Published in final edited form as:

Nat Immunol. 2005 August ; 6(8): 810–818.

Structure and Function of a Potent Agonist for the Semi-Invariant NKT Cell Receptor

Dirk M. Zajonc¹, Carlos Cantu III², Jochen Mattner³, Dapeng Zhou³, Paul B. Savage⁴, Albert Bendelac³, Ian A. Wilson^{1,5,*}, and Luc Teyton^{2,*}

¹ Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, California 92037, USA

² Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, California 92037, USA

⁴ Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, California 92037, USA

³ University of Chicago, Committee on Immunology, 5841 S. Maryland Av., Chicago, IL 60637

⁵ Brigham Young University, C100 Benson Science Building, Provo, UT 84602-5700

Abstract

NKT cells express a conserved, semi-invariant $\alpha\beta$ T cell receptor, which has specificity for a self-glycosphingolipids and microbial cell wall α -glycuronosylceramide antigens presented by CD1d molecules. Here we report the crystal structure of CD1d in complex with a short-chain synthetic variant of α -galactosylceramide at 2.2 Å resolution. This structure elucidated the basis for the high specificity of these microbial ligands and explained the restriction of the α -linkage as a unique pathogen-specific pattern recognition motif. Comparison of the binding of altered lipid ligands to CD1d and TCR shows the differential T_H1- and T_H2-like properties of NKT cells may originate primarily from marked differences in their loading in different cell-types and, hence, in their tissue distribution *in vivo*.

NKT cells are a conserved lymphocyte lineage expressing a semi-invariant TCR ($V_{\alpha}14$ - $J_{\alpha}18$ - $V_{\beta}8$, 7 or 2 in mouse and $V_{\alpha}24$ - $J_{\alpha}18$ - $V_{\beta}11$ in human). They are important in regulating a variety of microbial, allergic, autoimmune and tumor conditions through the rapid and substantial secretion of T_H1 and T_H2 cytokines and chemokines¹. Unlike other T cells, NKT cells are restricted to a non-MHC molecule, CD1d, which binds lipids and glycolipids instead of peptides. Whereas other CD1 isotypes in humans can present a large variety of bacterial compounds that stimulate individual T cell clones expressing diverse TCRs², CD1d appears to have specialized in the presentation of a limited set of lipids for recognition by the entire, or a large fraction of, NKT cell population. Two major classes of agonist ligands of mouse and human NKT cells have been uncovered, a self glycosphingolipid (GSL) isoglobotrihexosylceramide (iGb3)³, and a family of α -glycuronosylceramides that substitute for LPS in the cell wall of some Gram-negative LPS-negative bacteria including *Sphingomonas*^{4–6}. Although iGb3 appears to be the only required ligand for NKT cell development, the dual specificity for self and foreign ligands, a general feature of many innate-like lymphocyte subsets⁷, underlies the recruitment and activation of NKT cells in various disease conditions, including microbial infections⁵. Another microbial ligand present in the

*Corresponding authors: lteyton@scripps.edu, Phone: (858)784-2728, Fax: (858)784-8166, wilson@scripps.edu, Phone: (858)784-9706, Fax: (858)784-2980.

cell wall of mycobacteria, phosphatidylinositol mannoside (PIM₄) also appears to be a natural ligand of NKT cells⁸.

Microbial α -glucuronosylceramides are of particular interest because of their relevance in the context of infection *in vivo* and their very close structural homology with a highly potent agonist of human and murine NKT cells, α -galactosyl ceramide (α -GalCer)⁹, that was previously isolated from marine sponges. These molecules are both α -stereo isomers, a stereochemistry absent from mammalian glycosphingolipids, and phyto-ceramides due to the additional hydroxyl group at the C₄ position of sphingosine that is found in a limited fraction of mammalian glycolipids¹⁰.

Activation of NKT cells by these agonist ligands *in vivo* is initiated by CD1d-expressing antigen presenting cells (APCs), including dendritic cells (DCs), macrophages and B cells, and results in immediate reciprocal activation of the APC, through CD40L-CD40 interaction, and NK cells¹¹. These early events and the massive release of T_H1 and T_H2 cytokines and chemokines by activated NKT cells underlie the powerful adjuvant properties of these agonists for CD4⁺, CD8⁺ T cell and B cell immunity^{11, 12}. NKT cells appear, therefore, to constitute the main TLR-independent pathway leading to full DC maturation and immunity.

Because ligation of NKT cells induces both T_H1 and T_H2 cytokines and NKT cells are essential in regulating a variety of T_H1- and T_H2-mediated immune responses, much attention has been focused on developing variants of NKT cell agonists with biased T_H1 or T_H2 properties. All T_H2 variants reported so far are based on changes in the lipid rather than on the carbohydrate moieties of the glycolipid^{13, 14, 15}. One variant called OCH, produced by truncating the sphingosine moiety of α -GalCer to only 9 carbons, induces stronger interleukin-4 (IL-4) secretion and weaker interferon- γ (IFN- γ) secretion both *in vitro* and *in vivo* and can prevent experimental autoimmune encephalitis in mice¹³. We have recently extended this observation to other variants with either short fatty acid or sphingosine chains¹⁴.

Here we report the crystal structure of the complex between CD1d and one of these variant agonist ligands, which provides unique insights into the biology of innate lipid recognition by NKT cells and the molecular mechanism of NKT cell activation by α -GalCer and its variants. Furthermore, the combination of the structural comparisons and modeling of a series of ligands for V α 14 TCRs with pharmacological studies *in vivo* strongly suggest that the functional differences exhibited by the short lipid variants may be more related to differential uptake and presentation by CD1d-expressing APCs than to intrinsic differences in TCR recognition.

RESULTS

PBS-25 is a strong NKT cell agonist

To probe the biological properties of α -GalCer and α -glucuronosyl ceramide, we systematically changed the length of the sphingosine and fatty acid chains, the unsaturation of the acyl chain, the stereochemistry of the head group and the derivatization of the sugar¹⁴. The most striking series of compounds was obtained by shortening the length of either alkyl chain. One of these compounds, called PBS-25, has an eight carbonyl acyl chain instead of the long C26 fatty acid chain of the original α -GalCer. Otherwise, the phytosphingosine and α -galactose moieties of PBS-25 are similar to their counterparts in α -GalCer. This synthetic compound was tested *in vitro* for its ability to stimulate the canonical V α 14-bearing NKT hybridoma DN32.D3 along with α -GalCer and OCH (PBS-20 in our series). When loaded onto plate-bound CD1d, PBS-25 was as potent as α -GalCer in stimulating DN32.D3 and slightly more potent than PBS-20 (Fig. 1a). Similar results were obtained for a human NKT cell line (data not shown). CD1d tetramers loaded with either α -GalCer or PBS-25 also showed similar staining profiles when tested on V α 14 NKT hybridomas, murine NKT cells (blood, spleen and liver) and a human NKT cell

line (Fig. 1b and data not shown). Thus, like α -GalCer, PBS-25 can be loaded onto murine and human CD1d and bind the whole population of canonical V α 14 or V α 24 NKT cells. Importantly, PBS-25, like all other variants of α -GalCer with shorter fatty acid chains, exhibited an accentuated T_H2 profile as compared to α -GalCer 14.

PBS-25 solubility and loading properties

To further characterize this compound, we assessed its biophysical properties and binding characteristics. Unlike α -GalCer or OCH, which are poorly soluble and require either detergent and/or sonication for solubilization, PBS-25 is readily soluble in aqueous solutions. This physical property translates to efficient loading of PBS-25 onto CD1d, as measured by isoelectric focusing (Fig. 2a). This difference between PBS-25 and α -GalCer was best illustrated in an on-rate stimulation assay in which plate-immobilized CD1d molecules were loaded with a constant amount of lipid for various times and assayed for loading by T cell activation (Fig. 2b). Whereas α -GalCer required a full 24h incubation to reach suboptimal loading, PBS-25 reached equilibrium in about 60 minutes. The addition of lipid transfer proteins, such as murine saposin or murine GM2 activator, did not influence the kinetic behavior of PBS-25, but did improve dramatically the loading of α -GalCer (data not shown).

In the off-rate stimulation assay where, after loading, CD1d molecules were incubated at 37°C in buffer containing no lipid before cells were added, PBS-25 behaved similarly to α -GalCer with very long half-lives and no apparent dissociation over the period tested (Fig. 2c). This long half-life indicates that both lipids have very similar affinities for CD1d and that they mainly differ by their critical micellar concentration (CMC). Finally, CD1d- α -GalCer and CD1d-PBS-25 complexes were also tested for thermal denaturation by circular dichroism spectrum measurement. Thermal denaturation curves have been previously established as a faithful assay for the stability of peptide-MHC complexes¹⁶. Most lipids conferred identical thermal stability to murine CD1d molecules (Fig. 2d). In contrast, PBS-25 shifted the denaturation curve by about 5°C to higher temperatures, indicating that CD1d-PBS-25 complexes are more stable than CD1d- α -GalCer complexes or endogenous CD1d-lipid mixtures (so-called “empty” molecules), despite the substantially decreased length of the acyl chain.

CD1d-PBS-25 affinity for V α 14 TCR

The affinity of CD1d- α -GalCer for the semi-invariant V α 14 T cell receptor is in the low nanomolar range (30 to 90nM)¹⁷, which explains the exquisite sensitivity of NKT cells to minute concentrations of α -GalCer and making NKT cells very efficient sensors for α -GalCer-like bacterial compounds. Surface plasmon resonance measurements of the affinity of CD1d-PBS-25 complexes for recombinant V α 14-V β 8 T cell receptors gave similar results with fast on rates ($2.73 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) and slow off rates ($8.53 \times 10^{-3} \text{ s}^{-1}$) for a dissociation constant of $3.13 \times 10^{-7} \text{ M}$. This result translated into almost identical staining in the tetramer decay assay for CD1d- α -GalCer and CD1d-PBS-25 tetramers (Fig. 1b). The solubility of PBS-25 and its biophysical characteristics thus made it a prime candidate for structural studies with CD1d and V α 14 T cell receptors.

Structure determination of the CD1d-PBS-25 complex

For crystallographic studies, murine CD1d molecules were loaded with a 6-fold (molar) excess of PBS-25 in phosphate-buffered saline at room temperature for 16h. Monomeric lipid-CD1d complexes were purified by size exclusion chromatography, concentrated to $7.0 \text{ mg} \cdot \text{ml}^{-1}$ in 20 mM Hepes buffer pH 7.5 and crystallized for high-resolution diffraction data collection (see Methods). The structure was determined by molecular replacement using mouse CD1d as the starting model¹⁸ and refined to 2.2 Å resolution (Table 1 and Fig. 3) with R_{cryst} and R_{free} values of 24.2% and 29.3% respectively. In the Ramachandran plot 90.2% of the residues are

in the most favored region, 9.2% and 0.6% of the residues are in additionally allowed and generously allowed regions, respectively. The asymmetric unit of the crystal contains two CD1-lipid complexes (A and B) that are very similar, as judged by their root-mean-square deviation (rmsd of 0.31 Å). Thus, the CD1d-complex structure and ligand binding will only be described for molecule A, unless otherwise indicated.

Interaction of PBS-25 with CD1d

The basic architecture of mouse CD1d has been previously described¹⁸. The heterodimer is composed of the three CD1d heavy chain domains, $\alpha 1$, $\alpha 2$ and $\alpha 3$, which non-covalently associate with β_2 -microglobulin (Fig. 3a and b). The $\alpha 1$ and $\alpha 2$ helices sit on top of six β -strands and form a narrow, but deep, binding groove, which can further be divided into two large hydrophobic pockets termed A' and F', that merge to form the entry portal for lipid binding.

The short-chain PBS-25 (Fig. 3c) is bound to CD1 in a way such that the galactose headgroup is located at the boundary between the A' and F' pockets allowing the two alkyl chains to be inserted into each pocket (Fig. 3 and 4). Both alkyl chains are initially inserted perpendicular to the β -sheet platform and then extend more laterally toward the ends of the A' and F' pockets, respectively (Fig. 3b). Aromatic residues Tyr73 (A' pocket), Phe77 and Trp133 (F' pocket) make extensive van der Waals interactions with the glycolipid (Fig. 4a), stabilizing both alkyl chains upon insertion into the individual binding pockets. The phytosphingosine moiety fully occupies the F' pocket, which can accommodate linear alkyl chains of up to C₁₈. The F' pocket is shorter and less deeply buried than the A' pocket, which unexpectedly binds the short C₈-fatty acid. The A' pocket is the most conserved pocket in all of the CD1 isoforms that have been structurally characterized so far. All A' pockets contain a central pole formed by Phe70 and Val12 (CD1a and CD1b) or Cys12 (mCD1), which transects the pocket perpendicular to the β -sheet platform. The alkyl chains of each ligand have to circle around this pole.

More detailed analyses reveal that the A' pocket of mCD1d has more resemblance to CD1b than to CD1a (data not shown, see ¹⁹). Whereas the narrow, winding A' pocket of CD1a has a well-defined terminus, that of CD1d is shaped more like a flat dish, in which the alkyl chain can fully encircle the pole, as in CD1b²⁰. As the short chain fatty acid terminates at the entrance to this A' pocket, the current structure does not reveal in which orientation the pole is encircled by a long chain ligand. However, it seems reasonable to speculate that the long-chain (C₂₆) fatty acid of the full-length α -GalCer runs counter-clockwise, when looked down into the binding groove, as observed for an endogenous ligand bound to CD1d (data not shown). Tyr73 makes seven van der Waals contacts with the alkyl chain, thereby pulling it towards the $\alpha 1$ -helix, which would favor lipid entry into the A' pocket in a counter-clockwise orientation. No other obvious structural feature appears to hinder the alkyl chain encircling the pole in a clockwise orientation.

A spacer stabilizes the A' pocket

In addition to electron density corresponding to the glycolipid ligand, a well-defined tube of electron density was observed in the deeply buried region of the A' pocket that could not be accounted for by the short C₈-fatty acid (Fig. 4a). This density suggests the presence of a not yet characterized, linear hydrophobic compound, which acts as a "spacer lipid" similar to the detergent molecules initially observed in the CD1b-phosphatidylinositol structure²¹. Such a pocket factor could stabilize the hydrophobic binding pockets in the absence of an antigenic groove-filling ligand, such as full-length α -GalCer (C₄₄ total carbons, instead of C₂₆ used here) for CD1d, or GMM for CD1b²². A C₁₆-fatty acid chain could be built with confidence into the electron density and connected to the C₈-fatty acid via two methylene units to recapitulate the C₂₆ fatty acid of the full-length α -GalCer. The presence of this spacer lipid is reminiscent

of the assortment of endogenous peptides that are found in both MHC class I and class II molecules produced in the fly system in the absence of exogenous ligands^{23, 24}. This infers that all MHC and MHC-like molecules have grooves that must be occupied by endogenous ligands in order to maintain stability and prevent denaturation.

Otherwise, the electron density of PBS-25 is remarkably well-defined for the carbohydrate moiety and the polar region of the ceramide backbone compared to any of the other sphingolipids or lipopeptides bound to CD1a and CD1b molecules^{19, 21, 22, 25}. The tail end of the sphingosine (last 3 carbons) and the deeply buried spacer lipid are not quite as well ordered (Fig. 4a) as the headgroup or the rest of the alkyl chains (Fig. 4b). Closer analysis of the binding groove reveals that the end of the F' pocket is broad enough to accommodate the last three carbons in either a slightly upward or downward orientation (Fig. 4a).

Specificity of the lipid-CD1d interaction

Precise hydrogen-bonding between the glycolipid ligand and CD1d positions the ligand in an orientation which allows each of the two alkyl chains to be inserted into its respective pocket (Fig. 5). The 2' and 3' hydroxyl groups of the galactose headgroup are stabilized by Asp153 ($\alpha 2$ -helix) of CD1d and the 3'-, 4'-hydroxyl groups of the phytosphingosine hydrogen bond to Asp80. Such extensive hydrogen bonding between CD1 and its ligand has not been observed in other CD1 complex structures^{19, 21, 22, 25} and probably contributes to the stability, specificity and long half-life of the CD1d-PBS-25 and CD1d- α -GalCer complexes. Furthermore, interaction of the $\alpha 1$ -helix (Asp80) with the C₃, C₄-hydroxyl groups of the sphingosine backbone leads to a ligand orientation in which the sphingosine chain can only be accommodated in the F' pocket and not in the A' pocket, as observed for the sulfatide bound to CD1a¹⁹. Water molecules were not observed in the vicinity of the polar headgroup and, therefore, do not seem to play a critical role in binding of the galactose to CD1.

A total of 73 contacts (6 hydrogen bonds and 67 van der Waals) are made between the PBS-25 and CD1d, while 28 non-polar, van der Waals contacts are formed between the protein and the spacer lipid. The total surface area buried in the mCD1d binding groove for the α -GalCer antigen, spacer lipid and protein is 1,940 Å², where 1,150 Å² are contributed by the protein, 505 Å² by the glycolipid ligand and 285 Å² by the spacer lipid. The total volume of the groove is 1,410 Å³, slightly less than observed for the "empty" mCD1d binding groove (1650 Å³, Fig. 6a). This difference is mostly accounted for by a smaller entrance into the binding groove above the F' pocket in the PBS-25 bound structure (Fig. 6b). Although the rmsd between the "empty" CD1d and the CD1d-PBS-25 structure is only 0.89 Å (for C α), the $\alpha 1$ and $\alpha 2$ helices are closer together above the F' pocket in the PBS-bound CD1d structure, which has important structural consequences. For instance, Asp80 ($\alpha 1$) and Asp153 ($\alpha 2$), which form strong hydrogen bonds with the glycolipid, are positioned 1.3 Å closer to each other and Leu84 ($\alpha 1$) and Leu150 ($\alpha 2$) are situated 1.5 Å closer to each other than in the "empty" structure (Fig. 6c). In addition, these slight positional changes are accompanied by a conformational change of the side chain of Leu84, which is now located exactly above the F' pocket. These changes result in formation of a closed roof above the F' pocket, that buries the tail of the sphingosine chain inside the binding groove and prevents access to the CD1 surface (compare Fig. 6a-c). This situation is in contrast to the F' pockets of the CD1b-GMM²² and CD1a-sulfatide¹⁹ structures, where the tails of the respective glycolipids reach the CD1 surface and could, therefore, also interact directly with the TCR.

CD1 α -GalCer models and mutants

In order to assess the structural requirements of ligand binding to CD1d and NKT cell stimulation, we further interpreted the results of NKT cell stimulation assays based on CD1d mutants or α -GalCer derivatives^{9, 26–29} using the crystal structure of CD1d-PBS-25. All of

these mutants alter the hydrogen bonding network between CD1d and the ligand (Table 2). Also, the weaker reactivity of 4-deoxy α -GalCer and the absence of reactivity of 3,4-dideoxy α -GalCer can be explained by the loss of hydrogen bonding with Asp80 and the subsequent mis-positioning of the head group for recognition. The absence of reactivity of α -mannosyl ceramide (mannose is a C₂-epimer of glucose) can be explained by the loss of the hydrogen bond with Asp153 and the presence of an axial 2' hydroxyl group, which could clash with the TCR. Similarly, α -glucuronosyl ceramide (glucose is the C₄-epimer of galactose) is a weaker agonist of NKT than α -GalCer⁹, because its 4' hydroxyl group does not participate in hydrogen bonding with CD1d (Fig. 6 d and e). But, unlike mannose, it will not interfere with TCR recognition because its C₄-hydroxyl group is in an equatorial position.

The α -anomeric form of the galactose is critical for NKT cell recognition because β -GalCer has no stimulatory capability *in vitro* using purified CD1d- β -GalCer in a plate-bound assay even though some minimal activity has been reported *in vivo*^{9,30–32}. The overlay of the β conformer sulfatide (from the CD1a structure¹⁹) with PBS-25 shows the structural difference between α and β anomers (Fig. 6f). The β linkage positions the headgroup in a perpendicular orientation relative to the current structure, a change that is likely to interfere with the docking of V α 14 T cell receptors (Fig. 6f) and loss of intimate contact with CD1d.

The relatively straightforward modeling of α -GalCer based on the current PBS-25 structure, and the similar binding characteristics between α -GalCer and PBS-25 with respect to CD1d and TCR, argues that gross structural differences are not responsible for the different biological functions observed between short-chain (C₈) and the long-chain (C₂₆) α -GalCer compounds¹⁴. However, differential solubility and on rate could also translate into different pharmacological properties of the α -GalCer variants. This hypothesis was tested directly *in vivo* by injecting α -GalCer and PBS-25 to J α 18-deficient mice (to avoid interference with NKT cells) and purification of B cells, macrophages and dendritic cells from the spleen of these animals for stimulation of DN32.D3 cells (Fig. 7). Dendritic cells and macrophages presented both PBS-25 and α -GalCer whereas B cells presented only α -GalCer. Because B cells are by far the most abundant CD1d-expressing APCs, we would argue that these differences are likely to have significant consequences *in vivo* for the observed immunological outcomes of altered α -GalCer variants.

DISCUSSION

NKT cells are critical components of the adjuvant network that primes adaptive immune responses. In this respect, the molecular understanding of their TCR binding characteristics, activation and modulation of activation through altered ligands is essential to understand their complex behaviour in natural settings and to harness these functions *in vivo* by the rational design of NKT cell ligands optimized for vaccine therapies and the treatment of various forms of autoimmunity, cancer and allergy. The CD1d-PBS-25 structure provides important clues to elucidate the paradigms of NKT cell-mediated regulation. First, it appears that all variants of α -GalCer will anchor their alkyl chains similarly in both murine and human CD1d molecules³³. The sphingosine and acyl chains insert into the binding groove in opposite directions at the A' and the F' boundary to present the sugar head group for TCR recognition. This mode of binding is opposite to the orientation of the sphingosine and fatty acid chains adopted by sulfatide in human CD1a¹⁹, but it appears to be required for optimal interaction of the α -linked galactose through hydrogen bonding to Asp153 and for anchoring the phytosphingosine to Asp80 through its two hydroxyl groups.

This particular restriction of the conformation of the head group suggests why β anomers with only one sugar do not activate NKT cells. However, the radical re-orientation of β -anomers through β -linkage to the ceramide also brings up the issue of how β -linked head groups are

recognized by V α 14 TCRs. The prototype for a β -linked glycolipid is iGb3, the natural selecting ligand of NKT cells³. In this case, one (β -glucosylceramide (β 1-1glucose) or two sugar (lactosylceramide addition of a β 1–4galactose) variants do not stimulate NKT cells in plate-bound assays with purified CD1-lipid complexes. The third sugar, a galactose, is stimulatory only if branched at the α 1–3 position (iGb3), but not at the α 1–4 position (Gb3)³. This would suggest a particular interaction of the last galactose with the TCR, or perhaps even CD1d. However, this complex sugar cannot be easily modeled based on the present data and will require further structure analysis. Alternatively, the same V α 14 TCR might recognize the two complexes, CD1- α -GalCer and CD1-iGb3, with different docking solutions, but this seems unlikely. However, such forms of alloreactivity have been observed in MHC class I complexes for variant peptides³⁴.

The recognition of PIM₄⁸ is an even more complex issue. First, the backbone of PIM₄ is a phospholipid rather than a ceramide. The addition of the phosphoryl group will elongate the neck substantially, with the added flexibility possibly allowing the head group to be positioned to one side. It is, however, not currently possible to accurately model its four mannosyl groups based on the current structure. The same conclusions apply to ligands such as GD3³⁵ or lipophosphoglycan³⁶ that appear to stimulate small subsets of NKT cells.

The presence of a “spacer” or “filler” lipid in the A’ pocket is reminiscent of the collection of peptides found in association with MHC class I and class II molecules produced in the same insect expression system^{23,24}. Unexpectedly, the CD1 complex that binds PBS-25 and retains the spacer lipid is more thermodynamically stable than CD1- α -GalCer itself. Thus, one could argue that the additional spacer lipid adds stability by not being continuous with the fatty acid of PBS-25 but by behaving independently to stabilize the A’ pocket. This conclusion could also explain why the spontaneous loading of α -GalCer is difficult and requires the assistance of lipid transfer proteins³⁷ to extract this A’ pocket stabilizer and why PBS-25, with a shorter chain does not require this additional step. A filler lipid could also explain the stability of non-lipidic small molecules, such as PPBF³⁸. Similarly, the very weak agonist activity of compounds with short acyl chains (two to four carbons in length)^{14,26} could be linked to their inability to sufficiently stabilize the A’ pocket.

The structure of CD1d-PBS-25 also gives a glimpse at T cell recognition of this complex. It has long been known that the unique CDR3 region of the α chain is essential for ligand recognition by NKT cells³⁹, whereas CDR β loops are diverse⁴⁰, and that unrelated V β chains can be paired with canonical V α to recapitulate CD1- α -GalCer recognition¹⁷. These features would place the α chain over the A’ pocket and the β chain over the F’ pocket in an orientation very similar to MHC class I and class II-restricted TCRs⁴¹. As the A’ pocket is completely closed on top by a network of hydrogen bonds, CDR1 α and CDR2 α will see only CD1d and no part of the glycolipid. The CDR3 α will then be placed over and contact the tightly-embedded galactose where we could envisage specific interactions between this loop and the 4’ and 6’ (and possibly 3’) hydroxyl groups of the sugar, as these atoms are well exposed and less tightly complexed with CD1d. However, the hydroxyl group at position 6 can be derivatized without any influence on TCR recognition and is, therefore, not likely to directly influence binding to the TCR (P. B. Savage, unpublished). Galactose would position these two OH groups (2’ and 4’) optimally for recognition, whereas glucose would lose contact with its 4’ hydroxyl group and mannose would lose both 2’ and 4’ hydroxyl group contacts.

This simple recognition scheme, associated with the role of the same hydroxyl groups for CD1 binding, would explain the differences of recognition for the three variants. In this topology, V β will be placed over the F’ pocket of CD1, where binding of the sphingosine induces closure of the groove over the lipid and a substantial narrowing so that the CDR β loops would not have access to the sphingosine. V β would then primarily bind to CD1d residues, particularly for its

CDR1 α and 2 α loops. Thus, overall, most of the TCR interactions would be with CD1d residues, making the recognition unusually self-reactive and relying exclusively on CDR3 α , and perhaps CDR3 β , for ligand discrimination. This mode of recognition is simple and advantageous, limiting the need for scanning, docking and two-step recognition that is required for MHC-restricted TCRs⁴² that must recognize and differentiate more diverse set of bound ligands. In support of this model, our previous studies of CD1d- α -GalCer-V α 14 TCR interaction demonstrated that binding was of high affinity and required no accommodation of large proportions of various antigenic epitopes of the different glycolipid ligands between the CD1 and TCR surfaces¹⁷. The flip side of this mode of recognition is the high degree of self-reactivity that arises due to the extensive contacts with CD1d. Perhaps this propensity for self-reactivity underlies TCR recognition of the self ligand iGb3 as an agonist and its function as an alternate physiological ligand in disease.

Although α -GalCer and PBS-25 are synthetic ligands of NKT cells which are of pharmacological importance for the manipulation of immunity, their close relationship with microbial cell wall α -glycuronosylceramides emphasizes the physiological relevance of our study. In fact, it is possible that the α -GalCer purified from marine sponges based on its anti-tumor properties⁹ originated from bacterial symbionts known to colonize these marine sponges. A similar hypothesis was shown recently for an anti-tumor polyketide isolated from the marine sponge *Theonella swinhoei*⁴³.

The other important conclusion of this work relates to the T_H2 bioactivity of the short lipid variants of α -GalCer. As demonstrated here, structural differences are unlikely to explain the marked functional properties of these altered ligands. In the case of PBS-25, our combined structural and functional *in vivo* studies, suggest that other differences unrelated to TCR recognition, namely the cell types that differentially uptake and present these ligands, are also likely to be critical. Similar conclusions about the pharmacology and *in vivo* processing of α -GalCer derivatives have been reached by others^{15,44}. Differential solubility, access to lipid transfer proteins and receptor-mediated uptake could be involved. Indeed, a previous study already suggested that differences in presentation of short and long variants of mycolic acids are due to differential access to endosomal compartments⁴⁵ and the recently described di-unsaturated C20:2 fatty acid α -GalCer, which also elicits strong T_H2 responses both *in vitro* and *in vivo*, also appears to have a unique pharmacology¹⁵. It will be important to test this hypothesis systematically for compounds, such as OCH, that deviate from the classical α -GalCer behavior.

In conclusion, we report the first structure of the prototype CD1- α -glycosylceramide complex that physiologically activates human and murine NKT cell populations in the context of infection by Gram-negative LPS-negative bacteria, such as *Sphingomonas*. This structure highlights the α -branching of the microbial glycolipid as a unique pattern for recognition not only of the conserved NKT cell TCR, but also by CD1d. These data also provide a basis for future design of NKT cell ligands with selective T_H1 and T_H2 properties.

METHODS

Reagents and cell lines

The synthesis of α -GalCer and variants has been previously described¹⁴. The lipids were resuspended in DMSO at 1 mg/ml and then diluted to working stock solutions of 0.2 mg/ml with 0.05% Tween 20 in PBS buffer. Trisialoganglioside GT1b was purchased from Matreya (Pleasant Gap, PA). DN32.D3 NKT hybridoma cells and human V α 24 NKT cell lines have been previously reported^{5,39}. The cells were maintained in RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, Ca), 2mM L-glutamine (Cambrex, Walkersville, MD) and 20 mM HEPES buffer (Invitrogen).

Protein expression and purification

Recombinant soluble murine and human CD1d molecules were produced in a fly expression system, as previously described¹⁷. Proteins were affinity purified using nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) chromatography followed by anion exchange chromatography on a MonoQ 10/10 column (GE Healthcare, Piscataway, NJ). Purification was monitored by SDS-PAGE. Biotinylation of CD1d was done according to manufacturer's instructions (Avidity, Denver, CO). Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, ILL).

Isoelectric focusing (IEF) electrophoresis

The IEF assay to measure lipid loading onto mCD1d has been previously described¹⁷. Briefly, mCD1d was loaded with GT1b and purified to obtain a single species. A constant 2 μ M mCD1d/GT1b was used in measuring lipid interactions with α -GalCer and variants. Incubations of mCD1d/GT and lipids were for 1 h at 37° C prior to loading onto gels. For quantification, gels were scanned and digitized on an Agfa (Ridefield Park, NJ) scanner and quantified using the UN-SCAN-IT software program (Silk Scientific, Orem, UT). The amount of α -GalCer, or variant, bound to mCD1d is represented as a percentage of total mCD1d in the respective gel lane.

Surface Plasmon Resonance

A BIACORE 2000 instrument was used to determine interactions between purified CD1/lipid complexes and V α 14 TCR molecules. V α 14 TCR was immobilized by amine coupling chemistry on a CM5 research grade sensor chip. Successive injections of CD1/lipid complexes were performed in filtered and degassed PBS buffer at a flow rate of 20 μ l/min at concentrations of 2, 1, 0.5, 0.25, and 0.125 μ M. In all experiments, "empty" CD1d molecules were used as negative control and subtracted from experimental sensorgrams. On- and off-rates were obtained by non-linear curve fitting of subtracted curves using the 1:1 Langmuir binding model using the BIAevaluation program (version 3.0.2).

Thermal denaturation

Thermal denaturation experiments were done by circular dichroism on an AVIV 60DS spectropolarimeter (Aviv Associates, Lakewood, NJ) equipped with a thermoelectric cell holder. CD spectra were recorded in a 1.0 mm pathlength cell at a wavelength of 223 nm in temperature increments of 3°C with a 0.1 s time constant, 10 s averaging time, 3 min equilibration time and 1 nm bandwidth. Samples contained 8 μ M CD1d and 40 μ M lipid in 10 mM Tris pH 8.0 buffer.

T cell stimulation assays

The CD1d-restricted DN32.D3 NKT cell hybridoma was used in all T cell stimulation assays. Unloaded mCD1d molecules were coated for 16–24 h at 1 μ g/well in PBS on 96-well plates. Supernatants were harvested after 24 h to measure IL-2 release using a [³H]-thymidine uptake assay with an IL-2 dependent NK cell line¹⁷. For testing of loading of α -GalCer lipids onto CD1d, lipids were added after PBS washings at various concentrations from the working stock solutions and incubated for 24 h. The wells were washed three times with PBS prior to adding the hybridoma cells. For testing the association and dissociation requirements of α -GalCer lipids, CD1d was plate-coated at 1 μ g/well and loaded with 20 μ g/ml lipid. For dissociation, the plate was loaded for 24 h and then resuspended in PBS and washed at indicated times prior to addition of cells. For association, the respective wells of the plate were washed at indicated times prior to addition of the cells.

Decay experiments

CD1d tetramers were prepared as described using 1 mg/mL DMSO stock solution of α -GalCer, PBS-20 and PBS-25⁴⁶. Cells derived from human V α 24 NKT cell lines⁵ were mixed with a V α 24 negative T cell line as control and stained with the different tetramers and V α 24 (human) antibodies (Beckman Coulter). 1 mg/mL of the anti-human CD1d mAb 51 (obtained from S. Porcelli) was added to prevent rebinding of tetramers. At the indicated time points, an aliquot was washed and cells were analyzed on a FACSCalibur (BD Biosciences) using the CellQuest software.

In vivo tissue distribution

Glycolipids PBS-25, and α -GalCer were injected *i.v.* into J α 218 KO mice, at a dose of 1 μ g per mouse. 24 hours later, splenocytes were harvested. B cells, dendritic cells, and macrophages were purified by B220, CD11c, CD11b MACS beads (Miltenyi Biotech, CA) respectively. Dendritic cells and macrophages were further purified using the anti F4/80 antibody (BD Biosciences) and cell sorting to avoid cross-contamination. 20,000 of each cell type was mixed with 50,000 DN32.D3 NKT cell hybridoma for overnight stimulation. The stimulation was quantified by measuring IL-2 secretion.

Protein crystallization

The best crystals were grown overnight at 22° C, using the sitting drop vapor diffusion method, from 1 μ l protein drops (7 mg/ml) mixed with 1 μ l precipitant (20% PEG 4000, 0.2M calcium acetate).

Structure determination

Crystals were 'flash-cooled' at 100K in 'mother liquor' containing 25% glycerol. Diffraction data from a single crystal were collected at Beamline 8.2.2 (Advanced Light Source, Berkeley, USA) and processed to 2.2 Å with the DENZO-SCALEPACK suite⁴⁷ in spacegroup P2 (unit cell dimensions $a=59.45$ Å, $b=77.05$ Å, $c=111.01$ Å, $\beta=107.63^\circ$). Two CD1-lipid complexes occupy the asymmetric unit with an estimated solvent content of 49.2 % based on a Matthews' coefficient (V_m) of 2.42 Å³/Da. Molecular replacement solutions to a maximum resolution of 4 Å with the program MOLREP⁴⁸ identified the actual space group as P2₁ using the 2.67 Å resolution structure of mouse CD1d¹⁸ as the search model and resulted in an R_{cryst} of 46.2% and a correlation coefficient (CC) of 0.63. Subsequent rigid-body refinement to 3 Å resolution resulted in an R_{cryst} of 40.1%. Prior to maximum-likelihood restrained refinement using the full 2.2 Å resolution data in REFMAC 5.2⁴⁹, 3% of the reflections were set aside for cross-validation and the R_{free} was used to monitor refinement progress. The model was rebuilt into ρ_A -weighted $2Fo - Fc$ and $Fo - Fc$ difference electron density maps using the program O⁵⁰. At a later stage of refinement, N-linked carbohydrates were built at four out of the eight total Asn-X-Thr(Ser) motifs in molecules A and B. Starting coordinates for the PBS-25 ligand were obtained with the molecular modelling system INSIGHT II (Accelrys, Inc., San Diego, CA) and then energy minimized for 100 cycles using the Discover module of INSIGHT II. The ligand libraries for REFMAC⁴⁹ were created using the Dundee PRODRG2 Server⁵¹. Water molecules were assigned throughout the refinement for >3 σ peaks in an $Fo - Fc$ map and retained if they satisfied hydrogen-bonding criteria and returned $2Fo - Fc$ density >1 σ after refinement. A further drop of 2% in R_{free} was achieved by refining all protein atoms, including the N-linked carbohydrates and the lipid ligands for both molecules in the asymmetric unit, as a total of six independent anisotropic domains with the TLS parameterization in REFMAC. Tight NCS restraints were initially applied during the refinement process and the weight for these restraints was lowered gradually towards the final stages of refinement. The CD1d-glycolipid structure has a final $R_{\text{cryst}}=24.2\%$ and $R_{\text{free}}=29.3\%$, values that are slightly higher than typically seen for structures determined at 2.2 Å resolution ($R_{\text{free}} \approx 24-28\%$)⁵². These

higher *R*-values, which are generally an indication for the error between the refined structure and the experimental data, are likely due to an approximate non-crystallographic translation between the two CD1 molecules in the asymmetric unit (*x*=0.44, *y*=0.5, *z*=0.03), which results in pseudo *C*-centering and a large percentage of weak reflections, as previously observed for an Fab structure⁵³. The quality of the model was assessed with the program Molprobity⁵⁴. Buried molecular surface areas and van der Waals contacts were assessed as for the CD1a-sulfatide structure¹⁹.

Structure presentation

The program PyMOL⁵⁵ was used to prepare Figures 3 to 6. The programs Molscript⁵⁶, APBS⁵⁷ and Raster3D⁵⁸ were used to prepare Figure 6 using the coordinates 1CD1 as the “empty” CD1d structure.

Accession codes

Coordinates and structure factors for the mouse CD1d-PBS-25 complex have been deposited in the Protein Data Bank under accession code 1Z5L.

Acknowledgements

We thank the staff of the Advanced Light Source BL 8.2.1 for support with data collection, P. Wright and L. Tennant for help with the CD experiments and R. Stanfield for help with data analysis. This study was supported by National Institutes of Health grants AI053725 (L. T., A. B. and P. B. S.), GM62116 (I.A.W.), CA58896 (I.A.W.), and postdoctoral fellowships from the Skaggs Institute for Chemical Biology (D.M.Z.) and from CRI (J.M.).

BIBLIOGRAPHY

1. Kronenberg M. Toward an Understanding of NKT Cell Biology: Progress and Paradoxes. *Annu Rev Immunol.* 2004
2. Vincent MS, et al. CD1-dependent dendritic cell instruction. *Nat Immunol* 2002;3:1163–1168. [PubMed: 12415264]
3. Zhou D, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 2004;306:1786–1789. [PubMed: 15539565]
4. Kinjo Y, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 2005;434:520–525. [PubMed: 15791257]
5. Mattner J, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 2005;434:525–529. [PubMed: 15791258]
6. Wu D, et al. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc Natl Acad Sci U S A* 2005;102:1351–1356. [PubMed: 15665086]
7. Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol* 2001;1:177–86. [PubMed: 11905826]
8. Fischer K, et al. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci U S A* 2004;101:10685–10690. [PubMed: 15243159]
9. Kawano T, et al. CD1d-restricted and TCR-mediated activation of V_α14 NKT cells by glycosylceramides. *Science* 1997;278:1626–1629. [PubMed: 9374463]
10. Omae F, et al. DES2 protein is responsible for phytoceramide biosynthesis in the mouse small intestine. *Biochem J* 2004;379:687–695. [PubMed: 14731113]
11. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by α -galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 2003;198:267–279. [PubMed: 12874260]
12. Hermans IF, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* 2003;171:5140–5147. [PubMed: 14607913]

13. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing T_H2 bias of natural killer T cells. *Nature* 2001;413:531–534. [PubMed: 11586362]
14. Goff RD, et al. Effects of lipid chain lengths in α -galactosylceramides on cytokine release by natural killer T cells. *J Am Chem Soc* 2004;126:13602–13603. [PubMed: 15493902]
15. Yu KO, et al. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of α -galactosylceramides. *Proc Natl Acad Sci U S A* 2005;102:3383–3388. [PubMed: 15722411]
16. Rudolph MG, et al. The crystal structures of K^(bm1) and K^(bm8) reveal that subtle changes in the peptide environment impact thermostability and alloreactivity. *Immunity* 2001;14:231–242. [PubMed: 11290333]
17. Cantu C 3rd, Benlagha K, Savage PB, Bendelac A, Teyton L. The paradox of immune molecular recognition of α -galactosylceramide: low affinity and low specificity for CD1d, high affinity for alpha beta TCRs. *J Immunol* 2003;170:4673–4682. [PubMed: 12707346]
18. Zeng Z, et al. Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. *Science* 1997;277:339–345. [PubMed: 9219685]
19. Zajonc DM, Elsliger MA, Teyton L, Wilson IA. Crystal structure of CD1a in complex with a sulfatide self antigen at a resolution of 2.15 Å. *Nat Immunol* 2003;4:808–815. [PubMed: 12833155]
20. Moody DB, Zajonc DM, Wilson IA. Anatomy of CD1-lipid antigen complexes. *Nat Rev Immunol* 2005;5:387–399. [PubMed: 15864273]
21. Gadola SD, et al. Structure of human CD1b with bound ligands at 2.3 Å, a maze for alkyl chains. *Nat Immunol* 2002;3:721–726. [PubMed: 12118248]
22. Batuwangala T, et al. The crystal structure of human CD1b with a bound bacterial glycolipid. *J Immunol* 2004;172:2382–2388. [PubMed: 14764708]
23. Scott CA, Garcia KC, Carbone FR, Wilson IA, Teyton L. Role of chain pairing for the production of functional soluble I-A major histocompatibility complex class II molecules. *J Exp Med* 1996;183:2087–2095. [PubMed: 8642319]
24. Apostolopoulos V, et al. Crystal structure of a non-canonical high affinity peptide complexed with MHC class I: a novel use of alternative anchors. *J Mol Biol* 2002;318:1307–1316. [PubMed: 12083519]
25. Zajonc DM, et al. Molecular mechanism of lipopeptide presentation by CD1a. *Immunity* 2005;22:209–219. [PubMed: 15723809]
26. Brossay L, et al. Structural requirements for galactosylceramide recognition by CD1-restricted NK T cells. *J Immunol* 1998;161:5124–5128. [PubMed: 9820479]
27. Burdin N, et al. Structural requirements for antigen presentation by mouse CD1. *Proc Natl Acad Sci U S A* 2000;97:10156–10161. [PubMed: 10963678]
28. Sidobre S, et al. The V α 14 NKT cell TCR exhibits high-affinity binding to a glycolipid/CD1d complex. *J Immunol* 2002;169:1340–1348. [PubMed: 12133957]
29. Sidobre S, et al. The T cell antigen receptor expressed by V α 14i NKT cells has a unique mode of glycosphingolipid antigen recognition. *Proc Natl Acad Sci U S A* 2004;101:12254–12259. [PubMed: 15304644]
30. Stanic AK, et al. Defective presentation of the CD1d1-restricted natural V α 14J α 18 NKT lymphocyte antigen caused by β -D-glucosylceramide synthase deficiency. *Proc Natl Acad Sci U S A* 2003;100:1849–1854. [PubMed: 12576547]
31. Parekh VV, et al. Quantitative and qualitative differences in the in vivo response of NKT cells to distinct α - and β -anomeric glycolipids. *J Immunol* 2004;173:3693–3706. [PubMed: 15356115]
32. Ortaldo JR, et al. Dissociation of NKT stimulation, cytokine induction, and NK activation in vivo by the use of distinct TCR-binding ceramides. *J Immunol* 2004;172:943–953. [PubMed: 14707067]
33. Jones EY, Cerundolo V. *Nat Immunol*. 2005
34. Speir JA, et al. Structural basis of 2C TCR allorecognition of H-2L^d peptide complexes. *Immunity* 1998;8:553–562. [PubMed: 9620676]
35. Wu DY, Segal NH, Sidobre S, Kronenberg M, Chapman PB. Cross-presentation of disialoganglioside GD3 to natural killer T cells. *J Exp Med* 2003;198:173–181. [PubMed: 12847141]

36. Amprey JL, et al. A subset of liver NK T cells is activated during *Leishmania donovani* infection by CD1d-bound lipophosphoglycan. *J Exp Med* 2004;200:895–904. [PubMed: 15466622]
37. Zhou D, et al. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science* 2004;303:523–527. [PubMed: 14684827]
38. Van Rhijn I, et al. CD1d-restricted T cell activation by nonlipidic small molecules. *Proc Natl Acad Sci U S A* 2004;101:13578–13583. [PubMed: 15342907]
39. Lantz O, Bendelac A. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4–8– T cells in mice and humans. *J Exp Med* 1994;180:1097–1106. [PubMed: 7520467]
40. Apostolou I, Cumano A, Gachelin G, Kourilsky P. Evidence for two subgroups of CD4–CD8– NKT cells with distinct TCR $\alpha\beta$ repertoires and differential distribution in lymphoid tissues. *J Immunol* 2000;165:2481–2490. [PubMed: 10946274]
41. Garcia KC, Teyton L, Wilson IA. Structural basis of T cell recognition. *Annu Rev Immunol* 1999;17:369–397. [PubMed: 10358763]
42. Wu LC, Tuot DS, Lyons DS, Garcia KC, Davis MM. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature* 2002;418:552–556. [PubMed: 12152083]
43. Piel J, et al. Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *Proc Natl Acad Sci U S A* 2004;101:16222–16227. [PubMed: 15520376]
44. Bezradica JS, et al. Distinct roles of dendritic cells and B cells in $V_{\alpha}14J_{\alpha}18$ natural T cell activation in vivo. *J Immunol* 2005;174:4696–4705. [PubMed: 15814694]
45. Moody DB, Besra GS, Wilson IA, Porcelli SA. The molecular basis of CD1-mediated presentation of lipid antigens. *Immunol Rev* 1999;172:285–296. [PubMed: 10631954]
46. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med* 2000;191:1895–1903. [PubMed: 10839805]
47. Otwinowski Z, Minor W. HKL: Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.
48. Vagin AA, A T. MOLREP: an automated program for molecular replacement. *J Appl Cryst* 1997;30:1022–1025.
49. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum likelihood method. *Acta Crystallogr* 1997;D53:240–255.
50. Jones TA, Cowan S, Zou JY, Kjeldgaard M. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr* 1991;A47:110–119.
51. Schuettelkopf AW, van Aalten DM. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr* 2004;D60:1355–1363.
52. Kleywegt GJ, Jones TA. Homo crystallographicus--quo vadis? *Structure (Camb)* 2002;10:465–472. [PubMed: 11937051]
53. Stanfield RL, Ghiara JB, Ollmann Saphire E, Profy AT, Wilson IA. Recurring conformation of the human immunodeficiency virus type 1 gp120 V3 loop. *Virology* 2003;315:159–173. [PubMed: 14592768]
54. Lovell SC, et al. Structure validation by C_{α} geometry: ψ , ϕ and C_{β} deviation. *Proteins* 2003;50:437–450. [PubMed: 12557186]
55. DeLano W. The PyMOL Molecular Graphics System. 2002
56. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of proteins. *J Applied Crystallogr* 1991;24:946–950.
57. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A* 2001;98:10037–10041. [PubMed: 11517324]
58. Merritt EA, Bacon DJ. Raster3D: Photorealistic Molecular Graphics. *Meth Enzymology* 1997;277:505–524.
59. Jahng A, et al. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* 2004;199:947–957. [PubMed: 15051763]

60. CCP4. Collaborative Computational Project, Number 4. The CCP4 Suite: Programs for Protein Crystallography. Acta Crystallogr 1994;D50:760, 763.
61. Kamada N, et al. Crucial amino acid residues of mouse CD1d for glycolipid ligand presentation to V α 14 NKT cells. Int Immunol 2001;13:853–861. [PubMed: 11431415]

Figure 1. PBS-25 is an agonist of NKT cells. **(a)** Plate-bound CD1d molecules were loaded overnight with α -GalCer (α -GC, diamonds), PBS-20 (squares) or PBS-25 (circles) at various concentrations and used to stimulate DN32.D3 T cell hybridoma cells. IL-2 production was measured from triplicate cultures. A representative of 3 separate experiment is shown. **(b)** Binding properties of CD1d tetramers loaded with either α -GalCer or PBS-25. Biotinylated human CD1d molecules were loaded with either α -GalCer or PBS-25 and used to stain a human V α 24 T cell line. After staining, decay of the tetramers was examined at different times. The experiment was performed twice. .

Figure 2.

Kinetics of binding of PBS-25 to CD1d. **(a)** Binding of the various α -GalCer variants was examined by native isoelectrofocusing at various concentrations of lipid. PBS-25 (circles), α -GalCer (diamonds), PBS-20 (squares) and vehicle only (triangles) are shown side by side. **(b)** Association of PBS-25 with CD1d was measured in an on-rate assay. Plate-bound CD1d molecules were incubated with constant concentrations of lipids for various times and then washed before the addition of the α -GalCer variants and DN.32.D3 T cells (PBS-25 circles, α -GalCer: diamonds). **(c)** Stability of the CD1d- α -GalCer and PBS-25 CD1d complexes. Stability was measured using an off-rate assay in which lipid-CD1 complexes are incubated and washed at set times before the addition of DN32.D3 cells for activation. IL-2 production was measured from triplicate cultures. **(d)** Profiles of thermal denaturation of various CD1d-lipid complexes. Denaturation was measured by circular dichroism over a range of increasing temperatures as the change in molar ellipticity. CD1d-PBS-25 complexes (pink circles) are compared to PBS-20 (triangles), α -GalCer (squares), and "empty" CD1 complexes (diamonds). A minimum of 5 spectra were averaged for each sample. All experiments were carried out at least three times.

Figure 3.

Overview of the mouse CD1d structure with bound PBS-25 glycolipid. **(a)** Top view, looking down into the mCD1d binding groove with A' and F' pockets labeled. N-linked carbohydrates are shown at two of the four positions (N42 and N165) as grey sticks, with their nitrogens and oxygens coloured blue and red, respectively. **(b)** Front view of mCD1d ($\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta_2 M$ domains in grey) with the bound short-chain α -galactosyl ceramide (PBS-25) in yellow and a "C₁₆ spacer lipid" in orange. **(c)** Chemical representation of the glycolipid ligands PBS-25, α -glucuronosyl ceramide, α -galactosyl ceramide and OCH (PBS-20).

Figure 4.

Conformation of the short α -GalCer in the mCD1d binding groove. **(a)** A shake-omit map was calculated after omitting the lipopeptide ligand coordinates and contoured at $2.0\ \sigma$ as a green mesh around the glycolipid ligand (yellow) and the “spacer lipid” (orange) as shown in a side view, after removing the $\alpha 2$ -helix for clarity. **(b)** Top view, looking down into the groove (TCR view). Several important contact residues of the A' and F' pocket (A' and F' respectively) are depicted and. Whereas the charged residues Asp80 (D80), Arg79 (R79) and Asp153 (D153) contact to the polar regions of the glycolipid, the many hydrophobic residues are exclusively contacting the lipid backbone. The galactose is unusually well-ordered compared to the terminal carbons of the sphingosine or the deeply-buried spacer lipid.

Figure 5.

Stereo-view of the specific hydrogen-bond network between mCD1d and an α -GalCer ligand. The central part of the glycolipid is shown with the alkyl chains pointing down into the binding groove and the galactose nestled close to the CD1d surface between the $\alpha 1$ and $\alpha 2$ helices. Whereas the $\alpha 1$ -helix (Asp80 and Arg79) stabilizes the sphingosine backbone, the $\alpha 2$ -helix is mainly involved in hydrogen-bonding to the galactose headgroup. Hydrogen-bonds are depicted as blue dashed lines and distances (Å) between the hydrogen-bonding partners are labeled accordingly.

Figure 6.

CD1d antigen binding grooves with crystallized or modeled ligands. Comparison between the binding groove portals of (a) mCD1d to which no exogenous ligand has been added and (b) the CD1d-PBS-25 structure. (c) Ribbon representation of the “induced fit” after superimposition of the protein backbones (C α atoms) of both CD1 structures (PDB codes 1CD1 and 1Z5L) from panel a and b in the same view. (d) Molecular model of the microbial antigen α -glucuronyl ceramide bound to mCD1d. (e) Model of the full-length α -GalCer ligand (refined in REFMAC 5 against the mCD1d-PBS-25 data) bound by mCD1d, which mimics the combined overall conformation and location of the shorter PBS-25 ligand and the “spacer lipid” (compare to b, lower panel). (f) Comparison between the α -anomeric (yellow) and β -anomeric (green) configuration of the galactose. β -GalCer is not antigenic and would lose two hydrogen bonds with Asp153 (compare with Figure 5). The addition of a sulfate (orange) to β -GalCer results in sulfatide, which is a natural mCD1d ligand⁵⁹ and could introduce a new hydrogen bond and electrostatic interaction between the sulfate and Arg⁷⁹. Molecular surfaces are either shown with electrostatic potentials in a top view (a, b and d) and in a front view (f) or as transparent binding pockets with bound ligands in a side view (middle row and panel e). Note the α -anomeric galactose sits flatly atop the binding groove, compared to its β -anomer which sticks out away from the groove. Protein residues that are involved in hydrogen bonding or the formation of the roof above the F' pocket in (b) are labelled, as well as the A' and F' pockets (A' and F', respectively). Electrostatic surface potentials were calculated using the program APBS⁵⁷ Red is electronegative and blue is electropositive (–30 to +30 kT/e).

Figure 7.

Pharmacological distribution of α -GalCer and PBS-25. The two α -GalCer variants were injected *in vivo* and various antigen presenting cell (APC) subsets were tested for their ability to stimulate DN32.D3 IL-2 production (triplicates). CD11c⁺ were highly enriched DC cells (>98% purity), CD11b⁺ were highly enriched macrophages (>98% purity) and B220⁺ represented highly purified B cells (>98% purity). The experiment was performed 3 independent times.

Table 1
Data collection and refinement statistics for mCD1d

Parameter	mCD1d-PBS-25
Data collection	
Resolution range (Å) ¹	50.0–2.2 (2.25–2.2)
Completeness (%) ¹	93.8 (86.9)
Number of unique reflections	45,449
Redundancy	2.4
R _{sym} ^{1,2} (%)	7.5 (36.9)
I/σ ¹	20.8 (2.0)
Refinement statistics	
Number of reflections (f>0)	44,014
Maximum resolution (Å)	2.2
R _{cryst} ³ (%)	24.2 (33.7)
R _{free} ⁴ (%)	29.3 (44.0)
Number of atoms	
Protein	6,330
Glycolipid ligand	5,950
“Spacer lipid”	84
N-linked carbohydrate	32
Water	98
	166
Ramachandran statistics (%)	
Most favored	90.2
Additional allowed	9.2
Generously allowed	0.6
R.m.s. deviation from ideal geometry	
Bond length (Å)	0.015
Bond angles (°)	1.85
Average B values (Å²)⁵	
Protein	58.0
PBS-25 ligand	57.5
“Spacer lipid”	59.4
Water molecules	61.2
Carbohydrates	68.6

¹ Number in parentheses refer to the highest resolution shell.

² $R_{\text{sym}} = (\sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / (\sum_h \sum_i I_i(h))) \times 100$, where $\langle I(h) \rangle$ is the average intensity of i symmetry-related observations with reflections with Bragg index h.

³ $R_{\text{cryst}} = (\sum_{\text{hkl}} |F_o - F_c| / \sum_{\text{hkl}} |F_o|) \times 100$, where F_o and F_c are the observed and calculated structure factors, respectively, for all data.

⁴ R_{free} was calculated as for R_{cryst} , but on 3% of data excluded before refinement.

⁵ B values were calculated with the CCP4 program TLSANL⁶⁰.

Table 2
Interaction between crucial CD1d residues and α -GalCer

CD1d residue	Interacting partner	Type of contact	Mutant activity ^I
Arg79	3"-OH (sphingosine)/TCR	1 H-bond	none
Asp80	3", 4"-OH (sphingosine)	2 H-bonds	none
Glu83	None/TCR	n/a	none
Asp153	2", 3"-OH (galactose)	2 H-bonds	none

^I Mutant activity is based on previous V α 14 NK T cell stimulation studies using alanine mutants⁶¹. Only residues are shown which are critical for NK T cell recognition.