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Recognition of β -linked self glycolipids mediated by natural killer T cell antigen receptors

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Abstract

The most potent foreign antigens for natural killer T cells (NKT cells) are α -linked glycolipids, whereas NKT cell self-reactivity involves weaker recognition of structurally distinct β -linked glycolipid antigens. Here we provide the mechanism for the autoreactivity of T cell antigen receptors (TCRs) on NKT cells to the mono- and tri-glycosylated β -linked agonists β -galactosylceramide (β -GalCer) and isoglobotrihexosylceramide (iGb3), respectively. In binding these disparate antigens, the NKT cell TCRs docked onto CD1d similarly, achieving this by flattening the conformation of the β -linked ligands regardless of the size of the glycosyl head group. Unexpectedly, the antigenicity of iGb3 was attributable to its terminal sugar group making compensatory interactions with CD1d. Thus, the NKT cell TCR molds the β -linked self ligands to

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AUTHOR CONTRIBUTIONS

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resemble the conformation of foreign α -linked ligands, which shows that induced-fit molecular mimicry can underpin the self-reactivity of NKT cell TCRs to β -linked antigens.

Most receptors interact in a precise manner with defined ligand(s), thereby underpinning the specificity of a given biological system; however, such recognition events are further complicated in T cell biology. For example, in major histocompatibility complex (MHC)-restricted immunity, the $\alpha\beta$ T cell antigen receptor (TCR) recognizes both the peptide antigen and the MHC molecule¹. Therefore, TCR specificity must accommodate the highly polymorphic nature of MHC molecules and the variable peptide cargo. Moreover, thymic selection sets up host T cells to weakly recognize complexes of self peptide and MHC², while simultaneously the TCR's inherent flexibility capitalizes on chance improvements in this recognition, as self peptides are displaced by foreign peptides during infection. In this self–non-self recognition paradigm, self antigens and autoantigens are often considered to represent low-affinity mimics of the more potent foreign antigens^{3,4}.

Natural killer T cells (NKT cells), unlike conventional T cells, specifically recognize self lipid– or foreign lipid–based antigens bound to the monomorphic MHC class I-like molecule CD1d^{5,6}. NKT cells have been linked to microbial immunity, autoimmunity, allergy and cancer and, accordingly, they represent an important immunotherapeutic target with immense clinical potential⁷. Type I NKT cells express a semi-invariant TCR that has an invariant α -chain and a restricted TCR β repertoire (α -chain variable region 24– α -chain joining region 18 paired with β -chain variable region 11 ($V_{\alpha}24$ - $J_{\alpha}18$ - $V_{\beta}11$) in humans, and $V_{\alpha}14$ - $J_{\alpha}18$ paired with $V_{\beta}8$, $V_{\beta}7$ or $V_{\beta}2$ in mice)⁸. Nevertheless, despite this restricted TCR repertoire, NKT cell TCRs bind an array of different CD1d-restricted lipid-based antigens, including phospholipids⁹, as well as α -linked and β -linked glycolipids⁵. The α -glycosidic linkage that defines α -galactosylceramide (α -GalCer) and other bacteria-derived NKT cell agonists, including α -glycuronosylceramides⁵ and α -galactosyldiacylglycerols¹⁰, is considered to represent a 'microbial signature', as glycolipid antigens in mammals are typically β -linked anomers. The structures of NKT cell TCRs in complex with many α -linked glycolipids have been determined, including CD1d- α -GalCer and variants thereof^{11–15} and α -galactosyldiacylglycerol¹⁶. Although the NKT cell TCR has been shown to be relatively rigid, conformational adjustments in complexes of CD1d and α -linked antigen permit optimal engagement of NKT cell TCRs^{13,16}. That is, a conserved docking topology is observed whereby the NKT cell TCR shows a parallel docking mode above the F' pocket of the CD1d antigen-binding cleft, whereas the α -linked glycosyl moiety 'sits' snug and flat against CD1d, making many direct specificity-governing contacts with the NKT cell TCR⁵.

It is well established that CD1d-restricted recognition of self antigen is a central aspect of NKT cell biology, being involved in positive selection of NKT cell precursors in the thymus¹⁷ and underpinning the involvement of NKT cells in many diseases, including cancer and innate responses to infection^{8,18}. Self glycosphingolipid antigens are known targets of NKT cells in these responses¹⁸, despite their β -linked, rather than α -linked, sugar head groups. Indeed, NKT cell TCRs can recognize several different β -linked mammalian lipid molecules, including β -galactosylceramide (β -GalCer)^{19,20}, β -glucosylceramide²¹,

isoglobotrihexosylceramide (iGb3)²² and the disialoganglioside GD3 that stimulates a minor population of NKT cells²³. However, in the CD1d antigen-binding cleft, the α - and β -linked stereoisomers adopt markedly different conformations whereby the β -linked glycosyl moiety adopts a perpendicular or protruding orientation, in contrast to the flat α -linked glycosyl head group^{24–27}. How can the same semi-invariant NKT cell TCR recognize such structurally distinct forms of the glycosphingolipids? This recognition conundrum is even more intriguing given that iGb3 is a glycosphingolipid with three sugar groups that extend outward and prominently from the CD1d antigen-binding cleft. Here we provide a basis for understanding how the NKT cell TCR can recognize β -linked glycolipid antigens. Specifically, we show that human and mouse NKT cell TCRs can flatten CD1d-restricted β -linked self ligands to resemble the conformation of the foreign α -linked ligands.

RESULTS

Autoreactive NKT cell TCR– β -linked antigen complexes

The overall affinity of the NKT cell TCR for CD1d-antigen complexes is modulated by the TCR β chain²⁸. In an attempt to maximize NKT cell TCR affinity, we engineered a range of TCRs composed of the V α 14-J α 18 invariant chain associated with various TCR β chains in which the composition of the complementarity-determining region 2- β (CDR2 β) and CDR3 β loops were shuffled. We then independently transduced each NKT cell TCR combination into a TCR-negative hybridoma. In this way, we created six different hybridomas that each expressed an NKT cell TCR of differing affinities for the CD1d-antigen complexes. We stained each hybridoma with CD1d tetramers independently loaded with the α -linked ligand α -GalCer, the β -linked ligands β -GalCer, globotrihexosylceramide (Gb3), β -lactosylceramide (β -LacCer) or iGb3, or uncharacterized endogenous antigen(s) purified together with the CD1d molecules produced by the human embryonic kidney cell 293T cell line. We ranked the hybridomas according to their apparent avidity for the CD1d-antigen tetramer based on staining intensity (Fig. 1). In agreement with the affinity-scale hypothesis²⁸, the hierarchy of recognition was the same for each antigen; the NKT cell TCR with the highest apparent avidity for CD1d–endogenous complex also recognized the four β -linked glycolipid ligands, with iGb3 being the ligand of highest avidity. As these are mammalian antigens, we consider the NKT cell TCRs that bind to these antigens to be autoreactive. The engineered NKT cell TCR (autoreactive V α 14 (auto-V α 14)) that had maximal autoreactivity bound CD1d-antigen with a docking mode almost completely identical to that of published non-autoreactive NKT cell TCR–CD1d–antigen complexes²⁹. We also generated a soluble human V α 24-J α 18+V β 11⁺ NKT cell TCR (auto-V α 24) that had a CDR3 β selected for maximal reactivity to ligands of lower affinity²¹. We reasoned that the higher affinities afforded by these TCRs would facilitate structural analysis of the CD1d-restricted recognition of β -linked glycosphingolipid, as non-autoreactive NKT cell TCRs show weak affinity for self antigens that would make it more difficult to isolate the molecular complexes. First, we expressed and refolded auto-V α 14 and measured its affinity to a range of β -linked ligands (β -GalCer, Gb3, β -LacCer and iGb3). As expected, although a V β 8.2 NKT cell TCR¹² not selected for strong autoreactivity to CD1d had very low affinity for all the CD1d– β -linked antigen complexes (dissociation constant, >100 μ M; data not shown), the autoreactive NKT cell TCRs bound much more strongly to the same panel of β -

linked ligands bound to CD1d (dissociation constant, 3–10 μ M; Table 1 and Supplementary Fig. 1). Notably, the NKT cell TCR engaged CD1d- β -linked antigen complexes with much slower on rates and faster off rates than those of NKT cell TCR-CD1d- α -linked antigen interactions¹³, which suggests that substantial remodeling is required for the NKT cell TCR to engage β -linked ligands and that the resultant complexes formed are less stable.

Structures of NKT cell TCR-CD1d- β -GalCer complexes

Next we solved the structure of the auto-V α 24 NKT cell TCR and auto-V α 14 NKT cell TCR in complex with CD1d- β -GalCer to a resolution of 3.1 Å or 2.8 Å, respectively (Supplementary Table 1). The electron density at the respective interfaces, as well as that of the glycolipid headgroup, was unambiguous (Supplementary Fig. 2). The auto-V α 24 and auto-V α 14 NKT cell TCRs docked onto CD1d- β -GalCer in a manner analogous to that observed for the recognition of α -linked glycolipids by NKT cell TCRs (Fig. 2), which indicated that the presence of a monoglycosylated β -linked glycolipid does not cause substantial repositioning of the NKT cell TCR onto CD1d. NKT cell TCRs mediate autoreactivity to CD1d via their CDR3 β loop^{21,28,29}, and in both the auto-V α 24 NKT cell TCR-CD1d- β -GalCer and auto-V α 14 NKT cell TCR-CD1d- β -GalCer structures, their respective CDR3 β loops, although very different in sequence, directly contacted CD1d and did not contact the antigen. The sequence of the auto-V α 24 NKT cell TCR CDR3 β loop is CASSEFGGTEREQETQYFGPGTRLLVL, whereas the sequence of the auto-V α 14 NKT cell TCR CDR3 β loop is CASGSLLDVREVFVGKGTSLTVV. The CDR3 β loop of the auto-V α 14 NKT cell TCR abutted CD1d, spanning residues 145–152, whereas the corresponding loop of the auto-V α 24 NKT cell TCR ‘sat’ above an analogous region on human CD1d, which suggests that the autoreactivity of NKT cell TCRs is underpinned by direct interactions between the CDR3 β loop and CD1d. These CDR3 β contributions resulted in a buried surface area of the interaction that was about 100–160 Å² greater than that of non-autoreactive NKT cell TCRs (Fig. 2); that is, the buried surface area for the various autoreactive NKT cell TCR complexes ranged from 840 Å² to 900 Å², whereas for the published V β 8.2 NKT cell TCR-CD1d- α -GalCer complex¹², the buried surface area is 740 Å². As the footprints of the auto-V α 24 and auto-V α 14 NKT cell TCRs on CD1d- β -GalCer were similar (Fig. 2), the relative roles of the other CDR loops in mediating CD1d contacts were conserved. Specifically, two tyrosine residues in the CDR2 β loop (Tyr48 and Tyr50) contacted a focused stretch of the α 1 helix of CD1d, whereas the CDR3 α loop bridged the CD1d antigen-binding cleft (Supplementary Tables 2 and 3).

Most unexpectedly, the outward-pointing β -galactosyl moiety of β -GalCer was flattened by engagement of the auto-V α 24 or auto-V α 14 NKT cell TCR (Fig. 3). This ‘bulldozing’ of the β -linked antigen altered its orientation compared with that of the equivalent binary complex of CD1d and sulfatide (which is sulfated β -GalCer)²⁶ by about 46°, which resulted in a ligated conformation of β -GalCer analogous to that of α -GalCer. Accordingly, the β -GalCer head group ‘sat’ underneath the CDR1 α loop, adjacent to the CDR3 α loop of the autoreactive NKT cell TCRs (Fig. 3). In the auto-V α 14 NKT cell TCR-CD1d- β -GalCer complex, analogous to the V α 14 NKT cell TCR-CD1d- α -GalCer complex, contacts with the galactosyl head group included hydrogen bonds between the 2' OH and the main chain of the CDR3 α loop, and from 3' OH and 4' OH to Asn30 α of the CDR1 α loop, whereas

the 6'-OH group of β -GalCer was mostly exposed to solvent (Fig. 3). We observed analogous interactions with the respective galactosyl headgroups for the auto- $V_{\alpha}24$ NKT cell TCR-CD1d- β -GalCer and $V_{\alpha}24$ NKT cell TCR-CD1d- α -GalCer complexes (Fig. 3). Given that the affinity of the NKT cell TCR for β -GalCer was much lower than its affinity for α -GalCer, this suggests that a considerable energetic penalty is incurred in flattening the β -GalCer after ligation of the NKT cell TCR. Furthermore, the similarity between the human and mouse NKT cell TCR-CD1d- β -GalCer complexes suggests that a general mechanism of conformational deformation underpins the recognition of β -linked ligands by the NKT cell TCR.

Structure of the NKT cell TCR-CD1d-iGb3 complex

Having determined how NKT cell TCRs interacted with CD1d- β -GalCer, we next determined the structure of the auto- $V_{\alpha}14$ NKT cell TCR-CD1d-iGb3 complex to a resolution of 2.5 Å (Fig. 2 and Supplementary Table 1). Although it remains controversial whether iGb3 has a critical role in intrathymic NKT cell selection^{22,30–34}, there is evidence that iGb3 or possibly a related molecule is important in shaping the heavily biased V_{β} repertoire of NKT cells^{30,35}. Regardless of whether this is true, iGb3 is reported to be an agonist for mouse and human NKT cells^{22,30,36,37}. Because the tri-hexosyl head group (galactose- α 1-3-galactose- β 1-4-glucose) of iGb3 points directly outward from the CD1d antigen-binding cleft²⁷ (Fig. 4), in contrast to the flat conformation of α -GalCer, two docking models pertaining to NKT cell TCR recognition have been proposed: one suggests a divergent docking mode relative to that of the NKT cell TCR-CD1d- α -GalCer complex, and the other invokes a flattening of the iGb3 head group after ligation of the NKT cell TCR^{5,11,27}. The auto- $V_{\alpha}14$ NKT cell TCR docked onto CD1d-iGb3 in a manner analogous to the docking of the previously determined NKT cell TCR-CD1d-antigen complexes (Fig. 2), which immediately indicated that the tri-hexosyl moiety of iGb3 changes conformation after ligation. Unlike the CD1d-iGb3 binary complex, in which conformational mobility was associated with the terminal sugar group of iGb3, the entire glycosyl head group was visible in the electron density and thus was firmly locked in place in the auto- $V_{\alpha}14$ NKT cell TCR-CD1d-iGb3 complex (Supplementary Fig. 2). In the ligated state, the tri-hexosyl moiety extended toward the A' pocket, lying flat against CD1d, such that iGb3 'hand clasped' the α 2-helical axis, with the terminal sugar forming a 'finger grip' hold via a hydrogen bond to Thr159 and van der Waals interactions with Met162 of CD1d (Supplementary Table 4). The three sugar groups of iGb3 interacted with a span of the α 2 helix (residues 153–162) characterized by small aliphatic residues, which enabled the iGb3 head group to closely abut CD1d (Fig. 4). Indeed, the terminal sugar moiety of iGb3 contacted only CD1d and not the NKT cell TCR. Similarly, the second glycosyl moiety formed many contacts with CD1d, including a hydrogen bond to Thr159, but formed limited contacts with the NKT cell TCR, in which the 6'-OH group formed hydrogen bonds to the framework residue Lys68 α and to the carbonyl group of Asn30 α (Fig. 4 and Supplementary Table 5). Accordingly, the location of the second glycosyl moiety, as well as the NKT cell TCR-mediated contacts, were similar to those of the inositol moiety in the NKT cell TCR-CD1d-phosphatidylinositol complex²⁹ (Supplementary Fig. 3). The proximal sugar group of iGb3 was 'bent over', akin to that in the ligated β -GalCer conformation, forming interactions with Asn30 α of the CDR1 α loop and limited interactions with the CDR3 α loop. Thus, to enable

establishment of the consensus NKT cell TCR–CD1d–antigen docking topology, the iGb3 moiety was completely flattened after ligation of the NKT cell TCR.

The antigenicity of iGb3

Notably, although differences between iGb3 and Gb3 in the terminal sugar linkage suggest that this is critically important for iGb3 antigenicity²², the terminal sugar of iGb3 did not contact the auto-V α 14 NKT cell TCR. Indeed, published studies have demonstrated that iGb3 analogs with modifications in the terminal sugar group show much less potency against some NKT hybridomas in a V β -dependent manner^{37,38}. It has been shown that β -LacCer (which is similar to iGb3 but lacks the terminal sugar) and Gb3 (which differs only in the linkage of the terminal sugar: α 1–4-linked galactose for Gb3 versus α 1–3-linked galactose for iGb3) do not stimulate NKT cells²². This suggests that after engagement of the NKT cell TCR, the binding energy formed by interaction of the terminal sugar with CD1d is critical for the agonist activity of iGb3. To formally establish this, we took advantage of the high affinity of the auto-V α 14 TCR, formed complexes of the auto-V α 14 TCR with CD1d-Gb3 or CD1d- β -LacCer and determined these structures to a resolution of 3.1 Å or 3.0 Å, respectively (Fig. 4 and Supplementary Tables 1, 6 and 7). The electron density for the β -LacCer headgroup was unambiguous, and the first two sugar moieties adopted a conformation very similar to that of iGb3 (Supplementary Fig. 2). Similarly, the electron density for the Gb3 headgroup was distinct (Supplementary Fig. 2); the first two sugar moieties adopted a conformation very similar to that of iGb3 in the auto-V α 14 NKT cell TCR–CD1d–iGb3 complex. However, as a result of the terminal α 1–4 linkage, the third sugar moiety of Gb3 extended away from, and made limited contacts with, CD1d (Fig. 4). Accordingly, these data indicate that the antigenicity of β -linked NKT cell agonists can be attributed to induced interactions with CD1d rather than to direct interactions between the antigen and the NKT cell TCR itself.

DISCUSSION

Redundancy in receptor-ligand interactions is characteristic of many different biological settings. Indeed, during their development and maintenance, T cells must recognize self antigens bound to antigen-presenting molecules through their TCRs, which subsequently respond to foreign antigens during an immune response². In such systems, degeneracy of recognition is generally, but not always³⁹, linked to the recognition by receptors of common features in related but disparate ligands⁴⁰. However, although self peptides are generally considered to mimic foreign peptide-based antigens bound to the MHC, NKT cell TCRs recognize very different self and non-self lipid-based ligands restricted to CD1d^{8,33}. Here we have shown that for NKT cell TCRs, induced-fit molecular mimicry shaped self β -linked ligands to resemble foreign α -linked antigens. Furthermore, it has been shown that an α -linked microbial ligand can change conformation after ligation of the NKT cell TCR¹⁶. The ability of the NKT cell TCR to flatten the glycolipid antigen is reminiscent of the reshaping of bulged peptides bound to the MHC by TCRs⁴¹. However, in contrast to MHC-restricted immunity, the weak NKT cell agonist activity of glycolipid antigens is not related to suboptimal docking modes with the antigen-presenting molecule itself but seems to be related more to the conformational ‘gymnastics’ required of the self antigen that enables the

NKT cell TCR to converge onto a common, favored footprint on CD1d. Consistent with that, α -glycosidic anomers of iGb3 and Gb3 provide higher potency than do their β -linked counterparts⁴², which would reflect the natural adoption by these compounds of a flat orientation after engagement of the NKT cell TCR. Indeed, such conformational deformity was greatest for iGb3 recognition, in which the bulky head group was completely flattened against CD1d after ligation of the NKT cell TCR.

NKT cells show a range of reactivity to several different β -linked ligands. For example, the tumor antigen GD3 is recognized only by a small subset of NKT cells²³, whereas iGb3 is thought to activate a large proportion of NKT cells²². However, given that humans do not seem to express a functional iGb3 synthase required for the production of iGb3 (ref. 31), whether this glycolipid represents a physiological NKT cell antigen in humans remains controversial, although at least some human NKT cells seem to be able to respond to this antigen³³. To address the issue of weak NKT cell TCR reactivity to CD1d- β -linked self ligands, we used an engineered autoreactive mouse NKT cell TCR and a natural autoreactive human NKT cell TCR to more readily generate NKT cell TCR-CD1d- β -linked ligand complexes. Such TCRs use particular CDR3 β sequences that emphasize the role of this loop in mediating autoreactivity to CD1d in an antigen-independent manner and indicate that the self-reactivity to β -linked ligands can also be modulated by the CDR3 β loop^{21,29}. Indeed, the non-autoreactive V β 8.2 NKT cell TCR¹² interacted with β -linked ligands with much lower affinity, which suggests that such β -linked ligands might have only a limited role in stimulating most NKT cells, although the possibility of a role in the intrathymic selection of these T cells cannot be excluded. Future studies should quantify the affinity threshold of β -linked ligands to which NKT cells can respond. Furthermore, given that the iGb3 antigen does not contact the V β chain directly, studies should establish why V β 7 NKT cells seem to 'preferentially' respond to this antigen^{30,35}. We hypothesize, on the basis of published observations¹², that the β -chain can affect the α -chain-mediated interactions that lead to the 'preferential' recognition of iGb3 by V β 7 NKT cells.

Given that the affinity of the NKT cell TCR for the β -linked glycolipids was much lower than its affinity for their α -linked counterparts, our results suggest that a substantial energetic penalty is probably incurred after distortion of the β -linked ligands to resemble the shape of the α -linked ligands, consistent with the slower on rates needed to engage the β -linked antigens. Indeed, given the relative affinity values for the recognition of α -GalCer and β -GalCer by the V β 8.2 NKT cell TCR, we estimate the Gibbs free energy needed to engage β -GalCer to be over 4.4 kcal/mol. The 'energetic penalty' incurred will presumably depend on the degree of flexibility of the β -linked ligand itself. For example, given the CD1d-sulfatide binary complex²⁶, it is likely that the sugar moiety of β -GalCer is stabilized through multiple interactions with CD1d and is unlikely to be mobile and thus the NKT cell TCR must 'force down' this β -linkage. The two terminal sugars of iGb3, however, show greater mobility in the CD1d antigen-binding cleft²⁷ and thus may be pushed down more readily by the NKT cell TCR. It is possible that iGb3 can adopt the flattened conformation in the absence of the NKT cell TCR ligand, but this is less likely, as the β -linked proximal sugar moiety of iGb3 is tethered in the binary complex²⁷. Glycosylation has an important role in the immune system and, similar to the NKT cell TCR-CD1d axis, the flexible glycan moieties are generally reshaped after ligation by immunological receptors^{43,44}.

The antigenicity of a ligand has been attributed to its affinity for the antigen-presenting molecule, the ligand density and its interactions with the TCR. Our study has also shown that, after ligation of the NKT cell TCR, a ligand's antigenicity can be attributable to stabilizing interactions with the antigen-presenting molecule—namely, structural comparison of NKT cell TCR–CD1d complexes involving the non-agonist glycolipids Gb3 and β -LacCer showed that the antigenicity of iGb3 was determined by a compensatory mechanism involving interactions between its terminal sugar and CD1d itself. Collectively, by providing insight into the recognition of α -linked and β -linked glycolipids by the NKT cell TCR, our studies have also shaped understanding of the fundamental principles that govern the recognition of self by NKT cell TCRs.

ONLINE METHODS

Glycolipid antigens

The antigen β -GalCer with a 12-carbon acyl chain (for structural analyses) was from Avanti Polar Lipids; Gb3, iGb3 and α -GalCer were from Alexis Biochemicals; and β -LacCer and β -GalCer (for functional studies in Fig. 1) were from Matreya.

Functional studies

Reagents for functional studies were as follows: mouse CD1d monomers, produced in HEK293 cells, were from the US National Institutes of Health Tetramer Core Facility. CD1d tetramers were prepared as described²⁹ and the 'self' tetramer was prepared identically with the addition of vehicle only.

For tetramer staining, hybridomas were stained for 45 min at 20–25 °C with the appropriate tetramer plus antibody to TCR β (H57-597; eBioscience). Cells were analyzed by flow cytometry on a LSR II (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

For the generation of TCR $\alpha\beta$ constructs and retroviral plasmids, wild-type and mutant TCR β chains were generated as described⁴⁵. TCR constructs were cloned into mouse stem cell virus–based plasmids with an internal ribosome entry site plus sequence encoding either enhanced green fluorescence protein or human nerve growth factor receptor as a reporter. All TCR V-gene segment designations and amino acid numbering are according to the International Union of Immunological Societies–Arden compilation⁴⁶. Hybridomas engineered for this study expressed the following TCR β chains: a native V β 6 chain with the CDR3 β sequence derived from the DO11.10 TCR (GTTNT) was used as a negative control, as the combination of this TCR β chain with the invariant V α 14 does not allow interaction with CD1d molecules²⁸; the native V β 8.2 DO11.10 TCR chain; an engineered V β 6 chain with its CDR2 β sequence replaced with that in V β 8.2 chain and a specific CDR3 β sequence (LLDVR) that allows interaction with CD1d–self antigen tetramers (auto-V α 14)²⁹; a native V β 8.2 chain with that same CDR3 β sequence (LLDVR); a native V β 6 chain with that same CDR3 β sequence (LLDVR); and the engineered V β 6 chain with a single amino acid change in the CDR3 β loop (ALDVR) that largely abrogates binding to the CD1d–self antigen tetramer²⁹.

Production of NKT cell TCRs and CD1d-antigen complexes

The auto- $V_{\alpha}24$ NKT cell TCR²¹ composed of $V_{\alpha}24$ (TRAV10-TRAJ18) and $V_{\beta}11$ (TRBV25-1 and TRBJ2-5, with CDR3 β sequence and flanking residues CASSEFGGTERTQETQYFGPGTRLLVL) was produced as described¹¹. The auto- $V_{\alpha}14$ NKT cell TCR composed of $V_{\alpha}14$ -J α 18 (TRAV11*02-TRAJ18) and $V_{\beta}6$ (TRBV 19*02, with CDR2 β sequence YSYGAGSTEK and CDR3 β sequence and flanking residues CASGSLLDVREVFVGKGRLLTVV) was produced as described²⁹. Soluble mouse $V_{\alpha}14$, $V_{\beta}8.2$ and engineered $V_{\beta}6$ - $V_{\beta}8.2$ (auto- $V_{\alpha}14$) chains and human $V_{\alpha}24$ and $V_{\beta}11$ chains were expressed in *Escherichia coli* strain BL-21 and inclusion body proteins were prepared, refolded and purified essentially as described^{12,29}. Cloning, expression, purification and antigen loading of CD1d were done as described¹².

Crystallization and structure determination

For the auto- $V_{\alpha}14$ complexes, purified auto- $V_{\alpha}14$ NKT cell TCR and mouse CD1d-antigen were mixed and incubated at 20–25 °C, and the purified ternary complex was spun down to a concentration of 10 mg/ml. Hanging-drop vapor-diffusion crystallization experiments were done by mixture of 1 μ l protein and 1 μ l crystallization solution (11–15% (vol/vol) polyethylene glycol 6000 and 0.1 M sodium citrate, pH 6.0–6.3) at 20 °C. Crystals were cryoprotected with 20% (vol/vol) glycerol.

Diffraction data from the ternary complex crystals were collected with synchrotron radiation at the Australian Synchrotron MX2 beamline and were processed with the Mosflm program for integration of single-crystal diffraction data from area detectors⁴⁷ (Supplementary Table 1). Through the use of the NKT TCR auto- $V_{\alpha}14$ NKT cell TCR-CD1d-phosphatidylinositol structure²⁹ (with CDR loops, lipid and waters removed) the Phaser program for phasing macromolecular crystal structures by maximum-likelihood methods was able to yield interpretable experimental maps. The model was then rigid-body refined into the experimental map before iterative model building with the COOT program for macromolecular model building, completion and confirmation (Crystallographic Object-Oriented Toolkit)⁴⁸ and refinement with the Phenix software suite for the automated determination of macromolecular structures⁴⁹, often with implementation of simulated annealing, to yield a final model. The density of the glycolipid headgroups was unambiguous (Supplementary Fig. 2). The quality of the structure was assessed with programs in the CCP4 suite (Collaborative Computational Project number 4)⁵⁰.

For the auto- $V_{\alpha}24$ NKT cell TCR-CD1d- β -GalCer complex, the complex (5–10 mg/ml in 10 mM Tris, pH 8.0, and 150 mM NaCl) was crystallized at 20–25 °C in 30% (vol/vol) PEG 400, 0.1 M Tris, pH 8.5, and 0.1 M $MgCl_2$ by the hanging-drop vapor-diffusion technique. Crystals were flash-frozen in crystallization solution before data collection. Crystals diffracted to a resolution of 3.1 Å at the Australian Synchrotron MX2 beamline (Melbourne, Australia). Data were processed with the Mosflm program and were scaled with the Scala scaling and data-merging program from the CCP4 suite. The crystals belong to the space group C2 with two molecules of the ternary complex in the asymmetric unit. The crystal structure was solved with the MOLREP molecular-replacement program in the CCP4 suite, with the published human NKT cell TCR-CD1d- α -GalCer structure (Protein Data Bank

accession code 3HUI) lacking the lipid and CDR loops as a search model¹². The REFMAC macromolecular-refinement program in the CCP4 suite was used for initial restrained refinement, followed by further refinement through the use of the simulated annealing protocol implemented in Phenix. In subsequent rounds of refinement, restrained refinement included translation-libration-screw parameters. The quality of the structures was confirmed with the validation and deposition services of the Research Collaboratory for Structural Bioinformatics of Protein Data Bank.

Surface plasmon resonance measurements and analysis

These experiments were done at 25 °C on a Biacore 3000 with HBS buffer (10 mM HEPES-HCl, pH 7.4, 150 mM NaCl and 0.005% (vol/vol) surfactant P20 supplied by the manufacturer). Loaded CD1d was coupled to research-grade streptavidin-coated chips to a mass concentration of 700 resonance units. Increasing concentrations of auto-V α 14 TCR (1.42–90 μ M, except where described otherwise in figure legends) was injected over all flow cells for 30 s at a rate of 30 μ l/min. The final response was calculated by subtraction of the response of the streptavidin-coated chip alone from that of the auto-V α 14 NKT cell TCR–CD1d–antigen complex. BIAevaluation version 3.1 software (Biacore AB) was used to fit the data to the 1:1 Langmuir binding model, allowing local fitting of the binding maximum, for calculation of the kinetic constants. The equilibrium data were analyzed with the Prism program for biostatistics, curve fitting and scientific graphing (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Hierarchical recognition of multiple CD1d-antigen complexes by NKT cell TCRs. Flow cytometry of the TCRαβ⁻ 5KC mouse hybridoma transduced with the V_α14J_α18 invariant chain and various engineered TCRβ chains and stained with five different CD1d tetramers: α-GalCer, β-GalCer, β-LacCer, iGb3 or Gb3. Above, CDR2β and CDR3β sequences of the TCRβ chains; 'Self' (top row), CD1d tetramers made of CD1d monomers affinity-purified from the supernatants of human 293 cells transduced with a lentivirus encoding mouse CD1d with no external antigen added before the formation of tetramers; right, structures of antigens used in CD1d tetramers. β-GalCer was purified from bovine galactocerebrosides; β-LacCer was purified from porcine red blood cells; both contain heterogeneous fatty acid length and saturation (R). Numbers in top right quadrants indicate percent CD1d tetramer-positive cells. Data are representative of two independent experiments.

Figure 2.

Overview of the structures of NKT cell TCR-CD1d-β-linked antigens. (a) Auto- $V_\alpha 24$ NKT cell TCR-CD1d-β-GalCer. C_α , α-chain constant region; C_β , β-chain constant region; β_2m , β₂-microglobulin; hCD1d, human CD1d. (b) Auto- $V_\alpha 14$ NKT cell TCR-CD1d-β-GalCer. mCD1d, mouse CD1d. (c) Mouse NKT cell TCR-CD1d-α-GalCer12. (d) Auto- $V_\alpha 14$ NKT cell TCR-CD1d-iGb3. (e) Auto- $V_\alpha 14$ NKT cell TCR-CD1d-Gb3. (f) Auto- $V_\alpha 14$ NKT cell TCR-CD1d-β-LacCer. Below (a-f), associated footprints on the CD1d-antigen complexes: pink, CDR1α loops; purple, CDR3α loops; red, CDR2β loops; orange, CDR3β loops; black, framework (FW) contributions. (g,h) CDR3β loops in the binding of auto- $V_\alpha 14$ and auto- $V_\alpha 24$ NKT cell TCRs to mouse (g) or human (h) CD1d: gold, human NKT cell TCRα chain; green, human NKT cell TCRβ chain; blue, mouse NKT cell TCRα chain; brown, mouse NKT cell TCRβ chain; yellow, β-linked ligands and α-GalCer.

Figure 3.

Recognition of β -GalCer by human and mouse NKT cell TCRs. **(a)** Auto- $V_{\alpha}14$ NKT cell TCR-CD1d- β -GalCer. **(b)** Auto- $V_{\alpha}24$ NKT cell TCR-CD1d- β -GalCer. **(c)** $V_{\alpha}14$ - $V_{\beta}8.2$ NKT cell TCR-CD1d- α -GalCer¹². **(d)** $V_{\alpha}24$ - $V_{\beta}11$ NKT cell TCR-CD1d- α -GalCer¹². **(e)** Conformational adjustments of the β -GalCer head group (relative to sulfatide; Protein Data Bank accession code 2AKR²⁶) after ligation of the NKT cell TCR.

Figure 4.

Recognition of bulky β -linked ligands by NKT cell TCRs. (a) Auto- $V_{\alpha}14$ NKT cell TCR–CD1d–iGb3. (b) Auto- $V_{\alpha}14$ NKT cell TCR–CD1d–Gb3. (c) Auto- $V_{\alpha}14$ NKT cell TCR–CD1d– β -LacCer. (d) Conformational adjustments of the iGb3 head group after ligation of the NKT cell TCR (gray, not ligand bound; yellow, ligand bound). The position of the third sugar head group of iGb3 in the binary complex (green) was not resolved in the crystal structure of the CD1d–iGb3 complex²⁷.

Table 1

Affinity measurements and kinetic rate constants

Lipid	On rate ($\times 10^4 \text{ M}^{-1} \text{ s}^{-1}$)	Off rate (s^{-1})	K_{dcal} (μM)	K_{deq} (μM)	$t_{1/2}$ (s)
iGb3	22.2 ± 2.64	0.725 ± 0.025	3.35	3.03 ± 0.24	0.96
β -GalCer	4.19 ± 0.99	0.364 ± 0.029	9.05	5.24 ± 0.58	1.90
β -LacCer	8.79 ± 0.65	0.457 ± 0.047	5.19	4.84 ± 0.53	1.52
Gb3	ND	ND	ND	10.69 ± 0.62	ND

Surface plasmon resonance studies of the affinity of a NKT cell TCR for various CD1d- β -linked antigen complexes. K_{dcal} , calculated dissociation constant; K_{deq} , dissociation constant at equilibrium; $t_{1/2}$, half-life; ND, not detected. Data are representative of two independent experiments ($\pm$ s.e.m. of duplicates).