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Cutting Edge: Structural Basis for the Recognition of β-Linked Glycolipid Antigens by Invariant NKT Cells

Esther Dawen Yu,1 Enrico Girardi,1 Jing Wang, and Dirk M. Zajonc

Invariant NKT (iNKT) cells expressing a semi-invariant Vα14 TCR recognize self and foreign lipid Ags when presented by the nonclassical MHC I homolog CD1d. Whereas the majority of known iNKT cell Ags are characterized by the presence of a single α-linked sugar, mammalian self Ags are β-linked glycosphingolipids, posing the interesting question of how the semi-invariant TCR can bind to such structurally distinct ligands. In this study, we show that the mouse iNKT TCR recognizes the complex β-linked Ag isoglobotrihexosylceramide (iGb3; Galα1-3-Galβ1-4-Glcβ1-1-Cer) by forcing the proximal β-linked sugar of the trisaccharide head group to adopt the typical binding orientation of α-linked glycolipids. The squashed iGb3 orientation is stabilized by several interactions between the trisaccharide and CD1d residues. Finally, the formation of novel contacts between the proximal and second sugar of iGb3 and CDR2α residues of the TCR suggests an expanded recognition logic that can possibly distinguish foreign Ags from self Ags. The Journal of Immunology, 2011, 187: 2079–2083.

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nvariant NKT (iNKT) cells constitute a T lymphocyte population that is able to modulate the functions of several different cell types such as NK cells, B cells, and T cells, therefore bridging the innate and adaptive immune response to self and foreign Ags. Critical to this role is the ability of iNKT cells to rapidly secrete, upon stimulation, large amounts of both Th1 and Th2 cytokines, such as IFN-γ, TGF-β, IL-4, and IL-13 (1). iNKT cells express a semi-invariant αβ TCR (Vα14/Jα18-VP8.2/72 in mice; Vα24/Jα18-VP11 in humans) that recognizes glycolipid Ags presented by the nonclassical MHC class I homolog CD1d (2). Although mouse and human CD1d only share 60% sequence identity in the α1-α2 superdomain that presents the lipid Ag (3), the structures of human and mouse iNKT TCR in complex with CD1d and the potent sphingolipid Ag α-galactosylceramide (α-GalCer) demonstrated evolutionary conserved CD1d Ag-presenting and TCR-docking strategies that differ from classical TCR–peptide–MHC interactions (4, 5). Despite the progress made in characterizing novel iNKT ligands, the nature of the self Ags involved in the positive selection and development of NKT cells remains elusive (6). Interestingly, although the vast majority of known iNKT Ags are characterized by the presence of an α-linked glycosidic residue, mammals cannot synthesize α-linked glycolipids, but instead express β-linked Ags (6). One of the most well-characterized β-linked Ags is isoglobotrihexosylceramide (iGb3), in which the ceramide backbone is β linked to a trisaccharide (Galα1-3-Galβ1-4-Glcβ1-1-Cer), with the terminal sugar being important for antigenicity (7). Although the role of iGb3 as a self Ag is controversial (6), it represents to date the most complex iNKT ligand able to activate both mouse and human cells. The size and connectivity of the polar moiety of this Ag therefore pose interesting structural questions involving the specificity and mode of recognition of β-linked glycolipids by the iNKT TCR. The crystal structure of the mouse CD1d–iGb3–iNKT TCR complex described in this study illustrates how the iNKT TCR is able to recognize complex β-linked glycolipids by flattening the trihexosyl sugar of iGb3 between the CD1d and TCR surface to allow for the conserved TCR-binding mode on CD1d that has previously been observed for the α-linked Ags (4, 5, 8–11), therefore explaining how this conserved TCR can recognize a range of highly diverse Ags.

Materials and Methods

Lipids

β-lactosylceramide (β-LacCer) and β-glucosylceramide (β-GlcCer) were obtained from Avanti Polar Lipids. iGb3 was purchased from Enzo Life Sciences International. Lipids were dissolved at 0.4 mg/ml in 0.5% Tween 20 and 0.9% NaCl and stored at 4˚C.

Cell lines and culture conditions

iNKT hybridoma cell lines 1.2, 1.4, and 2C12 (12, 13) were cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 mg/ml each of penicillin and streptomycin, 50 mM 2-ME, and 10% FBS. The cell lines were maintained in an incubator with a humidified atmosphere containing 5% CO₂ at 37˚C.

Cell-free Ag presentation assay

The cell-free Ag presentation assay for stimulation of mouse iNKT cell hybridomas by soluble mCD1d was carried out following published protocols.
An additional interchain disulfide bond was engineered by mutating to cys-

Mouse CD1dβm expression and purification and Vα14-Vβ8.2 TCR refolding

The expression and purification methods of fully glycosylated mouse and human CD1d/βm heterodimer proteins were reported previously (11). Mouse TCR refolding was performed according to previously reported protocols, using the identical construct (11, 14). A representative human Vα24-Vβ11 iNKT TCR (CDR3a: CASSDPNEQFF) was cloned in pET22b (Vα24 chain) and pET30a (Vβ11 chain) Escherichia coli expression vectors. An additional interchain disulfide bond was engineered by mutating to cysteine Thr85 (TRBC 75) on the Cβ domain and Ser57 on the Cβ domain. Moreover, a cysteine residue (TRBC 75) on the Cβ domain was mutated to serine to avoid incorrect oligomerization. The two chains were expressed, refolded, and purified, as described for the mouse Vα14-Vβ8.2 iNKT TCR.

Glycolipid loading and ternary complex formation

Mouse CD1d was loaded overnight with 4–6 molar excess of iGb3 (0.4 mg/ml in 0.5% Tween 20 and 0.9% NaCl). iGb3-loaded CD1d was purified by size-exclusion chromatography and incubated with an equimolar amount of TCR for 1 h at room temperature. The mixture was concentrated to 4.8 mg/ml in 10 mM HEPES (pH 7.5), 30 mM NaCl before being used for crystallization.

Surface plasmon resonance studies

Surface plasmon resonance (SPR) studies were performed using a Biacore 3000 (GE Healthcare), according to the methods described previously (14). Human or mouse CD1d was expressed, purified, and biotinylated, as previously reported, and loaded with iGb3 (8). CD1d–iGb3 complexes and empty CD1d as a control were immobilized on a streptavidin sensor chip (CAPture chip; GE Healthcare), whereas increasing concentrations (0.02–20 μM) of the mouse or human TCR were passed over the chip at 25˚C with a flow rate of 30 μl/min. Experiments were repeated twice. Kinetic parameters were calculated in the BIA evaluation software (version 4.1) after background substraction.

Crystallization and structure determination

Crystals of mCD1d–iGb3–TCR complexes were grown at 27.6˚C by sitting drop vapor diffusion, mixing 0.5 μl protein with 0.5 μl precipitant (16% polyethylene glycol 3350, 8% v/v Tacsimate [pH 5.0]). Crystals were flash cooled at 100 K in mother liquor containing 20% glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource beamline 7-1. Data processing and structure determination by molecular replacement using the CD1d and TCR coordinates from PDB ID 3QUY were carried out as reported for other CD1d-glycolipid complexes (11). Model building and refinement were performed analogous to other CD1d-glycolipid–TCR structures (11). Data collection and refinement statistics are presented in Supplemental Table I.

Results and Discussion

Mouse NKT cells recognize iGb3 presented by mouse or human CD1d

We tested the ability of iGb3 and its biosynthetic precursors β-LacCer and β-GlcCer, which lack one or two distal sugar rings compared with iGb3 (Fig. 1A), to activate three Vα14 NKT hybridomas in a cell-free Ag presentation assay (Fig. 1B, Supplemental Fig. 1). Consistent with other studies (7, 15), the truncated ligands β-LacCer and β-GlcCer did not activate either hybridoma. However, iGb3 was able to induce significant IL-2 release from all three hybridomas. These results confirm the importance of the distal α1-3-linked sugar of iGb3 for its antigenicity. Interestingly, iGb3 was able to stimulate mouse iNKT cell hybridomas when presented by human CD1d, whereas presentation of iGb3 by the humanized mCD1d mutant G155W (in hCD1d, Trp153 is at the equivalent position compared with Gly155 in mCD1d) did not lead to mouse iNKT cell activation. Recognition of α-GalCer was unimpaired when presented by the G155W

![FIGURE 1](http://www.jimmunol.org/)
mutant, indicating that this mutant was still able to bind and properly present glycolipids. Most residues lining the entrance to the mCD1d-binding groove are conserved in hCD1d (except for the G155W exchange), but differences are observed in the area above the A’ pocket (i.e., Met69 in mCD1d to Ile, Gly155 to Trp, Met162 to Trp). The results obtained with the G155W mutant therefore suggest that the observed cross-reactivity of mouse iNKT cells toward human CD1d-presented iGb3 is not dependent on a single residue; it is in fact abrogated by the single amino acid substitution, and it is rather the result of several contacts unique to the human CD1d protein surface that compensate for the G155W substitution, suggesting an alternative mode of iGb3 presentation by mouse and human CD1d.

**iGb3 has a submicromolar affinity for the mouse iNKT TCR**

Previous SPR experiments carried out using a short-chain version of iGb3 and an iNKT TCR with a truncated CDR3α loop reported a low-affinity \( K_D \sim 50 \mu M \) interaction (16). In this study, we measured the affinity of both the mouse (Vα14/Vβ8.2 derived from the 2C12 hybridoma (14) and human (Vα24/Vβ11) iNKT cell TCR toward the full-length iGb3 presented by either mouse or human CD1d (Fig. 1, Supplemental Fig. 1B). The mouse TCR binds to mCD1d-iGb3 with submicromolar affinity \( K_D \) of 718 nM. Binding was characterized by a relatively fast on-rate \( k_1 9.5 \times 10^8 M^{-1}s^{-1} \) and a slow off-rate \( k_2 1.9 \times 10^{-2} s^{-1} \), not dissimilarly from other previously characterized α-GalCer analogs, but in contrast to microbial Ags that are characterized by much faster TCR dissociation rates (GalA-GSL from *Sphingomonas* spp., \( k_2 1.3 \times 10^9 M^{-1}s^{-1} \)).

**Crystal structure of the mCD1d-iGb3-iNKT TCR complex**

We determined the crystal structure of the mouse CD1d–iGb3–iNKT TCR complex at 2.8Å resolution (Fig. 3, Supplemental Table I). The overall docking orientation of the iNKT TCR on mCD1d is highly similar to other previously determined ternary complexes with bound α-GalCer (5), its various analogs (9, 11), as well as microbial Ags (8). In the mCD1d–iGb3–TCR complex, the TCR adopts a parallel orientation on mCD1d characterized by a footprint centered above the F’ pocket. Key residues in the CDR3α, CDR2β, and CDR3β loops (Supplemental Fig. 1C) mediate polar contacts with the α1 and α2 helices of mCD1d, whereas the TCR α-chain exclusively mediates contacts with the Ag.

**FIGURE 3.** Binding mode of iGb3 to mCD1d and TCR. A, iGb3 (in yellow) stick forms extensive polar contacts with CD1d residues D80, D153, T156, T159, and M162, mostly from the α2-helix. B, The TCR (in cyan) forms polar interactions with iGb3 through residues N30 (CDR1α), V50, K68 (CDR2α), as well as G96 (CDR3α). C, Superposition of the mCD1d–iGb3 and mCD1d–iGb3–iNKT TCR complexes. iGb3 is shown as green sticks before TCR binding (from PDB ID 2Q7Y) and in yellow after TCR binding. TCR is shown as a yellow surface with electrostatics surface potentials (from −30kT/e to +30kT/e) with the underlying CDR loops in cyan. D, A proposed “stairway” conformation involving the α2-helix residues of CD1d (D153, T156, T159, and M162) support and bind the trisaccharide head group of iGb3.
Consistent with all the sphingolipid Ags analyzed to date (3), the iGb3 molecule binds to mCD1d with its sphingosine chain in the F′ pocket and the C26 acyl chain in the A′ pocket. Surprisingly, upon TCR binding, the trisaccharide head group of iGb3 gets squashed over the α2 helix of CD1d, with each sugar making polar and VdW contacts with CD1d residues (Fig. 3A). In particular, the proximal β-Glc residue contacts Asp153 through its 2′-OH, whereas the second β-Gal sugar contacts Thr159 through its 2′-OH. In contrast, the terminal α-Gal residue nestles in a relatively hydrophobic pocket formed between Ala58, Thr59, and Met162, while packing against the side chain of Met162 (Fig. 3A). The flat binding orientation of the trisaccharide molds the proximal β-linked glucose of iGb3 to bind into a highly similar position compared with α-linked glycolipids such as α-GalCer, with the 2′-OH forming a H-bond with Asp153 of CD1d (Fig. 2C). The same residues on mCD1d (Asp153) and the TCR (Asn30α, Gly96) are involved in recognizing the iGb3 β-linked proximal sugar and the sugar of other α-linked Ags, therefore suggesting remarkably conserved recognition logic for both α- and β-anomeric glycosphingolipids. Surprisingly, considering the essential role of the terminal sugar of iGb3 in determining its antigenicity, contacts between the Ag and the TCR α-chain involve only the β-Glc and β-Gal sugars, but not the terminal α-Gal (Fig. 3B). In addition, unlike any previously characterized exogenous iNKT cell Ag, all three CDR loops from the Vα14 chain are involved in contacting the iGb3 molecule.

In particular, Gly96 and Asn30 on the CDR3α and CDR1α loops, respectively, form H-bonds with the 2′ and 3′ hydroxyl groups of β-Glc. At the same time, the carbonyl group of both Asn30 on CDR1α and Val50 on CDR2α, as well as the framework residue Lys68, is interacting with the 6′-OH of the β-Gal residue (Fig. 3B). Interestingly, a similar interaction pattern with the iNKT TCR was recently reported for the self Ag phosphatidylinositol (PI) (10), which is not a β-linked glycosphingolipid self Ag, but instead a phosphoglycerolipid and, as such, presented differently by CD1d. As is the case for iGb3, PI also contacts all three CDR loops of the invariant α-chain either directly or through water-mediated contacts. In particular, the novel interactions involving the residues near the CDR2α loop (Val50 and Lys68) are remarkably conserved, suggesting that those novel contacts, which are absent in all other studies of iNKT Ags to date, are used to discriminate self Ags, such as iGb3 and PI, from foreign Ags, notwithstanding that most of those interactions involve backbone atoms of the TCR and are therefore largely independent of the TCR sequence. Therefore, previous mutational studies of the TCR did not characterize CDR2α residues as important for Ag recognition (17). In summary, we speculate that the iNKT cell recognition logic toward self Ags is expanded compared with the recognition of α-GalCer or microbial Ags through those additional contacts involving CDR2α residues, whereas all other contacts involving CDR1α and CDR3α are conserved between self and foreign Ags.

Conformational changes on mCD1d and iGb3 upon TCR binding and implications for recognition of β-linked Ags

The availability of the crystal structure of the mCD1d-iGb3 complex (16) allowed us to compare the conformation of the ligand and mCD1d before and after TCR ligation (Fig. 3C). Superposition of the two structures on mCD1d shows how the TCR is able to squash the trisaccharide head group of iGb3 over the α2 helix of CD1d while maintaining the position of the ceramide backbone in a relatively conserved orientation. Consistent with the requirement of an additional energetic contribution for iGb3 upon complex formation, SPR measurements of the kinetics of binding in the murine complex show a considerably slower association rate for iGb3 compared with α-GalCer (Fig. 1). Interestingly, the well-defined density observed for iGb3 in the mCD1d–TCR complex suggests that in the presence of the TCR, the Ag adopts an ordered conformation involving, in particular, several contacts with the mCD1d α2 helix, as well as the TCR. In presence of the TCR, the trisaccharide moiety nicely packs against the surface of the helix, which forms a “stairway” generated by the side chains of Asp153, Thr156, Thr159, and Met162 to support and bind the three sugars of iGb3 (Fig. 3D). It therefore appears that when the TCR squashes the iGb3 head group onto the CD1d surface, the third sugar anchors the trisaccharide moiety of iGb3 to the CD1d surface, resulting in a stable conformation of the ligand, as implied by the relatively slow TCR dissociation rate and the well-defined electron density observed. Consistent with this binding mode, the shorter β-GlcCer and β-LacCer ligands that lack the terminal sugar are not antigenic. Moreover, the closely related compounds iGb4 (GalNAcβ1-3Galα1-3LacCer) and Gb3 (Galα1-4LacCer) are not antigenic because of the additional sugar moiety or the suboptimal orientation of their distal sugar that cannot fit or reach the anchoring pocket on the mCD1d surface. In conclusion, we hypothesize that complex β-linked glycolipids that cannot anchor the sugar moiety to the CD1d surface after TCR flattening require more binding energy to stabilize the CD1d–TCR complex and, as such, have significantly weaker potency.

Finally, as described for other iNKT Ags, this TCR is also able to force mCD1d in a conserved binding mode by inducing the formation of a hydrophobic roof above the F′ pocket (Supplemental Fig. 1D). The F′ roof formation involves the repositioning of the side chains of residues Leu84 and Val149 and Leu150 of mCD1d and was previously shown to affect the stability of the mCD1d–iNKT TCR complex (8). Although TCR-induced conformational changes for MHC-peptide and CD1d-glycolipid have been reported (8, 18), the extent of reorientation observed for iGb3 is unprecedented among TCR Ags, highlighting once again the unique nature of the iNKT TCR.

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Disclosures

The authors have no financial conflicts of interest.

References


Table SI. Data collection and refinement statistics.

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| Refinement statistics      |                |
| No. atoms                  | 6595           |
| Protein                    | 6256           |
| Ligand                     | 81             |
| Carbohydrate               | 210            |
| Waters                     | 48             |
| $R/R_{\text{free}}$        | 0.237/0.270    |
| Ramachandran plot (%)      |                |
| Favored                    | 98.2           |
| Allowed                    | 100.0          |
| R.m.s. deviations          |                |
| Bonds (Å)                  | 0.005          |
| Angles (°)                 | 0.835          |
| B-factors (Å$^2$)          |                |
| Protein                    | 46.1           |
| Ligand                     | 46.9           |
| Carbohydrate               | 62.9           |
| Waters                     | 35.8           |
**Supplemental Figure legends**

**Figure S1** Mouse iNKT cells recognize iGb3 presented by both mouse and human CD1d. (A) APC free Ag presenting assay demonstrating that iGb3 presented by both mouse and human CD1d is recognized by mouse iNKT cell hybridoma Hy2C12 and Hy1.4. Concentrations of used lipids are indicated in the figure insets. (B) Sensorgrams of SPR analysis used for the summary in figure 1. Equilibrium dissociation constant (K_{Deq}, bottom panels) were derived from steady state analysis (bottom).

(C) Conserved TCR footprint onto CD1d involving TCR residues from CDR2β, 3β (right panel) and 3α (left panel). In the mTCR-iGb3-mCD1d complex, the TCR adopts a parallel orientation on mCD1d characterized by a conserved footprint centered on the F’ pocket. Key residues in CDR3α, CDR2β and CDR3β loops mediate polar contacts with CD1d residues of the α1 and α2 helices. CDR3α shown in cyan, CDR2β and CDR3β shown in orange and mCD1d shown in grey. (D) The F’ roof is absent in the CD1d-iGb3 structure (left panel) but formed on mCD1d upon TCR binding (right panel) by an induced fit mechanism. Residues involved in the F’ roof formation are highlighted in red, while the corresponding molecular surfaces around the F’ pocket are shown in dark grey.