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Structural Evaluation of Potent NKT-cell Agonists: Implications for Design of Novel Stimulatory Ligands

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Abstract

Natural Killer T (NKT) cells are a subset of T cells that are activated by CD1d-glycolipid complexes through a semi-invariant $\alpha\beta$ T cell receptor (NKT TCR). Upon activation, NKT cells secrete regulatory cytokines that are implicated in T helper cell responses. α -Galactosylceramide (α -GalCer) is a potent NKT cell agonist when presented by CD1d. Phenyl ring substitutions of the α -GalCer fatty acid moiety were recently found to be superior in eliciting regulatory cytokines. Crystal structures of four new mCD1d-lipid complexes (5 structures), a new PBS-25 complex, and CD1d with an endogenous ligand, at 1.6–1.9 Å resolution, reveals that the α -GalCer phenyl analogues impart minor structural differences to the A'-pocket, while the sphingosine and galactose moieties, important for NKT TCR recognition, remain virtually unchanged. The observed differences in cytokine release profiles appear to be associated with increased stability of the CD1d-glycolipid complexes rather than increased affinity for the NKT TCR. Furthermore, comparison of mCD1d-glycolipid complexes in different crystallographic space groups reveals considerable conformational variation, particularly above the F'-pocket, the primary site of interaction with the NKT TCR. We propose that modifications of the sphingosine moiety or other substitutions that decrease α -GalCer flexibility would stabilize the F'-pocket. Such compounds might then increase CD1d affinity for the NKT TCR and further enhance the stimulatory and regulatory properties of α -GalCer derivatives.

Keywords

CD1; NKT cells; α -GalCer analogues; glycolipids; immune system

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi: XXX

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Introduction

CD1 proteins are non-polymorphic, class I antigen-presenting molecules, which present both self and foreign lipids as cognate antigens to T cells. The CD1 family consists of five isoforms that can be classified into three groups: group 1 comprises CD1a, CD1b and CD1c; group 2 comprises CD1d and group 3 comprises CD1e (1). Groups 1 and 2 are involved in antigen presentation, whereas group 3 is involved in lipid processing and trafficking. Group 1 CD1 molecules present lipid antigens to clonally diverse T cells that mediate adaptive immunity. In contrast, group 2 CD1d molecules present lipid antigens to natural killer T (NKT) cells. CD1d-reactive NKT cells comprise a unique subset of regulatory T lymphocytes that have been implicated in the regulation of immune responses associated with infectious diseases, cancer and autoimmunity (2,3). NKT cells express a semi-invariant T cell receptor that recognizes both self and foreign glycolipids presented by CD1d (4). Upon stimulation, NKT cells rapidly secrete large amounts of immune regulatory T helper cell 1 (Th1) and T helper cell 2 (Th2) cytokines, such as interferon- γ (IFN- γ) and interleukin 4 (IL-4), respectively. Th1 cytokines support cell-mediated immunity and are correlated with antitumor, antiviral/antibacterial and adjuvant effects. In contrast, Th2 cytokines are believed to either delay or prevent the onset of autoimmune diseases, such as type-1 diabetes or multiple sclerosis (5,6). α -Galactosylceramide (α -GalCer) is a potent CD1d stimulatory ligand that elicits secretion of these cytokines (7). However, its effectiveness is limited by the opposing effects of the Th1 and Th2 cytokines. Thus, the design of novel lipid molecules that could specifically stimulate NKT cells to secrete either IFN- γ (Th1) or IL-4 (Th2) cytokines would have tremendous therapeutic potential.

Crystal structures of the human CD1d- α -GalCer and mouse CD1d-PBS-25 complexes have established the mode of α -GalCer binding and presentation by CD1d (8,9). CD1d is a heterodimeric molecule consisting of the three heavy chain domains α 1- α 3 associated non-covalently with β 2-microglobulin (β 2m), as in MHC class I. The α 1 and α 2 domains form the antigen-presenting superdomain that is also common to class I antigen-presenting molecules. The α 1- α 2 superdomain contains a deep, hydrophobic ligand-binding groove that is assembled from an eight-stranded, β -sheet platform that is traversed by two anti-parallel α -helices, α 1 and α 2, which form the sides of the binding site (10). This binding groove is subdivided into two major pockets, A' and F' (Fig. 1). Glycosphingolipids access these pockets in a well-defined manner, such that the fatty acid moiety enters the A'-pocket and the sphingosine moiety inserts into the F'-pocket, whereas the polar head group is presented at the CD1d surface to the NKT TCR (8,9,11,12,13). Furthermore, the crystal structure of the human CD1d- α -GalCer-NKT TCR complex furnished general insights into CD1d-lipid recognition by the NKT TCR which binds above the F'-pocket and contacts the galactose head group of α -GalCer (14).

Based on these structural, as well as biological, data, several attempts have been undertaken to modify α -GalCer to alter its stimulatory profile. OCH, an α -GalCer analogue with a shortened sphingosine moiety, changes the NKT cell activation profile towards a Th2 response (15), whereas C-glucosides shift the profile towards Th1 responses (16). Substitutions of the galactose moiety by nonglycosidic compounds were also found to be effective ligands (17). However, substitutions of the fatty acid of α -GalCer with other functional groups have been the most extensively studied. In particular, phenyl analogues of α -GalCer have an increased affinity for CD1d and shift the cytokine-release profiles in NKT cell assays towards a Th1 response (18,19). The increase in ligand affinity was proposed to be associated with specific interactions between the phenyl moieties and the aromatic side chains in the A'-pocket of CD1d (18).

In order to assess the molecular basis of the enhanced Th1 responses, we set out to determine crystal structures of mouse CD1d (mCD1d) in complex with the α -GalCer analogues, PBS-25, C6Ph, C8Ph, C8PhF, C10Ph, as well as CD1d without addition of glycolipid ligand (see Fig.

1). In the C6Ph, C8Ph, and C10Ph ligands, the acyl chain is substituted by phenyl rings, which are linked to the sphingosine nitrogen by 6, 8 or 10 carbons atoms, respectively. C8PhF is a modification of C8Ph, where the phenyl ring hydrogen in the *para*-position is substituted by fluorine. The mCD1d-PBS-25 complex structure was described earlier in a different space group (9) and serves as the reference structure. To pinpoint subtle changes of biological significance, all structures were determined in the same space group at the highest resolution possible. Furthermore, to analyze the effect of crystal packing on the conformation of these complexes, C8Ph was also determined in a different space group and PBS-25 was compared to the previously published structure in yet another space group. Our results demonstrate that, although fatty acid analogues increase the stability of CD1d-glycosphingolipid complexes, they do not induce major conformational changes that would affect interaction with the NKT TCR to any significant extent. Nevertheless, even very subtle changes, as for agonist and antagonist ligands of MHC-I, (20,21) or, more obviously here, flexibility in the F'-pocket itself, may play a key role in the different observed immunological outcomes.

Results

Comparison of different mCD1d-glycolipid complexes in a given space group

The best diffracting crystals for mCD1d by itself and in complex with PBS-25, C6Ph, C8Ph, C8PhF, and C10Ph, were obtained in monoclinic space group $P2_1$. (for unit cell parameters, see Table 1). All six structures were determined to a resolution of ≤ 1.9 Å and show excellent geometry and statistics (Table 1). The final models lack residues 1-6 of $\alpha 1$ and 196-202 of $\alpha 3$ in the mCD1d heavy chain that are too flexible or disordered to be resolved in the electron density maps. Three out of five potential N-glycosylation sites show well-ordered electron density that permitted modeling of eleven sugar moieties per CD1d molecule (Fig. 1 and Table 1). Unambiguous electron density was observed for the ligands PBS-25, C6Ph, C8Ph, and C8PhF (Fig. 2), whereas C10Ph was partially disordered. Comparison of the B-values, which are dependent on the atom occupancy as well atom mobility, between the ligands and the surrounding mCD1d atoms, shows that the ligands all have 100% occupancy. The electron density for C10Ph has less well-defined features for the equivalent resolution; the electron-rich, phenyl ring was more disordered but, nevertheless, refines to a similar location as the phenyl rings of C8Ph and C8PhF. The B-values of the C10Ph acyl moiety are also considerably increased compared to the other ligands, which again indicate increased mobility.

The mCD1d structure without any added exogenous glycolipid ligand (mCD1d-nolig) does not show as strong and continuous electron density as the ligand complexes. However, disconnected, residual electron density within the binding groove indicates that it is not completely empty, but may be partially occupied by molecules either acquired from mCD1d expression in SF9 insect cells or during purification. All structures including mCD1d-nolig show clear density for an acquired lipid (AL) in the A'-pocket (Fig. 2). This cellular lipid likely prevents the large A'-pocket from collapsing in absence of external ligands (9,12,13), and was previously identified as palmitic acid (12).

In order to determine structural differences among the different mCD1d-lipid complexes, residues 8-180, which form the ligand-binding $\alpha 1$ - $\alpha 2$ superdomain, were superimposed. Overall, the five complexes, mCD1d-PBS-25, mCD1d-C6Ph, mCD1d-C8Ph, mCD1d-C8PhF, mCD1d-C10Ph and mCD1d-nolig, are very similar with no noteworthy variation in their main-chain conformations (Fig. 3). Conformational differences of the ligand-contacting side chains in mCD1d-PBS-25, mCD1d-C6Ph, mCD1d-C8Ph, mCD1d-C8PhF, and mCD1d-C10Ph were only observed for Gln14, Phe70, Tyr73 and Leu100 (Fig. 3). These five complexes exhibit overall root-mean-square displacements (rmsd) of 0.29 to 0.45 Å for all atoms and 0.11 to 0.16 Å for their respective C α -positions. In comparison, the mCD1d-nolig structure exhibits slightly higher rmsds of 0.62 Å (all atoms) and 0.22 Å (C α). The largest conformational difference of

ligand-contacting residues between mCD1d-nolig and the mCD1d-glycolipid complexes was observed for the Asp80 side chain (Fig. 3B).

To analyze ligand contacts and conformations, PBS-25, C6Ph, C8Ph, C8PhF, and C10Ph were subdivided in the following four constituents: the polar core, galactose head group, acyl moiety (hydrophobic part of the fatty acid), and sphingosine moiety (hydrophobic part of the sphingosine) (Fig. 4). The most significant differences among the five ligands are found in the A'-pocket, where the acyl moieties adopt different conformations due to their different size and space requirements (Fig. 3). Overall, interactions in the A'-pocket are dominated by weak hydrophobic or van der Waals (vdW) contacts. The total number of vdW contacts to the acyl moieties is 19, 22, 32, 40, and 37 for PBS-25, C6Ph, C8Ph, C8PhF, and C10Ph, respectively (Table S1). The number of vdW contacts per carbon in the A'-pocket is 2.7, 2.0, 2.5, 2.9, and 2.5, where the highest is for C8PhF. For all ligands, the majority of vdW contacts in the CD1d A'-pocket are contributed by Tyr73 that contacts mainly the lipid methylene groups rather than the appended phenyl ring moieties of C6Ph, C8Ph, C8PhF, and C10Ph. C6Ph is the only ligand that uses its phenyl ring to contact an aromatic CD1d side chain (Phe70). The phenyl rings of C8Ph, C8PhF, and C10Ph all bind in the same location with only minor conformational differences. All three ligands form vdW contacts between their phenyl rings and Cys12, Gln14, Leu100, and Ala102 (Fig. 4). While the fatty acid chains of C8Ph and C8PhF, like PBS-25 and C6Ph, adopt an all-trans lowest energy conformation in the A'-pocket, C10Ph, due to its length, has to adopt a slightly compressed structure. As a result, the polar core of C10Ph is slightly shifted with respect to the positions observed for PBS-25, C6Ph, C8Ph, and C8PhF (Figs. 2 and 3).

The polar cores and the galactose head groups of PBS-25, C6Ph, C8Ph, and C8PhF show only negligible conformational differences that are well within the coordinate error, and are the only components of the ligand that hydrogen bond to mCD1d. Four direct hydrogen bonds are formed between the ligands and Asp80, Asp153, and Thr156, with mCD1d always acting as the hydrogen bond acceptor. Three more hydrogen bonds are mediated through bridging water molecules. While the polar core contributes five of these hydrogen bonds, the galactose head group contributes only two. In contrast, the slightly shifted, polar core of C10Ph loses a water-mediated hydrogen bond in the mCD1d-C10Ph complex (Fig. 4).

The conformation of the ligand sphingosine moiety in the F'-pocket is similar for all ligands with an average total number of 25 vdW contacts which corresponds to 1.8 vdW contacts per carbon atom of the sphingosine moiety (Table S1). Proximal to the polar core, the positions of the sphingosine methylene groups are very well-defined and superimpose well and to the same extent as the atoms of the polar cores. However, further into the F'-pocket, the sphingosine moieties adopt slightly different conformations (Fig. 3), all of which are very close to the most favorable, all-trans conformation and appear to be exchangeable between the different ligands. There is no indication that this minor variation is caused by any real differences in the acyl moieties.

B-value analysis of the individual ligand constituents of PBS-25, C6Ph, C8Ph, and C8PhF shows that the hydrogen-bonded polar core has the lowest B-values that are equivalent to the surrounding protein residues. The sphingosine moieties and galactose head-groups exhibit, on average, 17% and 22% higher B-values, respectively, than the polar core. More noteworthy, the B-values of the terminal eight carbons of the sphingosine moieties are increased by 20% over the average and are comparable to those of the surface-exposed galactose head groups (Table S1). These analyses suggest that the terminal atoms of the sphingosine moieties have more conformational freedom than the polar core and acyl moieties.

Depending on the length of the acyl moiety of the added ligand, the AL adopts different conformations in the A'-pocket. The phenyl moiety of C6Ph terminates in the A'-pocket close to the methyl terminus of AL and pushes the AL forward so that its carboxylic acid head group can form a hydrogen bond with the Gln14 side chain. On the other hand, C8Ph, C8PhF, and C10Ph terminate in the A'-pocket on the carboxylate side of the AL and push it in the opposite direction. In the presence of the AL, the A'-pocket appears to be optimally filled by C8PhF.

Comparison of mCD1d-glycolipid complexes in different space groups

To address the question of whether any sites of dynamic variability are apparent in the mCD1d-glycolipid complexes, we compared mCD1d-C8Ph and mCD1d-PBS-25 in different space groups and crystal packing environments. To achieve that goal, the mCD1d-C8Ph complex was further determined in space group $P2_12_12_1$ at 1.9 Å resolution (Table 1) and the mCD1d-PBS-25 complex was compared to a previously described structure of the same complex in $P2_1$ with a different crystal packing (9). Residues 8-180 were again used for the superposition.

Overall, the two mCD1d-C8Ph complex structures in $P2_1$ and $P2_12_12_1$ are very similar with overall rmsd's of 0.72 Å, and 0.31 Å for their respective C α -positions (Fig. 5); the number of vdW contacts is comparable. The ligand acyl moiety superimposes best, but the polar core is slightly different resulting in the loss of a water-mediated hydrogen bond between C8Ph and Asp80 in the $P2_12_12_1$ structure. Minor conformational differences in the sphingosine moiety are comparable to those observed for different ligands in the same space group and indicate some conformational freedom within the F'-pocket. In contrast, the galactose head group is displaced by 0.7 Å (Fig. 5), that may be significant as the galactose head group of α -GalCer in the human CD1d- α -GalCer (PDB code 1ZT4) complex in comparison to its TCR complex (PDB code 2PO6) is also displaced by 0.7 Å, indicative that conformational flexibility of CD1d-glycolipid complexes may play a critical role for recognition by the NKT TCR.

Even larger conformational differences are observed when the mCD1d-PBS-25 complex presented here is compared to the same complex in space group $P2_1$ with unit cell dimensions of 59.5 Å, 77.1 Å, 111.0 Å and $\beta = 107.6^\circ$ and two molecules per asymmetric unit ($P2_1$ prev), as reported previously at 2.2 Å (9). Due to the high similarity between the two independent molecules (A and B) in the asymmetric unit, only molecule A of $P2_1$ prev is used in this comparison. The overall rmsd between mCD1d-PBS-25 complexes in $P2_1$ and $P2_1$ prev is 1.57 Å (1.09 Å for C α) (see Fig. 5). The C α -trace shows significant differences on both $\alpha 1$ and $\alpha 2$ helices at the end of the F'-pocket. In $P2_1$ prev, the F'-pocket adopts a more closed conformation. The acyl and sphingosine moieties also show considerable differences. In particular, the terminal methyl group of the sphingosine moiety is displaced by 2 Å. Otherwise, differences of the PBS-25 polar cores and galactose head groups are small and within the coordinate error. The only exception is the Asp80 side chain in $P2_1$ prev, which has a different conformation and forms two direct hydrogen bonds with PBS-25. These differences in the PBS-25 conformation are most likely caused by the more closed conformation of the F'-pocket in $P2_1$ prev.

Thus, conformational differences that are observed in different crystal packing environments suggest that this flexibility enables different conformers to be stabilized in these crystal forms and that these dynamic properties may play a role in recognition by the NKT TCR.

Alternative conformations and flexibility of mCD1d-glycolipid complexes

The observed conformational differences in these different space groups led us to carefully examine alternative conformations that may allow for more definitive conclusions about the observed flexibility in mCD1d-glycolipid complexes. To that end, we selected a set of eight mCD1d-ligand complexes that are unique with respect to their crystal-packing environment (see Materials and Methods). All PBS-25, C8Ph, C8PhF, iGb3, and sulfatide complexes with

CD1d contain glycosphingolipids that are very similar in their chemical nature and binding mode. The main differences arise from CD1d recognition of the sugar head groups (α - vs. β -sugar linkage), acyl moieties (fatty acid length and substitution), and polar core with an additional $-\text{OH}$ group instead of a double bond at the sphingosine C4 in PBS-25, C8Ph, and C8PhF. The sphingosine moiety (hydrophobic part of the sphingosine) is chemically identical for all ligands.

Despite their similarity, these complexes show considerable conformational variation when superimposed (Fig. 6). Major conformational differences are confined to three regions: the N- and C-terminal ends of helix $\alpha 1$, and a β -hairpin from the β -sheet platform. Helix $\alpha 2$ shows only small differences. Residues that form hydrogen bonds with the ligand or vdW contacts with the acyl moiety appear to be rather indifferent to the bound ligand. On the other hand, the C-terminal region of helix $\alpha 1$ adopts several different conformations in these various complexes, indicating that the F'-pocket can adopt more than a single, well-defined conformation in the presence of ligand. Since all of the compared ligands have the same length for the sphingosine moiety, some intrinsic flexibility is implied in this region of CD1d. Strikingly, the most flexible region closely matches the footprint of the NKT TCR on the CD1d- α GalCer complex, as determined by structural and mutational analyses (14,22,23). Analysis of the human CD1d- α GalCer-NKT TCR crystal structure reveals that the invariant α -chain recognizes the conformationally invariant part of the F'-pocket, whereas the flexible part, as we identify and define here, is bound by the variable β -chain. Comparison of mCD1d-ligand complexes with human CD1d- α GalCer in the ternary NKT TCR complex shows that the human structure is most similar to the mouse sulfatide complex (PDB code 2AKR), whereas the PBS-25 complex (PDB code 1Z5L) has a more closed F'-pocket, and the structures presented here have a slightly more open F'-pocket.

Discussion

Although the α -galactosyl sphingolipid ligands C6Ph, C8Ph, C8PhF, and C10Ph have significantly smaller fatty acid moieties compared to α -GalCer, they exhibit about 8, 10, 80, and 4 fold higher affinity for CD1d, respectively (19). In agreement with our structural findings, the affinity of these ligands seems to be correlated with an optimal linker length of eight carbon atoms. Presentation of C6Ph, C8Ph, C8PhF and C10Ph to the NKT TCR is clearly dominated by hydrogen bonds from CD1d to the polar core and galactose of the stimulatory ligands (Fig. 4). The accommodation of a phenyl ring in the A'-pocket causes only minor conformational changes. Thus, two features of the phenyl substituents seem to be responsible for the increased affinity of C6Ph, C8Ph, C8PhF and C10Ph to CD1d. First, the phenyl rings are bulkier than an acyl chain and, thus, decrease the ability of the ligands to move around or readily exit the A'-pocket. Furthermore, the appended phenyl atoms are restrained in conformation, which decreases the loss of entropy upon binding. Both effects lead to tighter binding of the phenyl derivatives that renders them as optimal fatty acid substitutions to anchor the relatively short, sphingolipid ligands to an even greater extent than the extended and hydrophobic α -GalCer. Interestingly, the addition of fluorine in C8PhF, as compared to C8Ph (Fig. 1), increases the affinity 8-fold. In addition to the vdW contacts, the fluorine interacts specifically with the backbone carbonyls of the peptide bonds between residues Cys12, Leu13 and Gln14 in an orthogonal disposition. Distances between the carbonyl and the fluorine are 3.1 Å for the first peptide and 3.5 Å for the second. The same type of interaction between fluorine and peptide bonds was observed, for example, with MAP kinase inhibitors (24). The free enthalpy contribution of such an interaction in apolar environments is about a third of a neutral hydrogen bond (25) and explains the significantly improved affinity of CD1d for C8PhF.

IFN- γ secretion induced by C6Ph, C8Ph and C8PhF is increased by 1.1, 1.8, and 2.4 fold, respectively, as compared to α -GalCer. Also, the ratio between IFN- γ and IL-4 secretion, which

is a measure for T-helper cell cytokine preference, changes by 1.5, 2.1, and 3.0 for C6Ph, C8Ph and C8PhF, respectively (19). Apparently, the cytokine-release profile does not correlate precisely with absolute ligand affinity, although it follows the same trend. This finding suggests that substitutions of the fatty acid moiety have greater impact on ligand binding to CD1d, rather than on direct recognition by the NKT TCR. This notion is supported by our structural observations that minor conformational differences of residues in the A'-pocket are neither propagated to adjacent residues, nor is there any indication that they lead to any major changes that could be readily discriminated by the NKT TCR. The NKT TCR recognizes only the galactose and the F'-pocket of CD1d in the human α -GalCer complex (14), which are conserved in all ligands; on that basis, the affinity for the NKT TCR would be expected to be very similar. This notion is further supported by a recent study on lipid lengths of α -GalCer, which showed that the length of the fatty acid does not significantly change the binding affinity for the NKT TCR (26). Observed changes in the cytokine-release profiles of NKT cells by C6Ph, C8Ph and C8PhF are likely due to more efficient loading and better stability of the resulting CD1d-ligand complexes. In contrast, Th2-type cytokine responses appear to be associated with more rapid kinetics and reduced requirement of endosomal loading (27). Thus, whether the α -GalCer analogs are biased towards either a Th1 or Th2 response may influence the likelihood of whether any given CD1d molecule is loaded with ligand and, therefore, its chance to be recognized by the NKT TCR when encountered by an NKT cell.

Earlier structural studies suggested that ligand binding to CD1d occurs via an induced fit mechanism (8,9). While this assumption might well be correct, the evidence must be carefully evaluated. In both analyses, structures in different crystal packing environments were compared. To further test this hypothesis, we set out to analyze 'unliganded' mCD1d in the same space group as the mCD1d-glycolipid complexes. Surprisingly, the conformation of mCD1d without added exogenous ligand was very similar to the mCD1d-C6Ph, mCD1d-C8Ph, mCD1d-C8PhF and mCD1d-PBS-25 complexes in the same space group. On the other hand, the mCD1d-PBS-25 complex analyzed here exhibits considerable conformational differences compared to that published in a different space group (9). Furthermore, conformational differences described between a liganded and an 'unliganded' (i.e. loaded with a short spacer-lipid) CD1d molecule in the same crystal lattice (8) could also be influenced by crystal packing. Extensive analyses of mCD1d-glycolipid conformations in different crystal forms revealed that some regions of the α 1- α 2 superdomain show larger conformational variation than others. The largest variation is found at the end of the F'-pocket and indicate that it may be more flexible than other parts of the α 1- α 2 superdomain. Most strikingly, the NKT TCR directly recognizes the F'-pocket (14,22). We propose that flexibility of the CD1d F'-pocket could correlate with the observed affinity of the NKT TCR to CD1d-glycolipid complexes. Therefore, CD1d-glycolipid complexes with a shorter sphingosine should be more flexible and, as a result, form less stable, NKT TCR complexes. Our hypothesis is supported by experimental data (26), which show that a short sphingosine moiety decreases the affinity for the NKT TCR.

Our findings have immediate implications for ligand design. While the ligand C8PhF is an excellent solution to anchor a glycosphingolipid in the A'-pocket of CD1d with exceptional affinity, it would not appear to significantly alter the affinity for the NKT TCR. In order to significantly increase the affinity for the NKT TCR, which is presumed to correlate with Th1 responses (28,29), chemical changes of either the galactose or the sphingosine are required. Based on our structures, we propose this could be accomplished by decreasing the conformational freedom of the sphingosine moiety, perhaps through introduction of double bonds, phenyl rings or other rigidifying modifications. These modifications should reduce CD1d conformational flexibility and dynamics around the F'-pocket. The consequences of CD1d flexibility and dynamics are difficult to discern from the crystal structures alone, but may, indeed, reflect the *in vitro* and *in vivo* repertoire of different CD1d conformers, some of which interact more readily and with higher affinity to the NKT TCR. Such flexibility could

also contribute to the observed differences in affinity among the different complexes. Modifications of the sphingosine, combined with the already excellent properties of C8PhF, should then yield interesting new ligands that not only form very stable complexes with CD1d, but have increased affinity to the NKT TCR, and, thus, exhibit enhanced Th1 versus Th2 responses.

Materials and Methods

Protein production

Heterodimeric mouse CD1d (mCD1d) was expressed in SF9 cells using the Baculovirus expression system. Cells were co-transfected with BaculoGold DNA (Invitrogen) and the Baculovirus transfer vector pAcUW51 (BD Biosciences), coding for mouse CD1d heavy chain residues 1-279, appended with a C-terminal, hexa-histidine tag, and mouse β 2-microglobulin residues 1-99. Virus amplification and protein expression was performed according to the Pharmingen protocol. Mouse CD1d was then purified from the medium using Ni-affinity and anion exchange chromatography at pH 8.0 according to standard procedures. The ligands PBS-25, C6Ph, C8Ph, C8PhF, and C10Ph were dissolved in dimethyl sulfoxide (DMSO) to 10 mg/ml. Loading of mouse CD1d was performed using a 5:1 molar ratio of ligand to protein in 100 mM NaCl, 20 mM HEPES pH 7.0 at 37°C for 3h, corresponding to a final DMSO concentration of <0.25 %. Mouse CD1d-ligand complexes were then purified by size exclusion chromatography and concentrated to 7 mg/ml in 30mM NaCl, 10mM Hepes pH7.0.

Structure determination

To minimize conformational differences between the mCD1d-ligand complexes that may result from crystal packing, we initially set out to determine all complexes in the same space group. Several crystal forms of mCD1d-ligand complexes in space groups P1, P2₁, P2₁2₁2₁, and P6₅22, were obtained and screened for diffraction quality. The best diffracting crystals were obtained from 0.2 M Malonate pH 4.5, 20 % (v/v) PEG3350, in sitting drops after two to three weeks at 295 K in monoclinic space group P2₁ with unit cell parameters on average of 41.7 Å, 97.7 Å, 55.4 Å and β = 106.5° (only minor variations observed in the various complexes – see Table 1). Sporadically, crystals grew in the orthorhombic space group P2₁2₁2₁ with unit cell parameters of 42.3 Å, 107.2 Å, and 109.4 Å under the same condition. Therefore, comparison of mCD1d-glycolipid complexes in these two space groups was as unbiased as possible. Crystals were cryo-cooled in liquid nitrogen using the reservoir solution supplemented with 20% ethylene glycol as a cryo-protectant. Datasets were collected at SSRL beamline 11-1 and reduced with XDS (30). The mCD1d-C6Ph complex structure was solved by molecular replacement (MR) using PHASER (31) with the mCD1d coordinates from PDB entry 2AKR. Initial phases for the other complexes were obtained by MR using the refined mCD1d coordinates from the mCD1d-C6Ph complex. Model building was done in COOT (32) followed by restrained TLS refinement in REFMAC5 (33).

Structure analysis

Van der Waals contacts were analyzed with CONTACSYM (34) using 4.1 Å as maximal contact distance. Hydrogen bonds were verified manually using 3.2 Å as maximum distance and strict geometry criteria. Pairwise comparison of mCD1d-glycolipid complexes was performed with LSQKAB (35), whereas superposition of multiple structures was achieved with ProFit2.6 (36). The selection criterion for the superposition of multiple mCD1d-ligand complexes was based on unique crystal packing. If more than one structure was determined in the same space group, only the one with the best resolution was included in the comparison. Furthermore, for space groups that contain two molecules per asymmetric unit, both molecules were included. The final set of structures analyzed here consists of eight complexes: two mCD1d-PBS-25 (molecule A and C; PDB code 1Z5L), two mCD1d-sulfatide (molecule A and

C; PDB code 2AKR), two mCD1d-iGb3 (molecule A and C; PDB code 2Q7Y), one mCD1d-C8Ph (this work) and one mCD1d-C8PhF (this work). All of these structures were determined at ≤ 2.2 Å. Multiple structure superposition for the residue range 8 to 180 was performed using ProFit2.6 (36). No coordinates were present for residues 108–111 (molecule A) and 109–111 (molecule C) for PDB entry 1Z5L. Therefore, the rmsd's for residues 108 to 111 were calculated only between the other six structures. The rmsd's were plotted on the mCD1d surface and colored according to the extent of displacement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

α -GalCer	α -galactosylceramide
mCD1d	mouse CD1d
hCD1d	human CD1d
NKT cells	natural killer T cells
TCR	T cell receptor
Th1	T helper cell 1
Th2	T helper cell 2
MHC	major histocompatibility complex
SF9	<i>Spodoptera frugiperda</i>
vdW	van der Waals
iGb3	isoglobotrihexosylceramide

Figure 1. Structural overview of mCD1d-glycolipid complexes

A schematic representation of the mouse CD1d-C8PhF complex is shown in side view (A) and top view (B) rotated by 90° around the x-axis from A. The CD1d heavy chain α 1- α 3 is highlighted in light blue, β 2m in light green, glycosylation sites as gray sticks with red oxygens, C8PhF ligand in yellow, and A'-lipid (AL), that is acquired from the cells during expression, in blue. C) Schematic drawings of the ligands used in this study. The template α -Galactosylceramide (α -GalCer) is shown in black for comparison. Ligands PBS-25, C6Ph, C8Ph, C8PhF, and C10Ph are derivatives of α -GalCer, in which the C₂₆ fatty acid is replaced by either a shorter fatty acid or a short fatty acid that terminates with a phenyl ring.

Figure 2. Experimental electron density of the ligands in the mCD1d-ligand complexes

CD1d is shown as light blue schematic, the ligands and AL's are shown as yellow and blue sticks, respectively. Upper panel shows the side view of the ligand-binding groove. For clarity, the $\alpha 2$ -helix was removed in this representation. The lower panel shows a close up of the A'-pocket rotated by 90° around the x-axis. Fo-Fc omit maps of the ligands and AL's are contoured at 3σ and represented as a green mesh. The ligands and AL's of the refined final models were superimposed to show the agreement between the refined model and the experimental data.

Figure 3. Superposition of presented mCD1d-glycolipid complexes

(A - C) Superposition of mCD1d without added ligand (salmon), in complex with PBS25 (blue), C6Ph (red), C8Ph (yellow) and C8PhF (green). (A) CD1d C α -traces are colored according to the bound ligand, but the ligands and AL's are all shown in gray, for clarity. (B) Top view of the ligands. The individual ligands and AL's are colored and mCD1d is presented as gray cartoon. Residues that are involved in hydrogen bonds with the ligands are highlighted as gray sticks for the ligand complexes and salmon for the 'unloaded' structure. (C) Side view of the complexes (this view is rotated by 90° around the x-axis as compared to B). Helix α 2 was removed for clarity. Ligands and CD1d residues that show conformational differences among the complexes are highlighted as sticks in the same color as the ligand. (D - F) Superposition of mCD1d in complex with C10Ph (purple) and C8Ph (yellow), shown in the same representation as the structures in the upper panel.

A Interactions of polar cores and galactoses

PBS-25, C6Ph, C8Ph, and C8PhF

C10Ph

C Interactions of the sphingosine moieties

PBS-25, C6Ph, C8Ph, C8PhF, and C10Ph

B Interactions of the acyl moieties

PBS-25

C6Ph

C8Ph

C8PhF

C10Ph

Figure 4. Ligand contact analysis of mCD1d-glycolipid complexes

All ligand contacts are represented schematically. The polar core of the ligands is highlighted as a light red box. Dashed lines represent hydrogen bonds, gray circles show van der Waals contacts, dashed circles indicate residues whose side chains adopt two alternate conformations, and filled gray circles highlight specific protein fluorine contacts. Ligand components are color coded according to Fig. 1. Apolar interactions with the F'-pocket are common to all ligands.

Figure 5. Comparison of mCD1d-glycolipid complexes in different space groups
 (A-C) Superposition of mCD1d-C8Ph in space groups $P2_1$ (yellow) and $P2_12_12_1$ (black) (see Table 1). (A) CD1d α -traces are colored yellow and black for $P2_1$ and $P2_12_12_1$ models, respectively, with C8Ph and AL shown in gray, for clarity. (B) C8Ph, AL and the residues involved in hydrogen bonds are shown as colored sticks according to the space group. (C) Side view of the complexes (this view is rotated by 90° around the x-axis as compared to B) with helix $\alpha 2$ removed for clarity. The same A'-pocket residues as in Fig. 3C are highlighted as colored sticks. (D - F) Superposition of mCD1d-PBS-25 in space group $P2_1$ (blue) (see Table 1) and $P2_1$ prev (orange) (PDB code 1Z5L; molecule A). In the lower panel, the same representations are used as in the upper panel.

Figure 6. Conformational differences of mCD1d-glycolipid complexes

(A) Root mean square displacement (rmsd) plot for all atoms of residues 8-180 of eight selected mCD1d-glycolipid complexes (see Materials and Methods). (B) Visualization of the rmsd as a surface representation of the mCD1d-C8PhF complex. The rmsd's correspond to the positional uncertainty of the atoms in the compared structures. Conformational differences are represented as colored surface according to the degree of positional displacement. Blue residues show only very small differences, green to yellow is intermediate, whereas red indicates that the residues show significant displacements. The three most different regions are labeled with numbers matching the peaks in panel A. (C) The footprint of the NKT TCR on CD1d-glycolipid complexes was projected on the surface of the mCD1d-C8PhF. Contacts with the α -chain are highlighted in blue, the β -chain in green and residues that are contacted by both in cyan- please note these colors do not represent B-values as they do in (B).

Table 1

Data collection and refinement of CD1d complexes

	PBS-25	C6Ph	C8Ph	C10Ph	C8PhF	No added ligand	C8Ph*
Resolution (Å)	30 - 1.7 (1.8 - 1.7)	30 - 1.7 (1.8 - 1.7)	30 - 1.8 (1.9 - 1.8)	30 - 1.7 (1.8 - 1.7)	30 - 1.6 (1.7 - 1.6)	30 - 1.8 (1.9 - 1.8)	30 - 1.9 (2.0 - 1.9)
Unit cell (Å; °)	41.71, 97.73, 55.49; 90, 41.66, 97.63, 55.27; 90, 41.74, 97.90, 55.42; 90, 41.67, 97.87, 55.41; 90, 41.74, 97.40, 55.40; 90, 41.63, 98.16, 55.97; 90, 42.27, 107.23, 109.42; 90, 90, 90	106.50, 90	106.57, 90	106.44, 90	106.51, 90	107.36, 90	90, 90, 90
Unique reflections	46,664 (7,300)	46,351 (7,294)	39,342 (5,893)	46,034 (7,207)	54,886 (8,935)	38,843 (5,747)	39,777 (5,609)
Completeness (%)	99.5 (98.8)	99.5 (99.7)	99.4 (99.3)	98.2 (97.7)	98.1 (96.8)	97.7 (97.0)	99.2 (99.7)
Multiplicity	4.1 (3.9)	3.7 (3.7)	3.8 (3.8)	3.8 (3.8)	4.2 (4.1)	4.3 (4.3)	3.7 (3.9)
Mean I/σ(I)	23.3 (4.0)	18.1 (3.1)	17.2 (2.8)	21.0 (2.6)	24.6 (3.9)	16.5 (2.1)	16.5 (3.8)
R _{meas} (%) ^a	3.5 (39.0)	4.4 (51.2)	5.0 (57.0)	3.7 (55.9)	3.3 (45.7)	4.8 (77.7)	6.9 (41.3)
Wilson B-factor (Å ²)	31.9	32.4	34.0	34.7	30.3	39.0	28.2
Refinement							
Resolution (Å)	30.0 - 1.7 (1.74 - 1.70)	30.0 - 1.7 (1.74 - 1.70)	30.0 - 1.8 (1.85 - 1.80)	30.0 - 1.7 (1.74 - 1.70)	30.0 - 1.6 (1.64 - 1.60)	30.0 - 1.8 (1.85 - 1.80)	30.0 - 1.9 (1.95 - 1.90)
Reflections (Working)	44,302 (3,247)	44,007 (3,268)	37,348 (2,768)	43,699 (3,244)	52,103 (3,708)	36,875 (2,688)	37,782 (2,781)
Reflections (Test)	2,361 (168)	2,344 (164)	1,993 (152)	2,334 (161)	2,782 (199)	1,966 (139)	1,995 (142)
R _{cryst} (%) ^b	17.6 (24.4)	17.7 (29.1)	17.5 (26.5)	17.9 (29.1)	17.7 (27.5)	18.2 (31.6)	18.8 (21.8)
R _{free} (%) ^b	21.6 (30.3)	21.6 (35.4)	21.9 (28.3)	21.8 (33.2)	21.6 (33.2)	23.1 (38.8)	22.9 (23.9)
Number of atoms							
Protein mCD1d / mβ2m	2,219 / 833	2,211 / 833	2,205 / 833	2,211 / 833	2,205 / 833	2,200 / 830	2,253 / 827
Carbohydrates	146	146	146	146	146	146	127
Ligand / palmitic acid	42 / 18	46 / 18	48 / 18	50 / 18	49 / 18	-- / 18	48 / 18
Waters / ethylene glycol	313 / 20	332 / 20	320 / 20	318 / 20	354 / 20	238 / 20	308 / 33
B-values (Å²)							
Protein mCD1d / mβ2m	22.0 / 19.8	22.0 / 20.5	23.6 / 21.3	25.1 / 23.0	20.7 / 17.9	28.1 / 24.7	14.3 / 14.1
Carbohydrates	44.6	49.2	54.3	50.2	44.3	56.4	50.5
Ligand / palmitic acid	31.5 / 43.6	33.0 / 41.1	34.7 / 46.9	48.4 / 55.0	28.7 / 34.7	-- / 59.9	29.6 / 36.8
Waters / ethylene glycol	32.2 / 27.8	33.6 / 29.8	34.9 / 30.2	36.2 / 33.5	31.2 / 25.9	35.6 / 35.7	23.1 / 28.5
Ramachandran plot							
Favored / Outliers (%)	98.1 / 0.0	98.1 / 0.3	97.2 / 0.3	98.1 / 0.6	97.8 / 0.0	97.8 / 0.3	97.6 / 0.3
Rmsd bond length (Å)	0.022	0.022	0.023	0.023	0.025	0.023	0.023
Rmsd bond angles (°)	2.1	2.1	2.1	2.1	2.3	2.1	2.0
PDB code	3GMP	3GML	3GMM	3GMN	3GMO	3GMO	3GMR

$$a - R_{meas} = \frac{\sum_{hkl} \sqrt{\frac{n}{n-1} \sum_{j=1}^n |I_{hkl,j} - \langle I_{hkl} \rangle|}}{\sum_{hkl} \sum_j I_{hkl,j}}$$

$$b - R_{cryst} = \frac{\sum_{hkl} |F_{hkl}^{obs} - F_{hkl}^{calc}|}{\sum_{hkl} F_{hkl}^{obs}}$$

R_{free} is calculated as for R_{cryst}, but for 5% of the reflections excluded from the refinement

* - ligand complex is determined in a different space group