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Human and Mouse Type I Natural Killer T Cell Antigen Receptors Exhibit Different Fine Specificities for CD1d-Antigen Complex*[S]

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Kwok S. Wun[‡], Fiona Ross[§], Onisha Patel[‡1], Gurdyal S. Besra[¶], Steven A. Porcelli[‖], Stewart K. Richardson, Santosh Keshipeddy**, Amy R. Howell**, Dale I. Godfrey[§2,3], and Jamie Rossjohn[‡2,4]**

*From the [‡]Australian Research Council (ARC) Centre of Excellence in Structural and Functional Microbial Genomics, Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria 3800, Australia, the [§]Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia, the [¶]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom, the [‖]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461, and the **Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269-3060*

Background: Natural killer T cell antigen receptors (NKT TCRs) are restricted to lipid antigens presented by CD1d.
Results: Fine specificity differences between human and mouse NKT TCRs toward CD1d-antigen complexes were observed.
Conclusion: A structural basis underpins the fine specificity differences between human and mouse NKT TCRs.
Significance: Understanding human NKT cell response to CD1d-restricted antigens has important therapeutic implications in developing NKT cell agonists.

Human and mouse type I natural killer T (NKT) cells respond to a variety of CD1d-restricted glycolipid antigens (Ags), with their NKT cell antigen receptors (NKT TCRs) exhibiting reciprocal cross-species reactivity that is underpinned by a conserved NKT TCR-CD1d-Ag docking mode. Within this common docking footprint, the NKT TCR recognizes, to varying degrees of affinity, a range of Ags. Presently, it is unclear whether the human NKT TCRs will mirror the generalities underpinning the fine specificity of the mouse NKT TCR-CD1d-Ag interaction. Here, we assessed human NKT TCR recognition against altered glycolipid ligands of α-galactosylceramide (α-GalCer) and have determined the structures of a human NKT TCR in complex with CD1d-4′,4″-deoxy-α-GalCer and CD1d-α-GalCer with a shorter, di-unsaturated acyl chain (C20:2). Altered glycolipid ligands with acyl chain modifications did not affect the affinity of the human NKT TCR-CD1d-Ag interaction. Surprisingly, human NKT TCR recognition is more tolerant to modifications at the 4′-OH position in comparison with the 3′-OH position of α-GalCer, which contrasts the fine specificity of the mouse NKT TCR-CD1d-Ag recognition (4′-OH > 3′-OH). The fine specificity differences between human and mouse NKT TCRs was attributable to differing interactions between the respective complementarity-determining region 1α loops and the Ag. Accordingly, germline encoded fine-specificity differences underpin human and mouse type I NKT TCR interactions, which is an important consideration for therapeutic development and NKT cell physiology.

NKT cells[5] are CD1d-restricted, lipid antigen-reactive T cells that are present in mice and humans. The most extensively studied NKT cells, known as type I NKT cells, are defined by their expression of an invariant TCRα-chain (Vα24Jα18 in humans and the orthologous Vα14Jα18 in mice) paired with a limited range of TCRβ chains (Vβ11 in humans and Vβ8, Vβ7, or Vβ2 in mice) (1). Type I NKT cells are also defined by their ability to recognize the synthetic glycolipid antigen α-galactosylceramide (α-GalCer) presented by CD1d (2). Type II NKT cells, by contrast, do not express the invariant TCRα chain that characterizes type I NKT cells, nor do they recognize α-GalCer, and they can adopt distinct CD1d-Ag docking modes when compared with type I NKT TCRs (3–5). This study will focus on type I NKT cells, which from here on will simply be referred to as NKT cells.

The structures of human and mouse NKT TCRs have been solved with a variety of CD1d-restricted Ags, including α-GalCer and analogues thereof, microbial ligands, phospholipid Ags, and β-linked self-Ags (6–18). Collectively, these NKT TCR-CD1d-Ag complexes exhibited a conserved docking strategy in which the NKT TCR adopted a parallel docking mode above the F′-pocket of the CD1d Ag-binding cleft (19–24). Within this common footprint, the NKT TCR α-chain dominated the interaction, binding to CD1d and Ag, whereas

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[S] This article contains supplemental Table S1 and Fig. S1.
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[1] Supported by an ARC Future Fellowship.
[2] Both authors contributed equally to this work.
[3] Supported by an NHMRC Senior Principal Research Fellowship. To whom correspondence may be addressed. Tel.: 613-8344-5689; Fax: 613-93471540; E-mail: godfrey@unimelb.edu.au.
[4] Supported by an NHMRC Australia Fellowship. To whom correspondence may be addressed. Tel: 613-99029236; Fax: 613-99025000; E-mail: jamie.rossjohn@monash.edu.

[5] The abbreviations used are: NKT, natural killer T cells; α-GlcCer, α-glucosylceramide; α-GalCer, α-galactosylceramide; Ag, antigen; CDR, complementarity-determining region(s); TCR, T cell antigen receptor; AGL, altered glycolipid ligand; MFI, mean fluorescence intensity; BSA, buried surface area.

the β-chain exclusively contacted CD1d. Nevertheless, variations on a theme were apparent in that there were differing roles of the complementarity-determining regions (CDR) of the NKT TCRs for some CD1d-Ag complexes (13, 14, 25). For example, NKT TCR-CD1d autoreactivity seems attributable to a greater role of the CDR3β loop, and altered juxtapositioning of the Vβ8/7/2 chains results in differing Vβ-mediated footprints that can impact on Vα-Jα interactions with the CD1d-Ag (6, 16, 21). Moreover, alterations in the Vα and/or Vβ usage within the NKT TCR can impact on the specificity toward CD1d-restricted Ags. For example, the Vα10 NKT TCR preferentially recognizes α-glucosylceramide (α-GlcCer) over α-GalCer (26), whereas Vβ7 NKT TCRs preferentially respond to iGb3 and α-GlcCer (9, 27, 28). Moreover, $V\alpha24^+$ and $V\alpha24^-$ NKT TCRs can respond differently to α-GalCer and the closely related α-GlcCer (29).

Although NKT cells are activated by a range of CD1d-restricted Ags, they also discriminate between closely related Ags (termed altered glycolipid ligands, AGLs) to generate distinct functional responses. For example, although some antigens can promote T_H1-biased responses downstream of NKT cell activation, others generate T_H2-biased responses (30, 31). Recently, we provided insight into mouse NKT TCR fine specificity against a panel of α-GalCer AGLs (9). The Vβ8.2 NKT TCRs were more sensitive to alterations at the 4′-OH position in comparison with the 3′-OH position of the galactosyl headgroup, and $V\beta7^+$ NKT TCRs preferentially proliferated to AGLs with modifications in the 4′-OH position. Studies using AGLs highlight the feasibility of manipulating the NKT cell response for therapeutic gain (reviewed in Ref. 32). However, to enable more rationally designed NKT-based therapeutics requires an understanding of the structural basis of antigenic modulation of the human NKT cell response. Although the Jα18-encoded loop is identical in the mouse and human NKT TCRs, sequence differences reside in the corresponding Vα14 and Vα24 chains, which is notable given that the CDR1α loop contacts the lipid Ag (6, 18, 33). Accordingly, we asked whether these differences in the Vα chains manifest in differing fine specificity profiles between the human and mouse NKT TCRs. We demonstrate that in contrast to mouse NKT TCR reactivity, the human NKT TCRs are more tolerant toward modifications at the 4′-OH of α-GalCer, yet are very sensitive to modifications in the 3′-OH motif. Accordingly, our data demonstrate that notable fine specificity differences exist between human and mouse NKT cells despite the high level of identity between these respective NKT TCRs.

EXPERIMENTAL PROCEDURES

Glycolipids—The glycolipid analogues used in this study have been previously described (9, 23, 31, 34).

Flow Cytometry-based Affinity Studies—Human CD1d tetramers were loaded with specific glycolipids used to label NKT cells from samples of human peripheral blood mononuclear cells. Human samples were derived from the Red Cross Blood Bank with ethics approvals from Red Cross and the University of Melbourne Human Ethics Committee (Ethics Application ID 1035100.1). Samples were selected based on NKT cell percentages being higher than 0.03%. In some experiments, NKT cells were enriched by CD1d-α-GalCer tetramer-phycoerythrin staining followed by enrichment using MACS anti-phycoerythrin beads and running over a MACS column (Miltenyi Biotec). Enriched NKT cells were sorted on a FACSAria (BD Biosciences) and then stimulated for 2 days in the presence of plate-bound anti-CD3 (clone UCHT1, 10 μg/ml), soluble anti-CD28 (clone CD28.2, 10 μg/ml), phytohemagglutinin (0.5 μg/ml), plus recombinant human IL-2 (100 units/ml, PeproTech) and IL-7 (50 ng/ml, eBioscience). After 2 days, NKT cells were washed and expanded in the presence of IL-2 and IL-7 but in the absence of further anti-CD3/CD28 stimulation for 2–3 weeks. In other experiments, fresh NKT cells were directly examined without *in vitro* expansion. For analysis of cultured or fresh NKT cells, cells were labeled with 7-aminoactinomycin D viability dye (Sigma), anti-Vβ11 (Beckman Coulter, clone C21), CD3 (eBioscience, clone UCHT1), and CD1d tetramer loaded with various glycolipids. Fresh NKT cells were electronically enriched by gating on $V\beta11^+$ cells, and then fresh and enriched NKT cells were examined for CD1d tetramer staining (mean fluorescence intensity, MFI) over a range of dilutions.

Purification of Vα24 NKT TCRs and CD1d-Ag—The method for cloning, expression, and purification of the human Vα24 NKT TCRs and human CD1d has been previously described (35). Lipid loading of CD1d was performed as described previously (6).

Protein Crystallization, Structure Determination, and Refinement—Purified human NKT TCR-CD1d-glycolipid complexes were concentrated to 10 mg/ml, and crystals were obtained at room temperature after 7 days via the hanging drop vapor diffusion technique with equal protein:mother liquor ratio. Crystals of the α-GalCer (C20:2) ternary complex were grown in 8% polyethylene glycol 10,000, 0.2 M magnesium chloride, and 0.1 M Tris, pH 8.8, and flash-frozen in the mother liquor containing 25% polyethylene glycol 10,000 as cryoprotectant. Crystals of the 4′,4″-deoxy-α-GalCer ternary complex were grown in 9% polyethylene glycol 10,000, 0.2 M magnesium chloride, and 0.1 M glycine, pH 10.0, and flash-frozen in the mother liquor containing 30% polyethylene glycol 10,000 as cryoprotectant.

Data for the human NKT TCR-CD1d-α-GalCer (C20:2) and 4′,4″-deoxy-α-GalCer ternary complexes were collected at the Advanced Photon Source facility, Chicago, IL, and the Australian Synchrotron Facility in Melbourne, Australia, respectively. Data were processed with programs from the CCP4 suite (36). Crystals of the ternary complexes diffracted to a range of 2.9–3.1 Å and belong to the space group C222. Crystal structures of the NKT TCR-CD1d-glycolipid complexes were solved via Phaser using the 2.5 Å human NKT TCR-CD1d-α-GalCer complex (Protein Data Bank (PDB) ID code 3HUJ) minus α-GalCer as the search model (6). To prevent model bias, the simulated annealing protocol in Phenix (37) was applied in the initial structure refinement. Glycolipid libraries were generated via the program Sketcher within the CCP4 suite, and the glycolipid models were subsequently built into the work models. Restrained refinement followed by the inclusion of translation libration screw parameters combined with rounds of model building with Coot (38) were used to improve the work model as monitored by the R_{free} values. Programs within the CCP4 suite were utilized to assess the quality of the structures. For

TABLE 1
Data collection and refinement statistics
Values in parentheses refer to the highest resolution bin.

	NKT TCR-CD1d-α-GalCer (C20:2)	NKT TCR-CD1d-4′,4″-deoxy-α-GalCer
Data collection		
Temperature	100 K	100 K
Resolution limits (Å)	100–3.10 (3.27–3.10)	80–2.90 (3.06–2.90)
Space group	C222	C222
Cell dimensions	$a = 145.93$, $b = 176.91$, $c = 82.95$; $\alpha = \beta = \gamma = 90°$	$a = 144.27$, $b = 177.26$, $c = 83.75$; $\alpha = \beta = \gamma = 90°$
Total number of observations	239,067	170,626
Number of unique observations	19,938	22,894
Multiplicity	12.0 (12.2)	7.5 (7.7)
Data Completeness	100 (100)	99.9 (100)
I/σ	4.8 (2.1)	11.3 (2.4)
R_{pim} [a] (%)	11.6 (38.3)	9.4 (30.5)
Mosaicity	0.40	0.70
Refinement statistics		
R_{factor} [b] (%)	22.8	19.1
R_{free} [b] (%)	29.2	25.0
Non-hydrogen atoms		
Protein	5869	6001
Lipid(s)	54	58
Water	17	20
Other	29	29
Ramachandran plot		
Most favored (%)	83.0	87.1
Allowed region (%)	16.7	12.6
Generously allowed region (%)	0.3	0.3
B-factors (Å^2)		
Average main chain	70.9	72.5
Average side chain	68.2	71.7
Lipid(s)	51.2	54.9
Water	35.2	57.2
r.m.s.d.[c] bonds (Å)	0.003	0.005
r.m.s.d.[c] angles (°)	0.714	0.903

[a] $R_{pim} = \Sigma_{hkl}(1/(N-1))^{1/2}\Sigma_i|I_{hkl,i} - \langle I_{hkl}\rangle|/\Sigma_{hkl}\langle I_{hkl}\rangle$.
[b] $R_{factor} = \Sigma_{hkl}\|F_o| - |F_c\|/\Sigma_{hkl}|F_o|$ for all data except ~5%, which were used for R_{free} calculation.
[c] r.m.s.d., root mean square deviation.

data collection and refinement statistics, see Table 1. All molecular graphics illustrations were generated using PyMOL (39).

Surface Plasmon Resonance Measurements and Analysis—The surface plasmon resonance (SPR) experiments were conducted at 25 °C on a Biacore 3000 instrument using HEPES-buffered saline buffer (10 mM HEPES-HCl (pH 7.4), 150 mM NaCl, and 0.005% surfactant P20) and have been previously described (23, 35). Approximately 3000 response units of biotinylated CD1d-glycolipids were coupled to an SA sensor chip and analyzed against a 2-fold serial dilution of the NKT TCRs. Concentration series for the α-GalCer, α-GalCer (C20:2), α-GlcCer (C20:2), 3′,4″-deoxy-α-GalCer, and 4′,4″-deoxy-α-GalCer glycolipids ranged from 39 nM to 40 μM, and concentration series for α-GlcCer glycolipid ranged from 98 nM to 25 μM. The analyte was passed over the sensor chip at 5 μl/min for 80 s at 25 °C, and the final response was subtracted from that of the unloaded CD1d. The affinity value was determined using the BIAevaluation software, and sensorgram plots were presented using GraphPad Prism. All experiments were carried out in duplicate.

RESULTS

Human NKT Cells Show Differential Reactivity with Distinct α-GalCer AGLs—We have previously observed that mouse NKT cells show differential recognition of α-GalCer AGLs with a high sensitivity to modifications in the 4′-OH moiety as detected using 4′,4″-deoxy α-GalCer and α-GlcCer (9, 16). To determine whether human NKT cells exhibit a similar fine specificity pattern, we used a CD1d tetramer dilution approach where CD1d tetramers were loaded with α-GalCer AGLs that varied in the composition of the galactosyl headgroup (3′,4″-deoxy-α-GalCer, 4′,4″-deoxy-α-GalCer, α-GlcCer (C20:2) and the acyl chain, α-GalCer (C20:2) (Fig. 1). The 3′,4″-deoxy-α-GalCer and 4′,4″-deoxy-α-GalCer analogues lack their 3′-OH and 4′-OH on the sugar moiety, respectively, and both also lack the OH group at position 4 of the sphingoid base. The latter modification does not impact on NKT TCR recognition (9, 16), and thus, the impact of the 3′,4″-deoxy-α-GalCer and 4′,4″-deoxy-α-GalCer AGLs can be attributed to the modifications in the galactosyl headgroup. These tetramers were used to measure staining intensity of heterogeneous human NKT cells. Two approaches were used. Firstly, human NKT cells were enriched and expanded *in vitro* for 2–3 weeks prior to tetramer staining. The results demonstrated some unexpected differences between the fine specificity of mouse and human NKT cells. Although the mean fluorescence intensities were highest for α-GalCer and α-GalCer (C20:2), modifications to the 4′-OH sugar moiety associated with 4′,4″-deoxy-α-GalCer and α-GlcCer (C20:2) resulted in only a slight reduction in staining intensity. In contrast, 3′,4″-deoxy α-GalCer labeling intensity was much lower and similar to "unloaded" (endogenous Ag-loaded) CD1d tetramer, which suggested that the 3′-OH moiety is critical for human NKT TCR recognition of α-GalCer (Fig. 2, *A* and *B*). To ensure that the MACS enrichment and *in vitro* expansion had not biased the TCR usage of

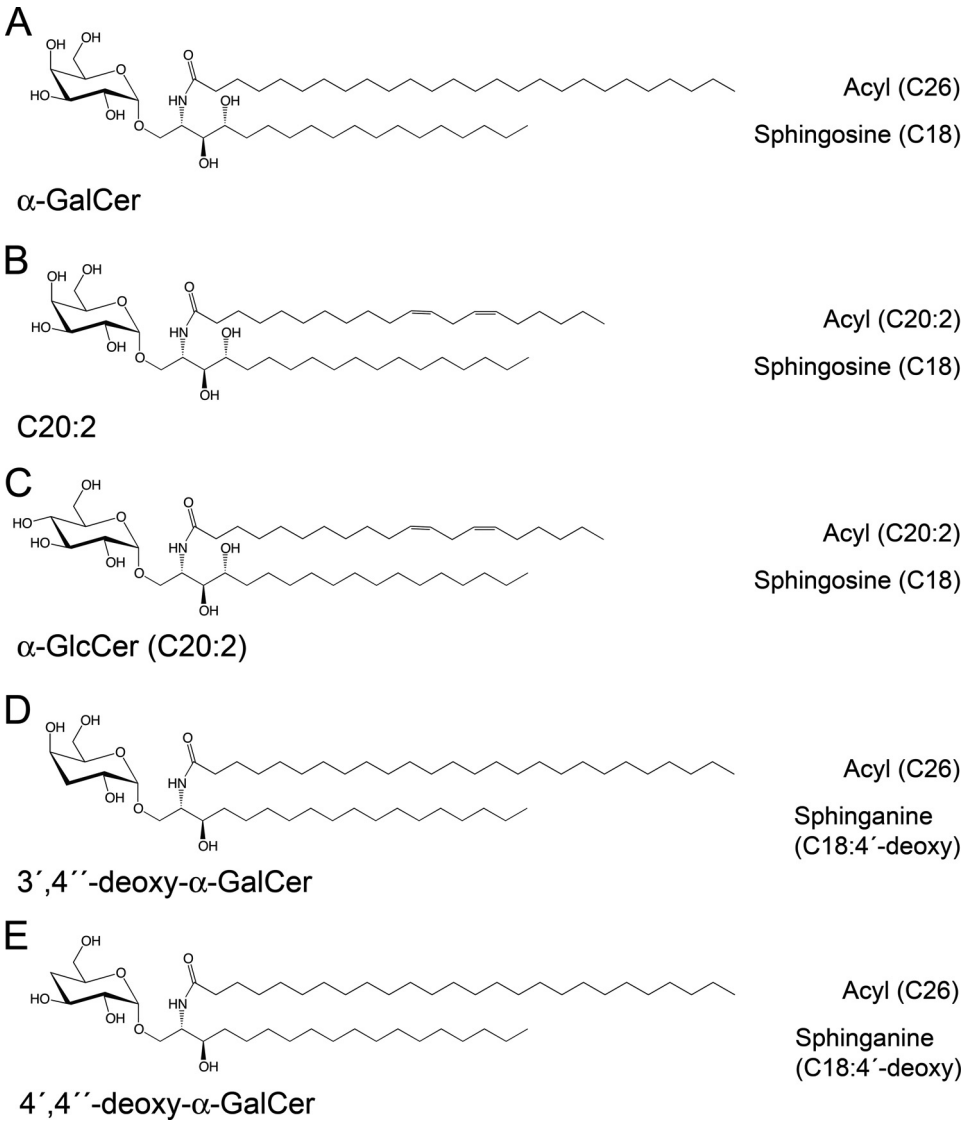

FIGURE 1. **Schematics of α-GalCer and AGLs used in this study.** *A*, α-GalCer; *B*, α-GalCer (C20:2); *C*, α-GlcCer (C20:2); *D*, 3′,4″-deoxy-α-GalCer; *E*, 4′,4″-deoxy-α-GalCer.

the NKT cells, fresh NKT cells from three separate blood donors (Fig. 2, *C* and *D*) were tested. A similar pattern was observed with freshly isolated NKT cells, where tetramer staining was almost completely abrogated with the 3′,4″-deoxy-α-GalCer AGL, whereas tetramer loaded with 4′,4″-deoxy-α-GalCer and α-GlcCer (C20:2) caused only a slightly reduced intensity. Thus, these results suggest that human NKT cells are far more sensitive to modifications to the 3′-OH moiety and relatively tolerant of modifications to the 4′-OH moiety, in contrast to mouse NKT cells (9, 16).

Affinity Measurements—Although the Vα14Vβ8.2 NKT TCR binds to CD1d-α-GalCer with high affinity (K_{Deq} ~60 nM) (6, 9), we previously established that the corresponding Vα24Vβ11 NKT TCR-CD1d-α-GalCer interaction was notably weaker (K_{Deq} ~460 nM) (23). Moreover, previous measurements using a distinct Vα24Vβ11 NKT TCR indicated that the interaction with CD1d-α-GalCer was even weaker (K_{Deq} ~7 μM) (40). The reasons for the differences in the affinity values of the two distinct NKT TCRs are unclear, but may be attributable to different methodologies in CD1d-α-GalCer purification, SPR-based methodology, or differing CDR3β usage (23, 40). Regardless, the observations suggest differing recognition characteristics of the mouse and human NKT TCRs. To address this, we first confirmed the relatively lower affinity of the Vα24Vβ11 (NKT15) NKT TCR-CD1d-α-GalCer interaction (K_{Deq} ~430 nM) and confirmed that cross-species reactivity onto mouse CD1d-α-GalCer was further reduced (K_{Deq} ~1.5 μM). Previously, we had characterized two other human NKT TCRs that differed only in their CDR3β composition (NKT12 and NKT18) and established that the CDR3β variability exhibited by these three human NKT TCRs either did not impact (NKT12) or impacted moderately (NKT18) on the CD1d-α-GalCer interaction (K_{Deq} for NKT12 and NKT18 was ~430 and 940 nM, respectively) (35).

Next, to address the fine specificity of the human NKT TCRs (NKT12, NKT15, and NKT18), we determined their affinity toward the panel of AGLs (Figs. 1 and 3, Table 2). Consistent with the mouse NKT TCR affinity measurements and with the

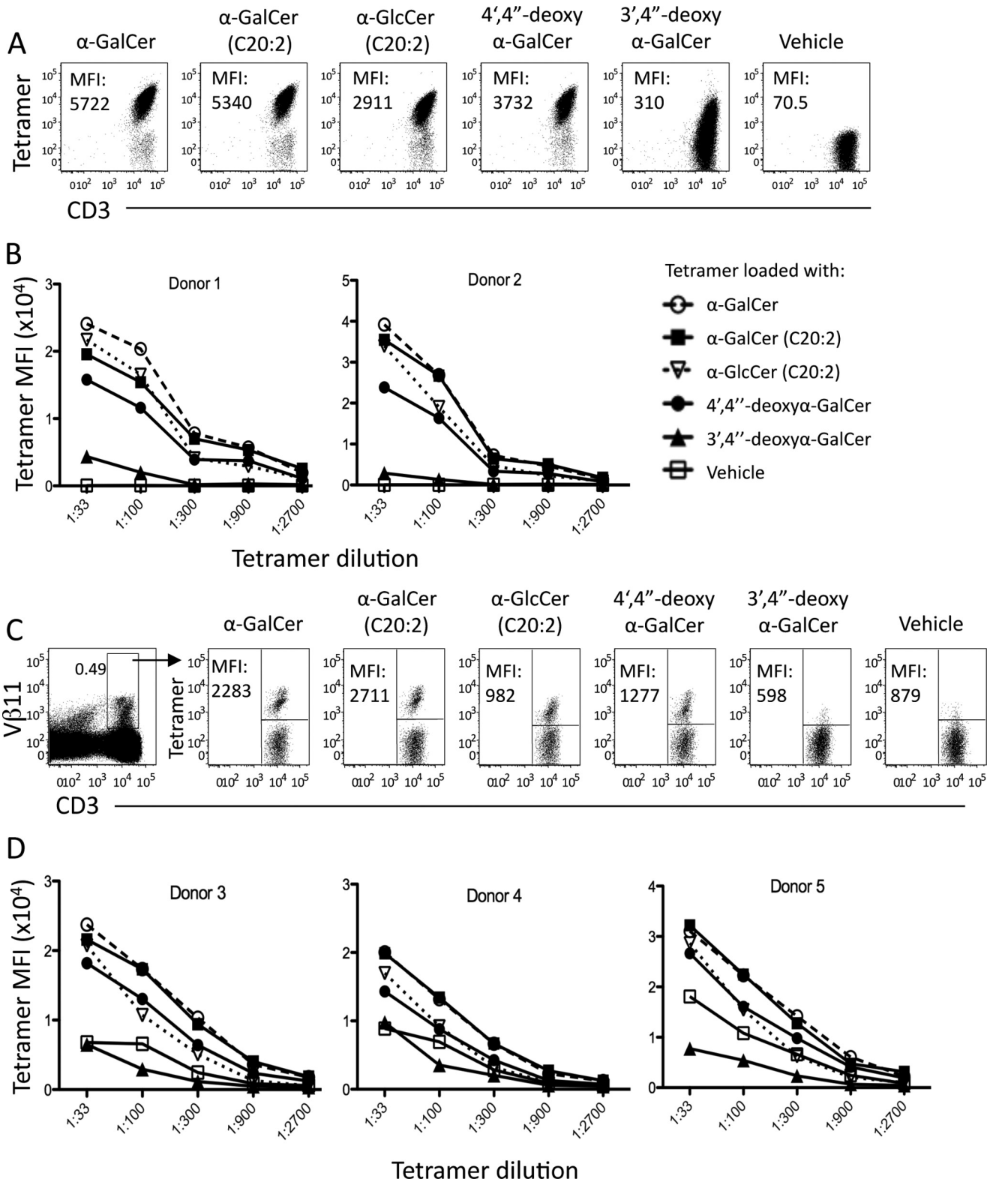

FIGURE 2. **CD1d tetramer staining of human NKT cells.** *A* and *B*, peripheral blood mononuclear cells of two donors were expanded *in vitro* for 3 weeks and stained with: anti-CD3 and serial dilutions of human CD1d tetramers loaded with α-GalCer, α-GalCer (C20:2), α-GlcCer (C20:2), 4′,4″-deoxy-α-GalCer, 3′,4″-deoxy-α-GalCer, or vehicle buffer. Representative flow cytometry plots are depicted for a 1:900 tetramer dilution for Donor 1 in *A*, and the MFI data from all dilutions and all donors are plotted in *B*. MFI depicts NKT cells derived from two patients studied in one experiment. *C* and *D*, Peripheral blood mononuclear cells were isolated from three donors (Donors 3, 4, and 5) and directly stained with: anti-CD3, anti-Vβ11, and serial dilutions of human CD1d tetramers loaded with α-GalCer, α-GalCer (C20:2), α-GlcCer (C20:2), 4′,4″-deoxy-α-GalCer, 3′,4″-deoxy-α-GalCer, or vehicle buffer. *C*, Vβ11$^+$ CD3$^+$ lymphocytes were electronically gated, and the MFI of tetramer staining on the positive (*upper region*) was determined as shown at the 1:900 dilution for Donor 4. *D*, MFI data from all three donors and all tetramer dilutions are plotted. Data in *D* represent three different donors acquired over two experiments.

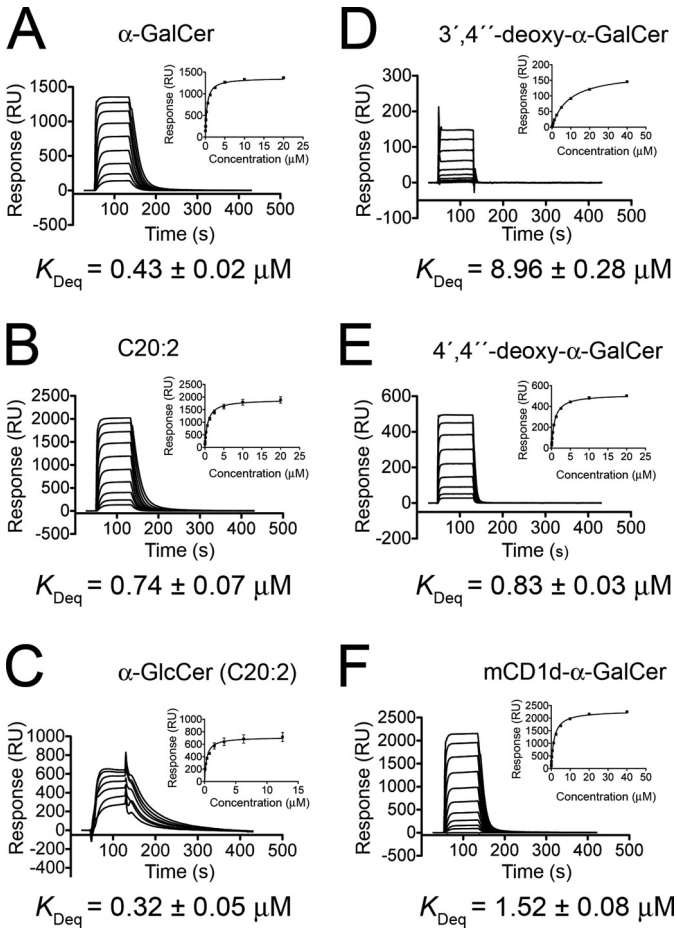

FIGURE 3. **Binding analysis of the human NKT TCR and CD1d-AGL interaction using surface plasmon resonance.** *A–F*, SPR sensorgrams demonstrating the interactions between the NKT15 TCR and human CD1d loaded with α-GalCer (*A*), α-GalCer (C20:2) (*B*), α-GlcCer (C20:2) (*C*), 3′,4′′-deoxy-α-GalCer (*D*), 4′,4′′-deoxy-α-GalCer (*E*), and mouse CD1d loaded with α-GalCer (*F*). Data represent duplicate runs from one experiment. *Error bars* in all panels indicate S.D. *Insets*, the equilibrium response *versus* concentration series. *RU*, response units.

human CD1d tetramer staining (Fig. 2), the AGL with the acyl chain modification, α-GalCer (C20:2), moderately affected the affinity of the interaction (K_{Deq} ~740 nM). Thus, analogous to mouse NKT cell reactivity, we suggest that acyl chain modifications that impact on human NKT cell activity are more likely attributable to mechanisms centered on Ag-loading/presentation (41). The affinity measurements confirmed that modifications at the 4′-OH position of α-GalCer AGLs (4′,4′′-deoxy and α-GlcCer (C20:2)) have moderate impact on the human NKT TCR affinity (K_{Deq} of 830 and 320 nM, respectively), indicating that the 4′-OH moiety is dispensable for the human NKT TCR-CD1d-α-GalCer interaction. Consistent with the CD1d tetramer staining data (Fig. 2), the affinity of human NKT TCR for 3′,4′′-deoxy-α-GalCer was markedly lower (K_{Deq} ~9 μm), confirming that the 3′-OH position was a critical determinant for human NKT TCR reactivity. Further, the heightened dependence of the 3′-OH position was also observed for the NKT12 and the NKT18 TCRs, whereas modifications in the 4′-OH group had a lesser impact (Table 2). Accordingly, in contrast to mouse NKT TCR recognition of CD1d-α-GalCer,

TABLE 2
Human NKT TCR affinity toward CD1d-Ag

Ligand	NKT12 TCR (K_{Deq})	NKT15 TCR (K_{Deq})	NKT18 TCR (K_{Deq})
	μM	μM	μM
hCD1d-α-GalCer	0.43 ± 0.03	0.43 ± 0.02	0.94 ± 0.04
hCD1d-α-GalCer (C20:2)	0.77 ± 0.09	0.74 ± 0.07	1.63 ± 0.12
hCD1d-α-GlcCer (C20:2)	0.34 ± 0.05	0.32 ± 0.05	0.76 ± 0.05
hCD1d-3′,4′′deoxy-α-GalCer	15.79 ± 0.52	8.96 ± 0.28	16.23 ± 0.46
hCD1d-4′,4′′deoxy-α-GalCer	0.93 ± 0.05	0.83 ± 0.03	1.90 ± 0.06
mCD1d-α-GalCer	2.83 ± 0.12	1.52 ± 0.08	2.31 ± 0.12

the 3′-OH position of α-GalCer is a critical determinant for human NKT TCR recognition.

The NKT TCR-CD1d-α-GalCer (C20:2) and 4′,4′′-Deoxy Complexes—To provide a structural basis of the human NKT TCR fine specificity, we determined the structure of the NKT15 TCR in complex with CD1d-α-GalCer (C20:2) and CD1d-4′,4′′-deoxy-α-GalCer (Table 1). These NKT TCR-CD1d-Ag complexes crystallized in the same space group with similar unit cell dimensions and were refined to a comparable resolution limit and refinement statistics, and unless explicitly stated, the electron density at the NKT TCR-CD1d interface was readily interpretable (supplemental Fig. S1). In both complexes, the NKT TCR adopted the canonical docking mode observed for the human NKT TCR-CD1d-α-GalCer complex (6, 7), indicating that the AGLs did not cause a repositioning of the human NKT TCR (Fig. 4). Thus, the NKT TCR bound approximately parallel to, and above, the F′-pocket of the CD1d-Ag-binding cleft.

For the two AGL ternary complexes, the buried surface area (BSA) upon complexation was ~850–870 Å^2, which compares closely to the BSA value of ~890 Å^2 at the human NKT TCR-CD1d-α-GalCer interface (7, 6). This indicates that, relative to α-GalCer, no substantial alteration in the BSA upon ligation was observed in accommodating these two AGLs. For these complexes, as observed for the human NKT TCR-CD1d-α-GalCer interaction, the TCRα chain contributed approximately three times more BSA than the TCRβ chain, whereby the Vα24- and Jα18-encoded contacts were mediated by the CDR1α and CDR3α loops, respectively (6). Further, the Vβ11 interactions were mediated primarily by the CDR2β loop interacting with the α1-helix of CD1d (~20% BSA). This conserved network of CDR2β-mediated contacts included Tyr-48β and Tyr-50β interacting with Glu-83 and Lys-86 of CD1d, with the latter residue also forming van der Waals contacts with Glu-56β (supplemental Table S1) (7). The CDR3β loop was located at the periphery at the interface, with one residue (Tyr-103β) making van der Waals interactions with Gln-150 from CD1d. The lack of substantial involvement of the CDR3β loop in contacting CD1d was consistent with the lack of impact of CDR3β variation in the staining of the total Vβ11 NKT cell population (Fig. 2) and the similar binding affinity of the NKT12 and NKT18 TCRs (Table 2).

α-GalCer (C20:2) differs from α-GalCer in its acyl chain, which resides within the A′-pocket of CD1d. In CD1d-α-GalCer, the tip of the 26C acyl tail curled back onto itself and formed intra-chain van der Waals interactions (42). Analogous to the crystal structure of the mouse NKT TCR-CD1d-α-GalCer (C20:2) complex (9), the electron density

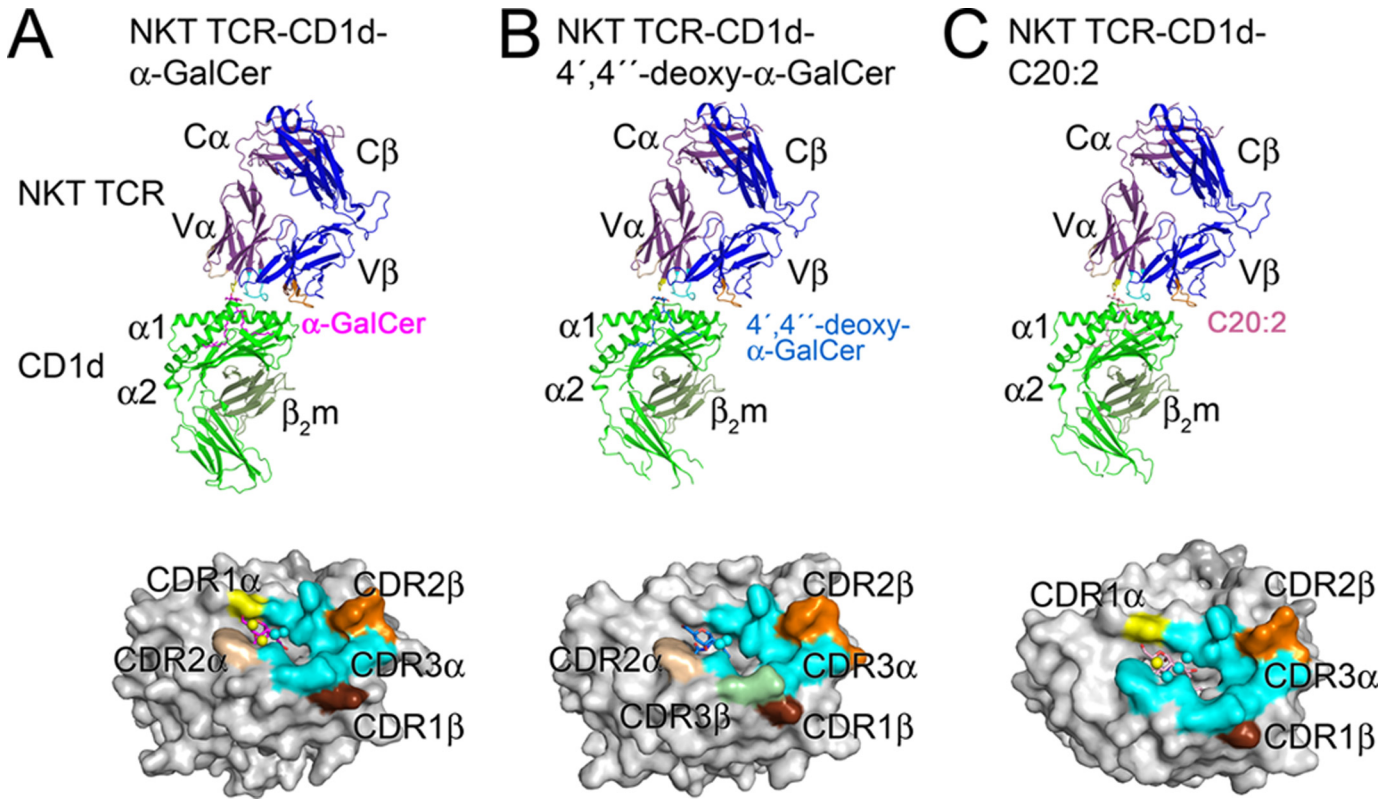

FIGURE 4. **Structure overview of the human NKT TCRs-CD1d-AGL complexes and their corresponding footprints.** *A*, NKT15 TCR-CD1d-α-GalCer (PDB code 3HUJ). *B*, NKT15 TCR-CD1d-4′,4″-deoxy-α-GalCer. *C*, NKT15 TCR-CD1d-α-GalCer (C20:2). Footprints are color-coded based on their CDR loop contributions. *Purple*, TCRα; *dark blue*, TCRβ; *yellow*, CDR1α loop; *beige*, CDR2α; *cyan*, CDR3α loop; *brown*, CDR1β; *orange*, CDR2β loop; *light green*, CDR3β loop; *green*, CD1d heavy chain; *dark green*, β_2m; *magenta*, α-GalCer; *pink*, C20:2; *blue*, 4′,4″-deoxy-α-GalCer.

corresponding to the majority of the 20 acyl tail carbons was not well resolved in the human NKT TCR-CD1d-α-GalCer (C20:2) ternary complex, suggesting that the acyl chain is mobile within the A′-pocket through lack of the intra-stabilizing interactions as observed for α-GalCer. Further, dissimilar to previous studies (43), no spacer lipid occupied the A′-pocket, presumably attributable to space restrictions. Nevertheless, the A′-pocket of CD1d, when bound to the α-GalCer (C20:2), adopted an essentially identical conformation when compared with NKT TCR-CD1d-α-GalCer. As such, the affinity of the NKT TCR-CD1d-α-GalCer (C20:2) interaction was matched closely to that of the NKT TCR-CD1d-α-GalCer interaction, and the interactions between that NKT TCR and the galactosyl headgroup at the respective interfaces were very similar. Namely, the galactosyl headgroup sat beneath the CDR1α loop and abutted the CDR3α loop. Although the 6′-OH moiety was solvent-exposed, the 2′-OH, 3′-OH, and 4′-OH moieties were sequestered by the NKT TCR, with Gly-96α and Phe-29α making hydrogen bonds to the 2′-OH and 4′-OH of the galactose moiety, respectively (Fig. 5). Thus, the altered NKT proliferation response to α-GalCer (C20:2) is more attributable to factors such as altered lipid loading/presentation as shown for mouse NKT cells (9, 41).

The 4′,4″-deoxy-α-GalCer AGL lacks the 4′-OH group on the galactosyl headgroup, and as a result, the hydrogen bond with the CDR1α loop is absent. Despite this, there is minimal perturbation to the positioning of the galactosyl headgroup in that it superposes very well onto α-GalCer. Possibly, the pres-

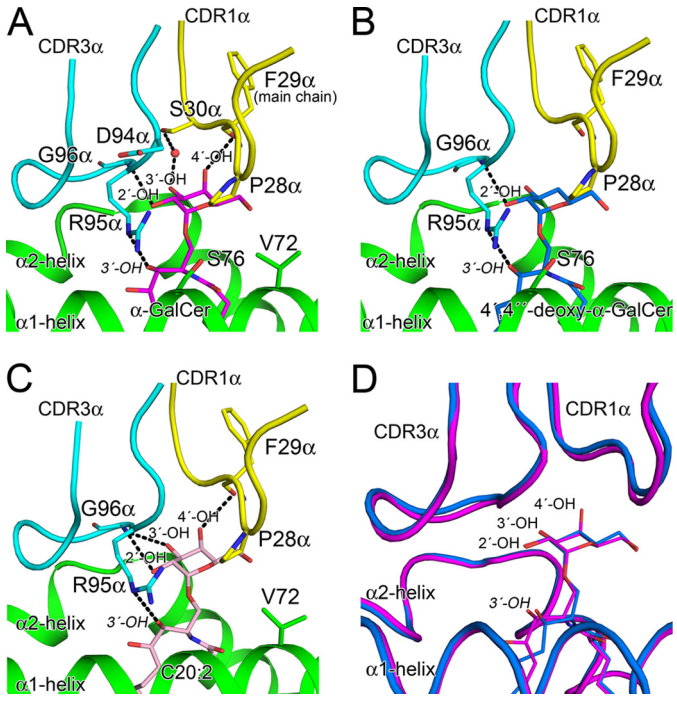

FIGURE 5. **Comparison of the glycosyl headgroup interactions between the AGLs with the NKT15 TCR.** *A*, α-GalCer; *B*, 4′,4″-deoxy-α-GalCer; *C*, α-GalCer (C20:2). *Yellow*, CDR1α loop; *cyan*, CDR3α loop; *green*, CD1d α-helices; *magenta*, α-GalCer; *pink*, C20:2; *blue*, 4′,4″-deoxy-α-GalCer. *D*, superpositioning of the α-GalCer ternary complex (*magenta*) onto the 4′,4″-deoxy-α-GalCer complex (*blue*) shows minimal repositioning of the glycosyl headgroup between the complexes.

ence of Trp-153 in human CD1d (Gly-155 in mouse CD1d), which packs against the galactosyl ring, helps stabilize its conformation within the Ag-binding cleft, thereby reducing the impact of the 4′-OH modification. The lack of a significant structural impact of the 4′,4″-deoxy-α-GalCer AGL was consistent with the minimal effect on the affinity of the interaction and contrasts the effect observed in the mouse NKT TCR-CD1d-4′,4″-deoxy-α-GalCer interaction (6). In the mouse system, the 4′-deoxy substitution caused a shift in the conformation of the galactosyl headgroup that resulted in a loss of interaction with Asn-30α and a weakening of the hydrogen bond between the 3′-OH and Asn-30α. Accordingly, the 4′-OH position has a major impact on mouse NKT TCR recognition (6, 16) and conversely a modest impact on human NKT TCR recognition.

DISCUSSION

A high level of sequence and structural identity exists between the Vα24Jα18 NKT TCR and the orthologous mouse Vα14Jα18 NKT TCR (44), and consequently, the structures of the corresponding NKT TCR-CD1d-α-GalCer complexes are very similar (6, 7). This conservation is underpinned by the Jα18-encoded CDR3α loop, which contacts CD1d and the Ag, and the CDR2β loop from the Vβ chain, which in the Vβ11 and Vβ8.2 NKT TCRs contains two tyrosine residues that sit above the F′-pocket of CD1d (6, 7, 19). Despite this consensus NKT TCR-CD1d docking, variations in the Vβ and CDR3β usage can modulate NKT TCR affinity and antigen specificity (6, 9, 13, 16).

A greater role in the Vα-encoded region in contacting the lipid Ag is evident in some NKT TCR-CD1d-Ag complexes (13, 14), suggesting that sequence differences within the germline-encoded Vα regions of NKT TCRs can potentially impact on antigen specificity. For example, the mouse Vα10 NKT TCRs preferentially recognizes α-GlcCer-containing ligands, whereupon the CDR1α and CDR2α loops play a role in contacting the Ag (26). Further, sequence differences in the CDR1α loop of the Vα14 and Vα24 NKT TCRs suggested that mouse and human NKT TCRs may exhibit altered fine specificity profiles. Within the mouse NKT TCR-CD1d-α-GalCer complexes, the 3′-OH and 4′-OH moieties are sequestered by Asn-30α, with the 4′-OH moiety of α-GalCer being more critical for the interaction than the 3′-OH moiety (9). This was attributable to the modification of the 4′-OH position leading to a greater disruption in the number of contacts between the galactosyl headgroup and the NKT TCR in comparison with the 3′-OH modification.

The human NKT TCR was more tolerant to modifications at the 4′-OH position and less tolerant to modifications in the 3′-OH position of α-GalCer. Within the human NKT TCR-CD1d-α-GalCer complex, the 4′-OH group hydrogen-bonds to the main chain of Phe-29α, whereas the 3′-OH hydrogen-bonds to Ser-30α. The human NKT TCR-CD1d-4′,4″-deoxy-α-GalCer complex showed that there were limited perturbations arising from the loss of the interaction between the 4′-OH moiety and Phe-29. Although we were not able to crystallize the NKT TCR-CD1d-3′,4′-deoxy-α-GalCer complex, we speculate that the 3′-OH modification results in a loss of the hydrogen bond with Ser-30α. Further, the 3′-OH modification may result in a loss of interactions with the CDR3α loop and with Asp-151 of hCD1d, which may impact on the Asp-151-mediated interactions with the CDR3α loop. The tetramer staining and affinity measurements show that the human NKT TCR is much less tolerant to modifications in the 3′-OH moiety than the 4′-OH moiety. Surprisingly, the three human NKT TCRs examined exhibited modest differences in the responses to some of the AGLs, indicating that CDR3β differences can subtly indirectly impact on CD1d-Ag-recognition, analogous to that observed in the mouse NKT TCR system (22, 24).

Our findings show that the human Vα24 NKT TCRs exhibit altered specificity profiles in comparison with the Vα14 NKT TCRs and suggest that non-Vα24 NKT TCRs may exhibit altered fine specificity profiles (40). Our data also provide a basis for understanding the conserved use of the Jα18-encoded CDR3α loop in the non-Vα24 TCRs (29) and suggest that these TCRs will adopt a similar docking mode to that of the Vα24 NKT TCRs. Our results also suggest that glucose-containing glycolipids may be more antigenic toward human NKT cells. In line with this view, it was recently established that β-GlcCer was a ubiquitous self-Ag for mouse and human NKT cells, with this Ag being more reactive toward human NKT cells (45). Of additional interest, some bacterial glycolipids seem to use glucose-based headgroups, suggesting that human NKT cells may be better at responding to such antigens (46, 47). Although the fundamental principles underscoring NKT TCR-CD1d-Ag recognition are conserved across mice and humans, fine specificity differences between the mouse and human NKT TCRs are apparent. Such differences are important for NKT cell physiology and will be critically important to understand in the context of rationally developing human-based NKT cell therapeutics (48, 49).

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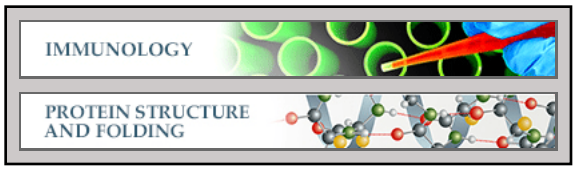
Immunology:

Human and Mouse Type I Natural Killer T Cell Antigen Receptors Exhibit Different Fine Specificities for CD1d-Antigen Complex



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