modulation of the NKT cell response. Recently, we have provided insight into NKT TCR fine specificity against a range α-GalCer AGLs, including two Th2-biased ligands (OCH and C20:2) (13). However, the molecular basis for TCR recognition of Th1-biased ligands, such as the prototypical α-C-GalCer, and analogs thereof, was less clear. To address this, we report in this paper the structural and functional correlates of NKT TCR recognition of CD1d-α-C-GalCer and analogs thereof.

**Materials and Methods**

**Glycolipid Ags**

The glycolipid analogs used in this study were synthesized as previously described, although α-1C-GalCer was prepared by a modification of a previously reported route (30, 31, 35). All of the synthesized compounds were characterized by [1H]- and [13C]-nuclear magnetic resonance spectroscopy and high-resolution mass spectrometry.

**Mice**

C57BL/6 mice were maintained in the animal facilities of the Department of Microbiology and Immunology, University of Melbourne. All animal experiments were approved by and carried out in accordance with the guidelines of the University of Melbourne Animal Ethics Committee.

**CFSE labeling and proliferation assay**

Splenocytes from C57BL/6 mice were labeled with 2 μM 5,6-CFSE (Molecular Probes) and incubated for 10 min at 37°C. Splenocytes were pulsed with glycolipids at the indicated concentration and cultured overnight (5 × 10⁵ cells/well). Culture media was replaced the next day without further addition of glycolipids. Cells and culture supernatants were harvested at 72 h for proliferation and cytokine analysis. For proliferation analysis, cells were stained with α-GalCer-loaded CD1d tetramer, and the CFSE signal of gated NKT cells was measured.

**In vivo stimulation of NKT cells**

Mice were injected i.p. with 1 μg α-GalCer or α-C-GalCer dissolved in 200 μl PBS and serum cytokines examined at 2 and 24 h using cytometric bead array (CBA) as described below.

**Cytometric bead array**

Splenocyte culture supernatants were collected after 72 h and analyzed using CBA flex sets for mouse IFN-γ, IL-4, IL-10, IL-13, IL-17A, TNF, and GM-CSF (BD Biosciences). Serum samples were collected at 2 and 24 h and analyzed for IFN-γ and IL-4.

**Intracellular cytokine staining**

Splenocytes from C57BL/6 mice were cultured (5 × 10⁵ cells/well) with glycolipid for 8 h, with the final 4 h in the presence of Golgi-
Stop (BD Biosciences). Four replicate wells were pooled for each determination, and cells were surface stained with α-GalCer–loaded CD1d tetramer-PE and TCRβ–FITC, fixed, and permeabilized (Cytofix/Cytoperm Kit; BD Biosciences) prior to staining with anti–IFN-γ–allophycocyanin.

Protein expression, refolding, and purification of Vα14 NKT TCRs

The method for cloning, expression, and purification of the mouse Vα14 NKT TCRs has been previously described (11). After the initial refolding

**FIGURE 2.** In vitro stimulation of spleen NKT cells with α-C-GalCer and associated analogs. Spleen cells were cultured with 100, 10, and 1 ng/ml of the glycolipids as indicated. A, After 8 h, cell cultures were harvested and surface labeled with anti–TCR-β and α-GalCer–loaded CD1d tetramer and then fixed and permeabilized for ICS for IFN-γ production. B, The mean ± SEM from three independent experiments testing IFN-γ production by ICS (as per A) are shown. C, Cytokine production at 72 h from spleen cells cultured with the various analogs was determined by CBA. The means ± SEM from three independent experiments are shown. The exception is IL-10, which was only measured in two experiments. D, Representative data showing the proliferation of NKT cells in response to the different ligands were determined by CFSE-labeled spleen cells gating on α-GalCer–CD1d tetramer+ cells after 72 h. E, The CFSE proliferation results for NKT cells (as depicted in C) from four independent experiments (mean ± SEM) are shown. F, Cytokines detected in the serum of mice determined by CBA following the i.p. administration of 1 mg of glycolipid. The combined results from three independent experiments (10 mice/group) are shown. G, The ratio of serum IFN-γ (24 h) to serum IL-4 (2 h). For B, C, and E, the data readings for each individual experiment were the mean of duplicate cultures. Error bars represent SEM.
and dialysis, the NKT TCR was purified using gel filtration, ion exchange, and hydrophobic interaction chromatography. The refolded Vβ8.2 NKT TCR in 10 mM Tris (pH 8) and 150 mM NaCl was concentrated to 7–10 mg/ml for crystallography experiments. The Vβ8.2 and Vβ7 NKT TCRs were also used in the surface plasmon resonance (SPR) experiments.

**Protein expression, purification, and loading of mouse CD1d with glycolipid**

Mouse CD1d cloned with a BirA and 6X His tag in a dual promoter baculovirus transfer vector, pBac10pH, was kindly provided by Dr. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, CA). Mouse CD1d expression, purification, and lipid loading has been described previously (11). Loading of α-C-GalCer was improved using Triton X-100 (Sigma-Aldrich) at a final concentration of 0.05%.

**Protein crystallization, structure determination, and refinement**

The Vα14-Vβ8.2 NKT TCR–CD1d–α-C-GalCer complex (adjusted to 7 mg/ml in 10 mM Tris [pH 8] and 150 mM NaCl) crystallized in 15–17% PEG 10K, 0.1 M ammonium acetate, and 0.1 M BisTris (pH 5.5) after 2 to 3 d. The crystal morphology was further improved by seeding in 22–25% PEG 400. The crystals were flash frozen in mother liquor containing 25% PEG 400 as the cryoprotectant. The data were collected at the MX2 beamline at the Australian Synchrotron Facility in Melbourne, VIC, Australia. The crystals diffracted to 3.2 Å and belong to the space group P2₁2₁2₁. The data were processed using Mosflm version 7.0.5 and scaled using SCALA from the CCP4 suite (36). The crystal structure was solved using the molecular replacement method with the PHASER program from the CCP4 suite. The Vα14-Vβ8.2 NKT TCR–CD1d–α-GalCer complex [Protein Data Bank identification code 3HE6 (11)] minus α-GalCer was used as the search model, and the structure was subsequently refined using REFMAC. To prevent model bias, the Rfree set of the Vα14-Vβ8.2 NKT TCR–CD1d–α-GalCer structure was used in the experimental intensities scaling using SCALA as well as the implementation of the simulated annealing protocol in Phenix (37). Model building was carried out using COOT, and, at a later stage of refinement, translation libration screw parameters were included. The quality of both structures was validated using the Protein Data Bank validation Web site. All molecular graphics

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**FIGURE 3.** Binding analysis of Vβ8.2 and Vβ7 to CD1d-Ag. Equilibrium binding response curve for Vβ8.2 TCR to CD1d–α-C-GalCer (A), CD1d–α-C-GalCer (C), CD1d–α-C-alkyne-GalCer (E), and CD1d–α-N-Cyc-Cer (G) and Vβ7 TCR to CD1d–α-GalCer (B), CD1d–α-C-GalCer (D), CD1d–α-C-alkyne-GalCer (F), and CD1d–α-N-Cyc-Cer (H). The equilibrium dissociation constant [Kd(eq)] by fitting to one-site binding model. All results are shown as one representative experiment performed in triplicate (representative of two to three experiments).
The equilibrium data were analyzed using GraphPad Prism (GraphPad). CD1d endogenous complex from the Vfinity measurements, loaded CD1d was coupled to research grade SA chips constants. All measurements were taken in duplicate. For steady-state fit the data to the 1:1 Langmuir binding model to calculate the kinetic showed that the strongest response was induced by GalCer only triggered ∼12% of the NKT cells to produce detectable levels of IFN-γ at this dose. Of the other AGLs, α-C-alkyne-GalCer and α-N-Cyc-Cer were ∼10-fold lower again, and α-1C-GalCer and α-C-O-GalCer did not induce IFN-γ staining above background (Fig. 2A, 2B). The CBA data showed a similar trend for other cytokines, with α-C-GalCer inducing 2–5-fold lower amounts compared with α-GalCer (Fig. 2C) and the other AGLs inducing still lower amounts of the other cytokines assayed. There was little difference in the proliferative response to α-C-

Table I. Affinity measurements of C-glycoside analogs with Vβ8.2 TCR and Vβ7 TCR

<table>
<thead>
<tr>
<th>Vβ8.2 TCR</th>
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<td>α-GalCer</td>
<td>1.62 x 10^4 ± 9.5 x 10^3</td>
<td>0.02 ± 4.5 x 10^-4</td>
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<td>α-C-GalCer</td>
<td>2.25 x 10^4 ± 4.1 x 10^3</td>
<td>0.29 ± 0.02</td>
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<tr>
<td>α-N-Cyc-Cer</td>
<td>5.67 x 10^3 ± 1.67 x 10^4</td>
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<td>3.68 ± 0.27</td>
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<tr>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>α-C-O-GalCer</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>Vβ7 TCR</td>
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<tr>
<td>α-GalCer</td>
<td>2.31 x 10^4 ± 3.15 x 10^4</td>
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<td>0.38 ± 0.01</td>
<td>0.47 ± 0.03</td>
<td>7.70</td>
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<tr>
<td>α-C-GalCer</td>
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<td>ND</td>
<td>ND</td>
<td>3.11 ± 0.23</td>
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<tr>
<td>α-N-Cyc-Cer</td>
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<td>4.06 ± 0.10</td>
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<tr>
<td>α-C-Alk-GalCer</td>
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<td>ND</td>
<td>ND</td>
<td>3.23 ± 0.29</td>
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<tr>
<td>α-C-O-GalCer</td>
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</table>

*Steady-state affinity determined in separate experiment (see Materials and Methods).

PRR measurements and analysis

All SPR experiments were conducted at 25°C on a Biacore 3000 instrument (Biacore) using HBS buffer (10 mM HEPES-HCl [pH 7.4], 150 mM NaCl, and 0.005% surfactant P20 supplied by the manufacturer). For kinetic experiments, loaded CD1d was coupled to research grade SA chips to a level of 300–500 resonance units. Increasing concentrations of Vβ8.2 (0.078–10 μM) and Vβ7 TCR (0.19–25 μM) was injected over all flow cells at 50 μl/min for 60 s. The final response was calculated by subtracting the response of the CD1d endogenous complex from the Vβ8.2 or Vβ7–CD1d–Ag complex. BIAevaluation version 3.1 (Biacore) was used to fit the data to the 1:1 Langmuir binding model to calculate the kinetic constants. All measurements were taken in duplicate. For steady-state affinity measurements, loaded CD1d was coupled to research grade SA chips to a level of 2000 resonance units. Increasing concentrations of Vβ8.2 and Vβ7 TCR (0.039–40 μM) were injected over all flow cells at 5 μl/min for 90 s. The final response was calculated by subtracting the response of the CD1d endogenous complex from the Vβ8.2 or Vβ7–CD1d–Ag complex. The equilibrium data were analyzed using GraphPad Prism (GraphPad).

Results

The proliferative and functional response of NKT cells to α-C-GalCer analogs

A series of α-GalCer analogs with modified α-glycosidic linkages were tested for their ability to stimulate splenic NKT cell proliferation and cytokine production. These α-glycoside analogs included α-C-GalCer (structurally identical to that used in previous publications) with a CH₂ group in place of the glycosidic oxygen atom (30); a nonisosteric analog that has one less CH₂ group than α-C-GalCer (31); a compound with a rigid triple bond in the link between the sugar and the sphingolipid (α-1C-GalCer) (31); a compound with a rigid triple bond in the link between the sugar and the sphingolipid (α-C-alkyne-GalCer) (35); a variant bearing an ether oxygen atom in the linker (α-C-O-GalCer); and aaminocyclitol variant (α-N-Cyc-Cer) (39). The structures of these compounds and that of the O-glycoside standard (α-GalCer) are displayed in Fig. 1. IFN-γ production was examined at 8 h by intracellular cytokine staining (ICS) (Fig. 2A, 2B), and several cytokines were examined at 72 h spleen cell cultures by CBA (Fig. 2C). IFN-γ production at 8 h showed that the strongest response was induced by α-GalCer with ~25% IFN-γ at the highest dose (100 ng/ml), whereas α-C-GalCer only triggered ~12% of the NKT cells to produce de

FIGURE 4. Structure of Va14-Vβ8.2 NKT TCRs in complex with CD1d–Ag. A, Va14-Vβ8.2 NKT TCR in complex with CD1d–α-C-GalCer (accession number 3TN0). α-C-GalCer, blue; CD1d heterodimer, gray; Va14, cyan; Vβ8.2, green; CDR1α, purple; CDR3α, yellow; CDR1β, teal; CDR2β, red; CDR3β, not modeled. B, Va14-Vβ8.2 NKT TCR in complex with CD1d–α-C-GalCer (from Ref. 11, accession number 3HE6). α-GalCer, magenta; CDR3β, orange; CD1d, Va14, Vβ8.2, CDR1α, CDR3α, CDR1β, and CDR2β color coding as in A. C, Footprint of Va14-Vβ8.2 on the surface of CD1d–α-C-GalCer. α-C-GalCer is shown in spheres. CD1d, α-C-GalCer, and CDR loops color coding as in A. D, Footprint of Va14-Vβ8.2 on the surface of CD1d–α-GalCer. CD1d, α-GalCer, and CDR loops color coding as in A and B.
GalCer compared with α-GalCer, with nearly all cells proliferating at the two highest doses (100 and 10 ng/ml) and only a slight drop in proliferation at the lowest dose (1 ng/ml) (Fig. 2D, 2E). Moderate proliferation was observed for α-C-alkyne-GalCer and α-NCyc-Cer, especially at the highest dose, where no proliferation (above background) was detected for α-1C-GalCer and α-C-O-GalCer. The reduced IFN-γ response following α-C-GalCer compared with α-GalCer stimulation in vitro seemed inconsistent with α-C-GalCer’s apparent ability to promote Th1-biased responses in vivo (30). Therefore, we compared the serum cytokine response to in vivo challenge with these two glycolipids. Consistent with a recently published study (34), we found that α-C-GalCer induced lower amounts of both IL-4 and IFN-γ production in vivo compared with α-GalCer (Fig. 2F), although the ratio of the peak IFN-γ response (at 24 h) to the peak IL-4 response (at 2 h) still revealed a Th1-like bias (Fig. 2G), similar to that in earlier reports. This is thought to be due to greater in vivo stability of α-C-GalCer and prolonged production of IFN-γ by downstream NK cells in vivo rather than more potent IFN-γ production by NKT cells (30, 34).

Taken together, these data are consistent with earlier studies suggesting that α-C-GalCer is a weaker NKT cell agonist than α-GalCer (34). Moreover, our data demonstrate that of the AGLs with modified glycosidic linkages tested in this study, α-C-GalCer is the most potent with respect to proliferative response and cytokine production, followed by α-C-alkyne-GalCer and α-NCyc-Cer, whereas the α-C-O-GalCer and α-1C-GalCer AGLs were greatly diminished in their potency.

Affinity measurements
Previous studies using a tetramer-binding assay with Vα14/β8.2+ NKT cell hybridomas showed that CD1d-α-C-GalCer tetramer bound to these cells with a much weaker avidity than the CD1d-α-GalCer complexes (34). We determined the affinity of the interaction between the Vβ8.2 and Vβ7 NKT TCRs and CD1d-α-C-GalCer complex (and analogs thereof) using SPR and compared these values to the CD1d-α-GalCer interaction (Fig. 3A–I). The affinity for the Vβ8.2 NKT TCR was ≈2 μM, which was determined by response at equilibrium (equilibrium dissociation constant, Kd(app)) (Fig. 3C) and was significantly weaker than that of the corresponding NKT TCR-CD1d-α-C-GalCer interaction (300 nM) (Fig. 3A) (Table I). The affinity of the Vβ7 NKT TCR for CD1d-α-C-GalCer was ≈3 μM (Fig. 3D) and 470 nM for the α-GalCer interaction; Fig. 3B), indicating little difference between the affinity of Vβ8.2 and Vβ7 NKT TCRs for α-C-GalCer. We also determined the rate constants of the interaction between the Vβ8.2 NKT TCR and CD1d-α-C-GalCer. For the NKT TCR-CD1d-α-C-GalCer interaction, the on-rate was approximately the same (k1 = 1.62 × 105 M−1 s−1), the off-rate was k2 = 0.02 s−1, and accordingly, the t1/2 was long, ~35 s (13) (Supplemental Fig. 2, Table I). In contrast, for the NKT TCR–CD1d–α-GalCer interaction, the on-rate was approximately the same (k1 = 2.25 × 105 M−1 s−1), yet the off-rate was faster (k2 = 0.29 s−1), such that t1/2 of the interaction was only 2.4 s (Supplemental Fig. 2, Table I). Recently, we have shown that for glycosyl-based modifications, the t1/2 on the interaction is linked to the potency of the ligand, which is consistent with α-C-GalCer being a less potent inducer of IFN-γ production from NK cells compared with α-GalCer (25) (13). We also measured the affinity of the interaction between the Vβ8.2 and Vβ7 NKT TCRs and the other AGLs depicted in Fig. 1 (Fig. 3E–H, Table I). For the five AGLs tested, the affinities between the Vβ8.2 and Vβ7 NKT TCRs were similar (ranging from ≈1.6–4 μM), indicating no marked hierarchical recognition properties, whereas Vβ8.2 NKT TCRs trended toward preferential

<table>
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<tr>
<th>CDR Loop</th>
<th>Vβ8.2 in α-GalCer Complex</th>
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<th>Bond</th>
<th>CDR2β in α-GalCer Complex</th>
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<td>Pro⁹⁰⁶¹</td>
<td>Ser⁹⁰⁶²</td>
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<td>Salt-bridge</td>
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<td>Arg⁶³</td>
<td>2'-OH, 3'-OH</td>
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</table>

Atomic contacts determined using the CCP4i implementation of CONTACT and a cutoff of 4.5 Å. VDW interactions defined as nonhydrogen bond contact distances of ≤4 Å. Hydrogen bond interactions are defined as contact distances of ≤3.3 Å. Salt bridge is defined as contact distance of ≤4.5 Å. α-GalCer complex data are derived from Ref. 11 and shown for comparison.
recognition of α-GalCer when compared with Vβ7 NKT TCRs (Table I) (24). The binding affinity for the α-C-alkyne-GalCer and α-N-Cyc-GalCer analogs were similar to α-C-GalCer (Table I), indicating that the modifications did not appreciably affect the affinity of the interaction, despite the differing stimulatory activity to these Ags. This finding is consistent with previous studies in which other factors (e.g., Ag processing and presentation mechanisms and ligand stability), besides binding affinity of the NKT TCR, can contribute to the biological responses of NKT cells (34, 40). Nevertheless, consistent with previous studies on α-GalCer analogs (13), the levels of cytokines produced by the NKT cells broadly reflects the NKT TCR affinity of these α-C-glycoside ligands.

No measurable binding affinity was observed for the α-1C-GalCer and the α-C-O-GalCer analogs (Table I), consistent with the lack of NKT cell stimulation in response to these ligands (Fig. 2). Taken together, our findings suggest that these two analogs are not CD1d-restricted Ags for mouse NKT cells.

The NKT TCR–CD1d–α-C-GalCer complex

Because the ceramide base and the α-galactosyl head group of α-C-GalCer is identical to α-GalCer, the only difference is the CH₂ versus O-bound glycosidic linkage; thus, it is not obvious why there would be such a large difference in TCR binding affinity to this glycolipid analog. To determine how the NKT TCR binds to CD1d–α-C-GalCer and to understand why the affinity of this interaction was much weaker than the NKT TCR–CD1d–α-GalCer interaction, we expressed and refolded the Vβ8.2 NKT TCR and ligated it to CD1d specifically loaded with α-C-GalCer. We subsequently determined the structure of Vα14-Jβ18-Vβ8.2 NKT TCR–CD1d–α-C-GalCer complex to 3.2 Å resolution to an Rfree of 22.6 and 28.9%, respectively (Fig. 4A, Supplemental Table I, accession number 3TN0). The initial phases clearly showed unbiased electron density for α-C-GalCer head group (Supplemental Fig. 1). The electron density at the NKT TCR–CD1d–α-C-GalCer interface was unambiguous, with the exception of a mobile CDR3 loop (residues 94–104) and side-chains of Leu⁹⁸α and Arg¹⁰³α of the CDR3α, which were not resolved in the final structure. Consistent with this, some previous Vβ8 NKT TCR–CD1d–Ag structures have exhibited mobility in the CDR3β loop (11, 13). The structure of the NKT TCR–CD1d–α-C-GalCer complex enabled us to compare it to the closely related NKT TCR–CD1d–α-GalCer complex that had been solved to similar resolution (11) (Fig. 4A, 4B). Further, the NKT TCR–CD1d–α-C-GalCer and NKT TCR–CD1d–α-GalCer complexes crystallized in identical space groups and isomorphous unit cell dimensions, and thus, any structural differences observed between these two NKT TCR–CD1d–Ag complexes can be attributed to the impact of the specific modification of the α-C-GalCer.

The NKT TCR adopted the docking mode observed for the NKT TCR–CD1d–α-GalCer complex (11, 12), indicating that the α-C-GalCer did not cause a significant repositioning of the NKT TCR. Namely, within the NKT TCR–CD1d–α-C-GalCer complex, the NKT TCR bound approximately parallel to, and above, the F-pocket of the CD1d–Ag binding cleft (Fig. 4C). The buried surface area (BSA) upon ligation is ≈690 Å², which is lower than BSA ≈760 Å² for the complex with α-GalCer (Fig. 4C, 4D). The lower BSA is attributable to the increased mobility of the CDR3α loop within this complex, in which the Leu⁹⁸α and Arg¹⁰³α side chains were not resolved in the electron density and thus were not included in the final refined model. In the NKT TCR–CD1d–α-GalCer complex, Leu⁹⁸α and Arg¹⁰³α make specificity-governing contacts with CD1d. Thus, the increased mobility of Leu⁹⁸α and Arg¹⁰³α in the NKT TCR–CD1d–α-C-GalCer complex would contribute to the lower affinity of this interaction. Similar observations were observed in the structure of the NKT TCR–CD1d–α-glucosylceramide complex, in which increased mobility of the CDR3α loop was also attributed to a lower affinity interaction (13).

Within the NKT TCR–CD1d–α-C-GalCer interface, the TCR α-chain contributes 68% of the BSA, in which the CDR1α and CDR3α contributed 20 and 48% of the BSA, respectively. As observed previously in the Vα14-Vβ8.2 NKT TCR–CD1d–Ag structures, the Vβ8.2 interactions were mediated solely via the CDR2β (32% BSA). These values are very similar to those in the NKT TCR–CD1d–α-GalCer interaction, with the CDR1α, CDR3α, and CDR2β loops contributing 17, 57, and 26%, respectively (11). The slightly higher BSA of the CDR2β in the NKT TCR–CD1d–α-C-GalCer complex arises from small differences in the Vα-Vβ juxtapositioning between the two ternary complexes. The CDR2β interactions include the conserved interactions mediated by Tyr⁴⁸β, Tyr⁸⁶β, and Glu⁸⁶β, which have been observed in all Vβ8.2 NKT TCR–CD1d–Ag complexes solved to date (11, 13).

Regarding the TCR α-chain, most of the interactions observed in the NKT TCR–CD1d–α-GalCer complex were preserved in the NKT TCR–CD1d–α-C-GalCer complex (11), thereby collectively providing a focused network of polar and salt-bridging interactions with CD1d (Table II). However, in comparison with the NKT TCR–CD1d–α-GalCer complex, there was a loss of an H-bond between main-chain Gly⁸α and Asp¹⁵β of the α2 helix of CD1d in the NKT TCR–CD1d–α-C-GalCer complex (Fig. 5A, 5B). The loss of this interaction was attributable to a 1 Å backbone shift at the Gly⁸α tip of the CDR3α loop, which was a result of
a small shift in the head group position at the C3 carbon, whereby α-C-GalCer sat in a higher position at this carbon compared with that of α-GalCer (Fig. 5C).

Interactions with the glycosyl head group

Similar to the CD1d–α-GalCer interactions observed in the NKT TCR–CD1d–α-GalCer complex, the 2′-OH and 3′-OH groups of the galactosyl moiety of α-C-GalCer H-bonded to Asp95, whereas the 3′-OH and 4′-OH of the sphingosine chain H-bonded to Asp390 (11). However, due the presence of a CH3 linkage in α-C-GalCer instead of a glycosidic O linkage in α-GalCer resulted in loss of the H-bond between Thr156 and the lipid (Fig. 5A, 5B). As previously observed with Vβ8.2 NKT TCRs, the CDR1α and CDR3α loops interact with the galactosyl head group of α-C-GalCer (11). Within the ternary complex, replacement of the glycosidic oxygen in α-GalCer with a CH2 group in α-C-GalCer caused no major reorientation of the head group (Fig. 5C). In comparison with NKT TCR–CD1d–α-GalCer, most of the interactions between α-C-GalCer and the TCR are maintained. Namely, the galactose ring sat underneath the CDR1α loop and abutted the CDR3α loop, forming van der Waals (VDW) contacts on one face of the sugar ring. Further, Gly96α H-bonded to the 2′-OH group, whereas Asn90α forms H-bonds to the 3′-OH group of the galactose ring. However, dissimilar to the NKT TCR–CD1d–α-GalCer complex, the H-bond between Asn90α and the 4′-OH group was absent, which was attributable to the slightly differing orientations of the respective galactosyl head groups (11) (Fig. 5D). As we have recently shown that the 4′-OH group of α-GalCer is more critical than the 3′-OH group in mediating contacts with the Vβ8.2 NKT TCR, the observation of a loss of a H-bond between the 4′-OH group of α-C-GalCer and the NKT TCR is consistent with the lower affinity of the interaction compared with the α-GalCer interaction (Table I) (13).

Discussion

The structures of a number of NKT TCR–CD1d–Ag complexes determined to date have shown that a conserved docking topology underpins the interaction, regardless of the nature of the bound antigenic ligand or, indeed, Vα-Jα and Vβ usage (13–18). Nevertheless, some CD1d-restricted ligands can exert markedly differing biological effects, and the α-C-GalCer ligand is considered the prototypical Th1-biasing ligand. Our results show that the NKT TCR–CD1d–α-GalCer and α-C-GalCer docking topologies are very similar. We provide a clear molecular basis for the lower affinity interaction observed with α-C-GalCer compared with α-GalCer, which was largely attributable to reduced contacts between the NKT TCR and the sugar head group of α-C-GalCer as well as the α-2 helix of CD1d. Similarly, in comparison with the Vβ8.2 NKT TCR–CD1d–α-GalCer complex, Aspeslagh et al. (14) reported a loss of an interaction between a Vβ8.2 NKT TCR and CD1d–C-GalCer, in which a loss of H-bond between Arg95α to the 3-OH of phytosphingosine was observed in comparison with the loss of H-bond between Asn90α of the Vβ8.2 NKT TCR to 4′-OH of galactose reported in this study. These differences could be attributable to the differing CDR3β sequences used in the respective studies, which is consistent with the much higher affinity (247 nM) for α-C-GalCer reported by Aspeslagh et al. (14) in contrast to the affinity measurements reported in this paper (2 μM). Regardless, the diminished contacts in the NKT TCR–CD1d–α-C-GalCer complex most likely explains the reduced effects on NKT cell activation and cytokine production against α-C-GalCer and its related analogs tested in this study. Moreover, these reduced contacts were consistent with the much shorter t1/2 of the interaction when compared with the NKT TCR interaction with CD1d–α-GalCer. However, despite this, the proliferative response of NKT cells to α-C-GalCer was only slightly reduced compared with α-GalCer, which is consistent with our earlier report showing that TCR affinity for glycolipid analogs does not directly correlate with proliferation. This may also reflect an earlier report demonstrating the sustained in vivo response to this analog despite reduced short-term cytokine production (34). Taken together, our molecular and functional data for α-C-GalCer and analogs thereof, in combination with an earlier report (34), highlight the fact that even subtle changes in glycolipid structure can have profound effects on TCR–glycolipid–CD1d contacts and the affinity of the NKT TCR interaction. In turn, this can clearly affect the initial stages of NKT cell activation and cytokine production, whereas the duration and downstream effects of NKT cell activation appear to be less associated with the affinity of the ligand and more closely linked to the stability of its presentation.

Collectively, our findings provide a basis for understanding the fine specificity of the NKT cell-mediated response that will be of enormous value in tailor making NKT cell AGLs for therapeutic benefit.

Acknowledgments

We thank the synchrotron staff at the MX2 beamline of the Australian synchrotron for assistance with data collection and David Taylor and the animal house staff at the University of Melbourne, Department of Microbiology and Immunology, for animal husbandry assistance. We also thank the National Institutes of Health tetramer facility for providing us with α-C-GalCer and Maria Sandoval for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


**Supplementary Data**

**Supplementary Figure 1. Omit map of α-C-GalCer in mouse CD1d binding cleft.**
Omit map contoured at 1 sigma. α-C-GalCer, blue; CDR1α, purple; CDR3α, yellow; mCD1d, grey.

**Supplementary Figure 2. Differential affinity binding kinetics of Vβ8.2 and Vβ7 to CD1d-Ag.**

Binding of increasing concentrations of Vβ8.2 TCR to CD1d-α-GalCer (A) or CD1d-α-C-GalCer (B). Binding of Vβ7 TCR to CD1d-α-GalCer (C) or CD1d-α-C-GalCer (D).
All results are shown as one representative experiment of two. Affinities derived in these experiments for α-GalCer/CD1d were generally lower than we have previously reported (refs 11 and 13), which is most likely to be due to the recognised differences (Rich et al, 2009) between the Biacore SPR instrument used in this study versus the Bio-Rad ProteOn used previously (refs 11 & 13)

**Supplementary Table 1**

Data collection and refinement statistics

Reference
### Supplementary Table 1 Data collection and refinement statistics

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1. \(R_{p.i.m} = \Sigma_{hkl} [1/(N-1)]^{1/2} \left| \Sigma_{hkl,i} - <I_{hkl}> \right| / \Sigma_{hkl} <I_{hkl}>\)

2. \(R_{factor} = (\Sigma |F_o| - |F_c|) / (\Sigma |F_o|)\) - for all data except as indicated in footnote 3.

3. 5% of data was used for the \(R_{free}\) calculation

Values in parentheses refer to the highest resolution bin.

4. The residues that could not be modelled in the structures were CD1d: residues 1-6, 300-302; β₂m: residue 1; TCR α chain: residues 1, 134-135, 209-210; TCR β chain: residues 1-2, CDR3β loop 96-102.
Supp. Fig. 2