Published in final edited form as:

Curr Pharm Des. 2009 ; 15(28): 3311–3317.

Presentation of lipid antigens by CD1 glycoproteins

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Summary

CD1 molecules are a family of non-polymorphic, class I antigen-presenting glycoproteins, which bind and present amphiphilic lipid antigens for recognition to T cells. Two groups of CD1 molecules are involved in presentation self and foreign lipid antigens: group 1 (CD1a, CD1b and CD1c) and group 2 (CD1d). Crystal structures of CD1a, CD1b and CD1d in complex with different ligands have revealed the key principles of the lipid presentation and defined unique binding groove architectures for the individual CD1 isoforms, which enable binding and presentation of an enormous variety of lipids. Structural and biochemical insights into presentation of glycolipids by CD1d have led to the discovery of novel lipid antigens and to a broader understanding of the underlying structural and mechanistic principles of NKT cell stimulation. Some of these glycolipids show enhanced and more specific stimulatory properties and crystal structures have suggested further design strategies for elicitation of immunostimulatory compounds that may enable selective control of the secretion of regulatory T helper cytokines.

CD1 molecules

A role for CD1 molecules in antigen presentation was proposed more than two decades ago [1]. However, it took several more years to realize that antigens presented by CD1 molecules are not peptides, as for major histocompatibility complexes MHC-I and MHC-II, but lipid molecules [2,3]. Now, fifteen years later lipid, antigen presentation by CD1 molecules is well-established, and also known as the third pathway of antigen presentation.

CD1 glycoproteins are expressed on most professional antigen presenting cells, such as B cells, macrophages and dendritic cells. The CD1 family consists of five isoforms that, based on sequence similarity, have been classified into three groups: group 1 comprises CD1a, CD1b and CD1c; group 2 consists only of CD1d and group 3 only of CD1e. Groups 1 and 2 are involved in antigen presentation, whereas group 3 is involved in lipid processing and trafficking [4]. According to their cytoplasmic targeting sequences, each of the group 1 and group 2 CD1 glycoproteins takes a distinct route through the endosomal compartments where they bind self or foreign lipids, glycolipids or lipopeptides, which are then presented at the cell surface to T cells [5]. All mammalian species that have been studied to date express CD1 molecules. However, the number of expressed CD1 isoforms varies among species. While humans express the five isoforms CD1a-e, rats and mice only express CD1d. On the other hand, the bovine CD1 family contains group 1 molecules, but no functional CD1d [6,7]. Recently, two CD1 homologs were discovered in avian species, suggesting the appearance of CD1 molecules in early terrestrial vertebrates [8].

CD1-lipid complexes

CD1 molecules are heterodimeric glycoproteins that consist of a membrane-anchored, heavy chain, with three domains $\alpha 1$ - $\alpha 3$, which are non-covalently associated with $\beta 2$ -microglobulin ($\beta 2m$). The $\alpha 1$ and $\alpha 2$ domains form the antigen-presenting $\alpha 1$ - $\alpha 2$ superdomain that is also common to MHC class I antigen-presenting molecules. In contrast to MHC-I, the ligand-binding groove of the CD1 $\alpha 1$ - $\alpha 2$ superdomain is much deeper, more hydrophobic and narrower. Otherwise, it is formed in the usual way from an eight-stranded, β -sheet platform as the base that is traversed by two anti-parallel α -helices, $\alpha 1$ and $\alpha 2$, which form the side walls of the binding site [9]. Most of the antigens presented by CD1 molecules are amphiphilic molecules, such as glycolipids or lipopeptides, that have distinct hydrophobic and hydrophilic moieties (Fig. 1). CD1 molecules bury their hydrophobic moieties in the interior of the $\alpha 1$ - $\alpha 2$ superdomain whereas the carbohydrate, or peptide, hydrophilic head groups are anchored at the CD1 surface, such that they can be recognized by the TCRs [10-23]. To date, crystal structures of 17 different CD1-antigen complexes including a trimolecular complex of CD1-antigen-TCR have been published (see Table 1).

CD1-presented lipid antigens

The first lipid antigen discovered to specifically stimulate T cells, when presented by CD1, was mycolic acid [3]. Since then, the range of known self and foreign lipid antigens that are presented by CD1 molecules has now substantially expanded to include extremely diverse types of lipids including lipopeptides, diacylglycerolipids, sphingolipids, mycolates, phosphomycoketides, but also small molecules (Fig. 1) [4,24,25]. Among these are self-lipids, such as sulfatide or isoglobotrihexosylceramide (iGb3) [26,27], but also many microbial antigens from pathogenic bacteria, such as didehydroxymycobactin or glucose monomycolate [28,29].

The ability of non-polymorphic CD1 molecules to bind diverse lipids is accomplished in several ways. First of all, many species express several CD1 isoforms, which have distinct binding groove shapes and volumes that can accommodate lipids of different length and complexity. Most importantly, the antigens are bound mainly by relatively non-specific, apolar interactions between the hydrophobic groove and the lipid antigen, whereas only a small number of hydrogen bonds are formed with the hydrophilic head groups that protrude from the binding groove. These largely apolar interactions occur mainly between the repeating methylene units of the alkyl chains and the hydrophobic surface of the interior of the binding pockets. Since hydrophobic interaction do not require as precise positioning as hydrogen bonds or salt bridges, and the single-bonded methylene groups have a certain degree of rotational freedom, the acyl chains thread along the groove and adopt their most favorable conformations to optimize the binding energy within the binding pockets. Binding strength then appears to be correlated with the number of favorable apolar interactions within the binding pocket.

Another mechanism for an individual CD1 to accommodate lipids of different sizes, which may not completely fill the hydrophobic groove, is to promiscuously sequester small lipid molecules or spacer lipids deep in binding groove in addition to the exogenous lipid antigen. Furthermore, these endogenous spacer lipids appear to function as stabilizing factors for CD1 molecules that prevent the collapse of an empty binding groove in the absence of self or foreign lipid antigens, or occupy remaining space in cases where the lipid antigen does not fully occupy the available binding pockets [12,14,17,20,22]. The largest variation of the lipids that can be accommodated in CD1 molecules, is found for CD1b, which span from C12 to C80 atoms, and, consequently, exhibits the largest overall pocket volume [30].

Comparison of CD1 binding grooves

The dimensions and shape of the binding grooves are determined by three structural elements: 1) the relative distance apart and orientation of the $\alpha 1$ and $\alpha 2$ helices with respect to each other; 2) the distance between the α -helices from the β -sheet platform; and, most importantly, 3) the side-chain composition of the groove-forming residues. Each CD1 isoform has a characteristic groove shape and volume varying from 700 - 2200 Å³ [9,10,12,15,23] (Fig. 2). The CD1 binding grooves can be sub-divided into individual, but largely continuous, binding pockets termed A', F', C', and T'. Pockets A', F', and C' are named after corresponding pockets in MHC-I molecules. The A' pocket is common to all human and mouse CD1 structures determined to date (Fig. 2). It is also the only binding pocket in the more distantly related chicken CD1. The A' pocket is almost completely buried inside the CD1 molecule by the A' roof, which forms a barrier between the CD1 surface and the pocket interior. It is also the pocket that shows the highest sequence identity within the antigen binding $\alpha 1$ - $\alpha 2$ superdomain. The A' pocket itself can be thought of as a continuous channel that describes a full circle in hCD1b, hCD1d, and mCD1d around a pole consisting of the side chain(s) of CD1 residues, while in hCD1a and chCD1, it forms an incomplete circle, which restricts the length of the bound lipid moiety.

The F' pocket is a deep, long and extended, hydrophobic pocket in hCD1b, hCD1d and mCD1d, while it is shallower in hCD1a. The F' pocket forms a portal that connects the hydrophobic groove with the solvent-exposed surface of the CD1 molecules. At the rim of the F' pocket, the lipid antigens can specifically interact with the CD1 side chains by hydrogen bonding. This fixing of the polar part of the lipid framework leads to a well-defined positioning of the head groups for presentation to T cells. CD1b is the only CD1 molecule that has two additional pockets, which have been termed C' and T' [12]. The C' pocket originates from the F' pocket, which is not completely enclosed in CD1b compared to hCD1a, hCD1d and mCD1d. It connects the F' pocket with the solvent exposed surface underneath the $\alpha 2$ helix and, thereby, creates another portal in CD1b that allows presentation of lipid antigens that would otherwise exceed the volume of the binding groove [12,13,30]. The T' pocket is also unique to CD1b and connects the A' and F' pockets, so as to create an unusually long and winding tunnel, termed the A'T'F' superchannel, that can accommodate extremely long alkyl chains [12].

CD1-lipid complex recognition by reactive T cells

In all CD1-lipid structures determined to date, the hydrophilic moiety or head group of the amphipatic ligands protrude from the binding groove for presentation to the TCR. However, the hydrophilic epitope presented to T cells varies among the CD1 isoforms. Glycolipids and lipopeptides presented by CD1a barely protrude from the groove, while in CD1b and CD1d these hydrophilic parts are considerably exposed and accessible to the TCR. Consequently, the epitope structure of a particular CD1-lipid complex is not only determined by the lipid antigen itself, but also by the CD1 isoform.

Group 1 CD1 molecules present lipid antigens to clonally diverse T cells that mediate adaptive immunity. Group 1 restricted T cells that are specific for microbial lipid antigens have highly diverse TCR α - and β -chains, which are indistinguishable from the comparable, peptide-specific TCRs [31]. So far, no structural information is available for CD1a-, CD1b- or CD1c-restricted TCRs and their trimolecular, CD1-lipid-TCR complexes.

In contrast to group 1 CD1, group 2 CD1d molecules present lipid antigens to natural killer T (NKT) cells. CD1d-reactive NKT cells are regulatory T lymphocytes that have been shown to play an important regulatory role for immune responses associated with infectious diseases, cancer and autoimmunity [32,33]. NKT cells recognize both self and foreign

glycolipids presented by CD1d and use a semi-invariant T cell receptor (NKT TCR). These NKT TCRs are expressed with an invariant α -chain and a restricted set of β -chains [6]. Stimulation of NKT cells induces the rapid secretion of large amounts of immune regulatory T helper 1 (Th1) and T helper 2 (Th2) cytokines, such as IFN- γ and IL-4, respectively. Th1 cytokines support cell-mediated immunity, and are correlated with antitumor, antiviral/antibacterial and adjuvant effects, whereas Th2 cytokines are believed to protect against autoimmune diseases, such as type-1 diabetes or multiple sclerosis [34,35].

NKT cells get stimulated by glycosphingolipids from *Sphingomonas* spec. [36,37], glycerolipids from *Borrelia burgdorferi* [38], phosphoinositolmannosides from Mycobacteria [39], but most potently by the glycosphingolipid α -galactosylceramide (α -GalCer), which was originally isolated from the marine sponge *Agelas mauritanus* [40]. Structures of the glycosphingolipids α -GalCer (see Fig. 4) in complex with hCD1d, and mCD1d in complexes with PBS-25 (a short chain α -GalCer) (see Fig. 4), GalA-Gsl (see Fig. 1), sulfatide, as well as iGb3, have been determined (see Table 1). These glycosphingolipids enter the two pockets of CD1d in a well-defined manner, such that the fatty acid moiety inserts into the A' pocket and the sphingosine moiety enters the F' pocket, while the polar head group is presented at the CD1d surface to the NKT TCR [15,17,19,20,22].

However, the most extensively studied CD1d antigen is α -GalCer and its analogs (see next section). Several structures of NKT TCRs in the unbound state have been determined [22,41,42]. Most importantly, the recent crystal structure of the human CD1d- α -GalCer-NKT TCR complex established fundamental insights into CD1d-lipid recognition by the NKT TCR [16]. The mode of antigen recognition is strikingly different to that of MHC-I-peptide-TCR complexes (Fig. 3). In contrast, the NKT TCR is skewed to one end of the groove and binds only above the F' pocket where it contacts the galactose head group of α -GalCer exclusively with the invariant α -chain, while its β -chain contacts CD1d. The structure illustrates why different β -chains can recognize the same antigen with comparable affinities. Nevertheless, the question as to how the invariant TCR α -chain achieves high affinity recognition of structurally and compositionally diverse sugar moieties, such as α -GalCer and iGb3, remains to be elucidated.

Therapeutic implications of CD1d-glycolipid complexes

α -Galactosylceramide (α -GalCer), when presented by CD1d, is an extremely potent stimulatory ligand that elicits secretion of regulatory cytokines by NKT cells [40]. However, the effectiveness of α -GalCer is limited in two ways. α -GalCer stimulates the secretion of Th1 and Th2 cytokines at the same time, which oppose each other, and rapidly lead to NKT cell anergy [43]. Thus, the design of α -GalCer variants that can specifically stimulate NKT cells to secrete either IFN- γ (Th1) or IL-4 (Th2) cytokines and reduce such NKT cell anergy would have tremendous therapeutic potential. Attempts to selectively control the secretion of these cytokines by NKT cells have led to the discovery of several α -GalCer analogs.

Basically, all three components of the α -GalCer molecule were targeted by chemical synthesis: the galactose moiety, the sphingosine moiety and the fatty acid moiety (see Fig. 4). Several sugar analogs were tested such as α -glycosyl ceramide (α -GlcCer) exhibited slightly reduced activity, while α -mannosyl ceramide (α -ManCer) or β -GalCer showed no activity [40]. Also several disaccharide analogs were tested, but none of them demonstrated greater stimulation than α -GalCer. Some of the disaccharides are processed to monosaccharides by lysosomal α -galactosidase A [44].

These results indicated a crucial role of the galactose α -linkage and the galactose 2'-OH group. Therefore, modifications of the galactose 2'-OH group were extensively studied. Any 2'-OH modifications to the 2'-fluoro or 2'-acetoamino groups abolished stimulation of NKT

cells [45], whereas the 3'-O-sulfo group showed almost comparable activity [46]. In contrast, at the 6'-OH position, even large substitutions, such as fluorophores and biotin, are well tolerated, which makes these compounds useful tools to study CD1d-mediated, glycolipid trafficking [47].

All of these findings are consistent with the crystal structure of the trimolecular hCD1d- α -GalCer-NKT TCR complex where only 2'-OH, 3'-OH and 4'-OH of the galactose are contacted by the NKT TCR. The analysis of this complex led to a new generation of non-glycosidic, NKT cell agonists, such as threitolceramide (ThrCer) [48], which shows comparable stimulatory activity, but reduced activation-induced anergy of NKT cells and reduced lysis of dendritic cells, as compared to α -GalCer.

Structural and biochemical studies pointed to the importance of the sphingosine 2-amino 3-, 4-diol as essential components for the positioning of ceramide ligands in the binding groove of CD1d. While isosteric replacement of the 2-amino by a triazole yielded comparable stimulatory properties with bias towards a Th2 response compared to α -GalCer, the 3-OH is crucial for antitumor activity and binding of α -GalCer analogs to CD1d [49-51]. On the other hand, the absence of a 4-OH, as naturally found in GalA-Gsl, or a double bond, as in sulfatide, affect the positioning of the sphingosine moiety, and result in a shifted position of the sugar head group at the CD1d surface (Fig. 5). Both α -GalCer and the *Sphingomonas* glycolipid GalA-Gsl activate NKT cells, but α -GalCer is much more potent, which can be attributed to the more complex hydrogen bond network for α -GalCer analogs that contain both 3-OH and 4-OH groups.

A direct relationship has been demonstrated between α -GalCer lipid tail length and bias of the cytokine-release profile toward a Th2 response [52]. OCH, a sphingosine and fatty acyl truncated analog of α -GalCer, protects mice against the development of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. This finding was attributed to a bias toward IL-4 secretion [53]. The shortening of the sphingosine reduces the binding affinity of the NKT TCR for CD1d-glycolipid complexes and, therefore, modulates NKT cell response [54]. Another analog that biases toward a Th2 response is C20:2. This variant has a shortened fatty acid moiety with two introduced *cis* double bonds [55]. A possible explanation for the Th2 bias is the relative stability of the CD1d-glycolipid complex [34]. Conversely, α -C-GalCer, in which the glucosidic O-linkage between the sugar and the ceramide is replaced by a C-linkage, showed an increased Th1 response and a 100 to 1000 fold improved activity against melanoma metastases and malaria compared to α -GalCer [56,57]. The increased Th1 response may be a result of increased *in vivo* stability caused by decreased enzymatic degradation of the C-linkage.

In another study, the introduction of terminal aromatic groups into the fatty acid tail of α -GalCer enhanced the stability of the CD1d-glycolipid complex and biased the cytokine profile towards a Th1 response [58,59]. These compounds form complexes that are more stable than those by α -GalCer, which has a much longer fatty acid moiety [60]. Lipids that yield an increased Th1 response showed greater anticancer efficacy in mouse lung and breast cancer models than α -GalCer itself, *in vivo* [59].

Concluding remarks

Over the past two decades, CD1-mediated presentation of lipid antigens has been unequivocally established as a fundamental mechanism for the presentation of antigens other than peptides. The architecture of CD1 molecules allows binding of hydrocarbon chains of varying length and substitution in the hydrophobic binding pockets that enable presentation of the hydrophilic head groups at the CD1 surface for survey by effector T cells.

Although rapid progress has been made in our understanding of lipid antigen presentation by CD1 glycoproteins, many questions remain: how are lipid antigens that are presented by group 1 CD1 molecules recognized by TCRs? How are more complex and larger sugar head groups accommodated by the same NKT TCR that recognizes only single sugar moieties? Finally, how do small changes in lipid antigen structure alter the sensing of the CD1-lipid complex by the TCR. Notwithstanding, the crystal structures of this family of previously enigmatic antigen receptors, as well as identification of both self and foreign antigens has significantly advanced our understanding of the immunological function in innate and adaptive immunity, and of the possible therapeutic uses.

Acknowledgments

Support for the CD1 studies in our laboratory was obtained through the National Institutes of Health (CA58896 and GM62116 to I.A.W.) and the Skaggs Institute for Chemical Biology (A.S. and I.A.W.).

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Figure 1. Lipid presentation by CD1 molecules

A schematic representation of a CD1-glycolipid complex is shown in side view (A) and in top view, rotated by 90° (B). The CD1 heavy chain domains $\alpha1$ - $\alpha3$ are highlighted in light blue. The overall structure of the CD1 heavy chain is shown in light blue, $\beta2m$ in light green, glycosylation sites as gray sticks with red oxygens, and lipid ligands in the binding groove in yellow. C) The structural diversity of lipid antigens presented by CD1 molecules. Didehydroxymycobactin (DDM-838), Glucose Monomycolate (GMM), Mannosyl-1 β -phosphomycoketide (β -MPM), and Phosphatidylinositol mannoside-4 (PIM-4), are mycobacterial lipid antigens that are presented by CD1a, CD1b, CD1c and CD1d, respectively [28,29,61,39]. Glycosylsphingolipids, such as α -galacturonosyl ceramide (GalA-Gsl) from non-pathogenic *Sphingomonas* bacteria [36,37], and diacylglycerol glycolipids from the pathogenic bacterium *Borrelia burgdorferi* (BbGl-2c) [38], or artificial, non-lipidic compounds, like phenyl 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonate (PPBF) [62], can also be presented by CD1d.

Figure 2. Architecture of the CD1 binding grooves

Dimensions and shapes of the individual CD1 isoform binding grooves are illustrated for comparison. The $\alpha 1\alpha 2$ antigen-binding superdomain in panels A and C are shown as gray cartoons in orthogonal views and the corresponding grooves, in panels B and D, are shown as a gray mesh. Ligands are highlighted in yellow. Promiscuously bound lipids or spacer lipids that were acquired during expression or refolding and that function as pocket stabilizers are shown in blue. The binding groove volumes of the CD1 isoforms vary considerably from 700 Å³ for chicken CD1 [23] to 2200 Å³ in CD1b [12], 1300 Å³ in CD1a [10], 1400 Å³ in hCD1d [15] and 1350 Å³ in mouse CD1d [9].

Figure 3. Structure of the ternary hCD1d-αGalCer-NKT TCR complex

Comparison of the ternary CD1d-αGalCer-NKT TCR complex (left- PDB code 2PO6) with MHC-peptide-TCR complex (right- PDB code 2CKB). CD1 and MHC-I heavy chains are in light blue, β2m in light green, and the antigen is highlighted in red. The TCR α-chain is in blue and β-chain in yellow. To illustrate the strikingly different mode of interaction by the NKT TCR, CD1-αGalCer and MHC-I-peptide complexes are in the same orientation. The NKT TCR is significantly shifted and rotated with respect to the α1α2 antigen-presenting platform compared to the αβ TCR in an MHC-peptide-TCR complex [16].

Figure 4. Synthetic NKT cell agonists

Illustrated are elements of α -GalCer that have been targeted by chemical synthesis to obtain novel glycolipids with altered stimulatory properties. Selected variations of the galactose moiety, sphingosine and fatty acyl moieties are highlighted in red, blue and black, respectively.

Figure 5. Structures of CD1d with synthetic ligands

Superposition of mCD1d-PBS25 (yellow) and mCD1d-GalA-Gsl (blue). (A) Protein backbones of the glycolipid complexes are shown in gray. Residues Asp80, Asp153 and Thr156, which form direct hydrogen bonds to the ligands in all published mCD1d-glycolipid structures, are shown in sticks and colored according to the ligand. (B) Ligands (without the surrounding protein residues) are shown rotated by 90° around the y-axis. The superposition illustrates that polar contacts are important for the positioning of the galactose head group.

Table 1

CD1-lipid complex structures

CD1	Ligand molecules	Resolution	PDB code	Reference
Human CD1a	Sulfatide	2.15 Å	1ONQ	[10]
Human CD1a	Synthetic lipopeptide	2.80 Å	1XZ0	[11]
Human CD1b	Ganglioside GM2 + C ₂₂ + C ₁₂	2.80 Å	1GZP	[12]
Human CD1b	Phosphatidylinositol + C ₂₂ + C ₁₂	2.26 Å	1GZQ	[12]
Human CD1b	Glucose monomycolate	3.10 Å	1UQS	[13]
Human CD1b	Phosphatidylcholine + C ₄₀ O ₂	1.80 Å	2H26	[14]
Human CD1d	α -galactosylceramide	3.00 Å	1ZT4	[15]
Human CD1d	No ligand	3.00 Å	1ZT4	[15]
Human CD1d	α -galactosylceramide + NKT TCR	3.20 Å	2PO6	[16]
Mouse CD1d	No ligand added	2.67 Å	1CD1	[9]
Mouse CD1d	PBS-25 + C ₁₆	2.20 Å	1Z5L	[17]
Mouse CD1d	Phosphatidylcholine	2.80 Å	1ZHN	[18]
Mouse CD1d	Sulfatide	1.90 Å	2AKR	[19]
Mouse CD1d	α -galacturonosyl ceramide + palmitic acid	1.80 Å	2FIK	[20]
Mouse CD1d	Synthetic dipalmitoyl-PIM-2	2.61 Å	2GAZ	[21]
Mouse CD1d	Isoglobotrihexosylceramide + palmitic acid	1.95 Å	2Q7Y	[22]
Chicken CD1-2	Palmitic acid	2.00 Å	3DBX	[23]