Cholesteryl esters stabilize human CD1c conformations for recognition by self-reactive T cells

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Cluster of differentiation 1c (CD1c)-dependent self-reactive T cells are abundant in human blood, but self-antigens presented by CD1c to the T-cell receptors of these cells are poorly understood. Here we present a crystal structure of CD1c determined at 2.4 Å revealing an extended ligand binding potential of the antigen groove and a substantially different conformation compared with known CD1c structures. Computational simulations exploring different occupancy states of the groove reenacted these different CD1c conformations and suggested cholesteryl ester (CE) and acylated steryl glycosides (ASG) as new ligand classes for CD1c. Confirming this, we show that binding of CE and ASG to CD1c enables the binding of human CD1c self-reactive T-cell receptors. Hence, human CD1c adopts different conformations dependent on ligand occupancy of its groove, with CE and ASG stabilizing CD1c conformations that provide a footprint for binding of CD1c self-reactive T-cell receptors.

Structures of membrane-bound CD1c with lipid antigens, a mycobacterial phosphomycoketide (PM) or mannosyl-β1-phosphomycoketide (MPM) occupied the A′ channel, whereas an undefined short ligand was present in the F′ channel (7, 12). The spatial arrangement of these ligands in the CD1c groove was very similar to and virtually overlapping in CD1d (Fig. S1 A and B). Because CD1c and CD1d are known to traffic to the same intracellular compartments for antigen sampling (13), these CD1c-PM and CD1c-MPM structures did not readily explain how CD1c and CD1d could functionally differentiate. Furthermore, the F′ channel in both CD1c-PM and CD1c-MPM was widely open to solvent, which was strikingly different from known structures of CD1a, CD1b, and CD1d and reminiscent of CD1e (7, 12). Based on these facts we hypothesized that human CD1c might undergo substantial conformational transformations.

Significance

T cells autoreactive to cluster of differentiation 1c (CD1c) are abundant in human blood but lipid antigens recognized by these T cells remained poorly understood. A new 2.4 Å structure of CD1c and computational simulations thereof indicated substantial conformational plasticity of CD1c with ligand-induced formation of an F′-roof and G′-portal, as well as the potential of CD1c to present acylated sterols. Confirming these predictions we demonstrated CD1c loading and biophysical interaction of CD1c-lipid complexes with self-reactive human T-cell receptors for two lipid classes: cholesteryl esters similar to those accumulatog in foamy macrophages (e.g., in atherosclerosis) and acylated steryl glycosides from Borelia burgdorferi. These findings differentiate CD1c from other CD1 isoforms and open up new avenues for research into the role of CD1c in human immunity.


The authors declare no conflict of interest.

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Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 5C9I).

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in the F′ channel region upon binding of more optimal ligands, with relevance for T-cell receptor binding.

Results

Structural Features of Human CD1c with Extended-Ligand Binding and Closure of the F′ Channel Roof. Based on our experience with human CD1b and CD1d (2, 3), we first attempted to refold the extracellular α1-α3 domains of human CD1c (“wild-type CD1c,” CD1cwt) together with β2m, using detergents as surrogate ligands. Although CD1cwt/β2m complexes of correct size and stoichiometric composition could be purified, these complexes rapidly disintegrated after buffer exchange at 4 °C. This suggested an unstable quaternary conformation of the complex with loss of the noncovalent association between CD1cwt and β2m.

Thus, we decided to use a strategy similar to that used by Scharf et al. (7), by grafting the α3 domain of human CD1b onto the antigen binding α1-α2 domain of human CD1c (CD1cbα3). Using CD1cbα3 instead of CD1cwt, with otherwise identical refolding conditions as before, resulted in stable soluble CD1cα3/β2m complexes that ultimately yielded diffracting protein crystals. The structure was determined by molecular replacement and refined with the use of data to 2.4 Å (Table 1).

The structure shows the typical MHC class I-like domain arrangement that is common to all known CD1/β2m complexes (Fig. 1A). Clear electron density is seen for three hydrocarbon chain spacer ligands (SL) filling the A′ and F′ channels (Fig. 1B). We refer to this new ligand occupied state as CD1c-SL. Similar to other known CD1/ligand structures, including CD1c-PM and CD1c-MPM, a single C18 stearic acid ligand saturates the A′ channel of CD1c-SL and protrudes slightly into the F′ portal (Fig. 1). In striking difference from previous CD1/ligand structures, including CD1c-PM and CD1c-MPM, the F′ channel of CD1c-SL is occupied by two C12 SL, likely corresponding to lauric acids present in the refolding mixture, that are stacked in a parallel manner (Fig. 1). These differences in ligand saturation between the previously solved CD1c structures and CD1c-SL are paralleled by radical differences in the conformation of the F′ channel. In contrast to either CD1c-PM or CD1c-MPM, the F′ channel in CD1c-SL is shielded from solvent by an F′ channel roof (F′ roof). Tyr155 and Leu150 on α2 as well as Glu83 and His87 on α1 provide tethering interactions between the two alpha-helices that result in the formation of this F′ roof above the F′ channel (Fig. 2A and Fig. S2A). Consequently, a new portal

Fig. 1. Overview of human CD1c structure with aliphatic lipid spacers. (A) Cartoon representation of the CD1c-SL structure (α1–α3 domains in blue and β2m orange) in two orientations with bound lipid spacers represented as Van Der Waals spheres (yellow in A′ channel and pink in F′ channel, oxygen atoms shown in red). (B) Ligands bound in the CD1c antigen binding cavity shown with the F0–F3 electron density calculated from an omit map and contoured at 1.5σ (gray mesh). (C) Chemical structure of bound ligands in CD1c-SL, C12 lauric acid, and C18 stearic acid.

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Table 1. Data collection and refinement statistics

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Data obtained from a single crystal.
*Highest-resolution shell is shown in parentheses.
into the side of CD1c, which we propose to call the G′ portal, can be clearly defined in CD1c-SL (Fig. 2A and Fig. S2A). Furthermore, the E′ portal (7), which is an open conduit from the F′ channel to the exterior of the protein described in CD1c-PM and CD1c-MPM, is virtually absent in CD1c-SL (Fig. S2B).

**Molecular Dynamics Simulations of CD1c-SL.** The above differences between CD1c-SL and the previously determined CD1c-PM and CD1c-MPM structures (7, 12) indicated that ligand occupancy of the F′ channel is a major determinant of human CD1c protein conformation. To further address this hypothesis we performed molecular dynamics (MD) simulations in the presence and absence of CD1c ligands at neutral pH. As expected, MD simulations performed on CD1c-SL with all observed bound ligands were fully consistent with a stable CD1c protein complex (Fig. 3A and Movie S1). In contrast, removal of all bound ligands from CD1c-SL led to a rapid inward motion of both alpha-helices with progression to a complete collapse of both A′ and F′ channels (Fig. 3A and B and Movie S2). Next, we examined the behavior of the complex with more hydrophilic ligands. For this we chose to exchange the two aliphatic lauric acids within F′ channel with PEG molecules that were present in the crystallization buffer. In these MD simulations, the PEG molecules rapidly evacuated the F′ channel (Movie S3). In consequence, the antigen binding domain went through a rapid succession of changes from the initial closed F′ roof conformation to a transiently open conformation (Fig. 3C–E) before collapse of the F′ channel. Notably, the MD simulations with PEG ligands closely reenacted the observed conformational differences between CD1c-SL and CD1c-MPM in the region of the F′ channel roof. Rapid disengagement of α1-α2 roof-tethering interactions upon F′ channel ligand evacuation greatly increased the flexibility of these residues, at times leading to the adoption of configurations highly similar to CD1c-PM and CD1c-MPM (Fig. 3C and D). Therefore, human CD1c can adopt both open and closed F′ roof conformations depending on F′ channel ligand occupancy.

**The CD1cα3β2m Complex Used for CD1c-SL Is Recognized by Human CD1c Self-Reactive T Cells.** In light of the major conformational differences between CD1c-SL and either CD1c-PM or CD1c-MPM, we aimed to confirm the functional validity of the soluble CD1cα3β2m complexes that were used for crystallization and structure determination of CD1c-SL. Fluorescent-conjugated CD1cα3β2m tetramers produced from the same protein batch that was used for crystallization (CD1c-SL tetramers) were used to generate CD1c-SL tetramer-positive T-cell receptor (TCR) αβ+ T-cell lines and clones from human blood by FACS sorting (Fig. 4A). Although these T cells were brightly stained with CD1c-SL tetramers, they failed to bind either CD1b or CD1d tetramers (Fig. 4A). Conversely, CD1c-SL tetramers failed to bind to CD1d-restricted human invariant natural killer T cells (iNKT) (Fig. S3A). In cellular assays, these T cells exhibited strong CD1c-dependent cytokine secretion in the absence of added exogenous ligands (Fig. 4B). To further examine the specific binding of CD1c-SL tetramers to CD1c-restricted TCRs, we generated a human Jurkat T-cell line with stable expression of both TRAV22 and TRBV6.2 TCR chains from a CD1c self-reactive T-cell clone (clone NM4) (Fig. S3B and Table S1). These Jurkat-NM4 cells brightly stained with CD1c-SL tetramers, whereas CD1c-SL tetramers failed to stain CD8-1 Jurkat.
cells expressing the mycoketide-specific CD1c-restricted CD8-1 TCR (14), or other Jurkat cell lines expressing CD1a-, CD1b-, and CD1d-restricted TCRs (Fig. 4 and Fig. S4). These results were highly consistent with a physiologically relevant and functionally differentiated state of CD1c-SL, and thus they suggested that the 3D conformation exhibited by CD1c-SL represents a valid model to interrogate the ligand binding potential of the F′ channel of human CD1c.

Ligand Binding Potential of Human CD1c. To start exploring the potential spectrum of CD1c bound ligands, we carried out molecular docking simulations using the antigen binding cavity of CD1c-SL as the template (Fig. 5).

In initial experiments, steroids such as cholesterol and cholesteryl-like detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) showed favorable docking poses within the F′ channel of CD1c-SL, suggestive of their potential as CD1c ligands. Following from these results, we envisaged cholesteryl esters (CE) and acylated steryl glycosides (ASG) as possible groove-stabilizing ligands for CD1c because they might simultaneously engage both CD1c′s A′ and F′ channels via binding of their fatty acid and cholesteryl moieties, respectively. Indeed, molecular docking simulations produced favorable docking poses for different natural CE and ASG, thereby supporting the notion that human CD1c could present these lipids to T cells (Fig. 5B).

To address this hypothesis, we refolded CD1c proteins with acylated cholesteryl β-D-galactoside (ACGal), an ASG of the human pathogen Borrelia burgdorferi sensu lato that is soluble in aqueous buffers (15, 16). Both CD1cβGal and CD1cεGal proteins could be successfully refolded in the presence of ACGal, enabling the generation of CD1c-ACGal tetramers that specifically stained Jurkat T cells expressing the CD1c self-reactive

![Fig. 3. Molecular dynamics modeling of CD1c-SL. (A) Pocket volumes for different simulation setups against time, protein without ligand (red), protein with PEG ligands (blue), and protein with lauric acid ligands (green). (B) Comparison of CD1c-SL (light blue) versus a configuration from MD with empty channels (blue). Significant displacement of the α2 helix leads to collapse of the A′ and F′ channel in CD1c with empty channels. (C and D) Configurations of the CD1c-SL 40-ns trajectory with PEG with its F′ roof residues shown in green and F′ roof residues of CD1c-MPM in red. (C) Configuration at 0 ns of MD shows the tethered arrangement of the roof residues as seen in CD1c-SL (green). (D) As simulation progresses, at 40 ns the structure of the F′ roof has been lost, giving configurations that more closely resemble CD1c-MPM (compare red and green). (E) Root mean square deviation (RMSD) of the four F′ roof side chains with respect to their starting tethered arrangement. Small RMSD values indicate configurations highly similar to the CD1c-SL, whereas larger values indicate loss of this configuration. Data are representative of three independent experiments.](https://www.pnas.org/cgi/doi/10.1073/pnas.1519246113)
NM4-TCR (Fig. 6A). To further characterize the molecular interaction between CD1c and NM4-TCR, we produced soluble recombinant NM4-TCR (Fig. S5) and measured its binding to both CD1c-SL and CD1c<sup>wt</sup>-ACGal in surface plasmon resonance (SPR) (Fig. 6B). Consistent with the results obtained with Jurkat-NM4 T cells, soluble recombinant NM4-TCR exhibited binding at equilibrium similar to CD1c-SL ($K_d = 6.05 \pm 0.4 \mu M$) and CD1c-ACGal ($K_d = 6.13 \pm 0.4 \mu M$) (Fig. 6B).

**CE and ASG Are CD1c-Stabilizing Ligands.** The above results indicated that CE and ASG could provide CD1c with conformational stability, thereby enabling recognition by CD1c autoreactive TCRs. To address this hypothesis we devised a cell-free assay for direct testing of the ligand-dependent binding of NM4-TCR to CD1c. We first produced soluble fluorescent NM4-TCR tetramers and confirmed their CD1c-dependent binding to CD1c-expressing T lymphoblasts (T2-CD1c) (Fig. 7A). Binding of NM4-TCR tetramers to T2-CD1c lymphoblasts was effectively blocked by an anti-CD1c blocking antibody (Fig. 7A). Consistent with the autoreactive nature of the interaction, binding of NM4-TCR tetramers to T2-CD1c cells did not require the addition of antigens to T2-CD1c cultures. Next, we established a bead-based assay to measure the binding of NM4-TCR tetramers to CD1c after in situ ligand exchange. First, CD1c-SL-coated MACSi beads (CD1c-beads) but not unconjugated MACSi beads stained brightly with NM4-TCR tetramers (Fig. 7B). We then tested several ligand-stripping procedures and found that Triton X-100 was most effective in abolishing NM4-TCR tetramer binding to the CD1c-beads (Fig. S6A). Finally, we assessed different lipids for their effectiveness in abolishing NM4-TCR tetramer binding. In CD1c-SL, CD1c-PM, and CD1c-MPM a single mycoketide molecule was bound to the A′ channel, in analogy to the arrangement seen for the stearic acid in CD1c-SL. Together CD1c-SL, CD1c-PM, and CD1c-MPM thus illustrate a certain promiscuity of the A′ channel, which is the most conserved region of the CD1 groove for ligand binding.

**CD1c-PM and CD1c-MPM complexes are exclusively recognized by mycoketide-specific human T cells, but not by CD1c self-reactive T cells (12).** The specificity of this interaction was shown to be determined by subtle structural features of the mycoketide (12, 19). Conversely, it had remained unclear whether and how ligands bound to the F′ channel of CD1c could influence T-cell receptor binding. In CD1c-PM and CD1c-MPM, a small undefined ligand was observed in the F′ channel. In striking difference to other known antigen-presenting CD1 proteins, the F′ channel in these structures showed a widely open conformation, thus potentially exposing its contents to solvent (12). In stark contrast, two lauric acid molecules, stacked on top of each other, fill the F′ channel in the new CD1c structure presented here, CD1c-SL, where tethering interactions between the two alpha-helices provide for a roof over the F′ channel, thereby shielding the bound ligands from solvent. Based on its position above the F′ channel, this new roof structure in CD1c-SL is called the F′ roof. Below the F′ roof, a well-defined large portal, here proposed to be called the G′ portal, provides an open conduit from the exterior into the F′ channel of CD1c-SL (20).
The clear conformational differences between CD1c-SL and the previous CD1c structures suggested that the F′ channel of CD1c behaves similarly to a venus flytrap, dramatically adapting its conformation with closure of the roof over the channel in response to a sufficient degree of ligand occupancy. The results of our MD simulations of CD1c-SL supported this model. In these MD simulations, ligand evacuation from the F′ channel rapidly induced a seamless transition from a closed F′ roof conformation, as seen in CD1c-SL, to an open F′ groove conformation, as seen in CD1c-PM or CD1c-MPM. Together, these findings identify an F′ roof and G′ portal in CD1c-SL as new structural features of the CD1 protein family (20).

Prompted by the observed extended-ligand binding in CD1c-SL, computational docking simulations with CD1c-SL as a template indicated the possibility that CD1c may present two new ligand classes, CE and ASG, to T cells. Indeed, the docking simulations suggested that both CE and ASG could engage the A′ and F′ channel of CD1c simultaneously via their acyl chains and steryl moieties, respectively. Using several complementary experimental approaches we confirmed that both CE and ASG can be presented by CD1c to human T cells. First, we generated recombinant CD1c-ASG complexes by using a synthetic ASG from B. burgdorferi, ACGal (7, 15), which could be used in aqueous refolding conditions. We used these CD1c-ASG complexes in tetramer-based flow cytometry experiments as well as in SPR-aided measurements of CD1c-TCR binding affinity, showing their specific binding to a CD1c self-reactive T-cell receptor, NM4-TCR. To extend these findings to other ASG and to CE, and to assess whether ASG and CE support CD1c recognition by the CD1c self-reactive NM4-TCR we devised a novel cell-free, MACSi bead-aided, flow cytometry-based assay. Starting from CD1c-SL-coated MACSi beads we found that NM4-TCR tetramer binding to these beads was lost upon washing with the non-ionic surfactant Triton X-100 but could be reconstituted after pulsing of the same beads with either ACGal or CE. These findings demonstrate that ASG and CE are ligands for CD1c, and, together with the above discussed structural findings in CD1c-SL and CD1c-PM/MPM, they suggest a model for CD1c recognition by self-reactive TCRs. In this model, endogenous ligands, such as CE that fill both the A′ and F′ channels of CD1c, induce a closed F′ roof conformation of CD1c, providing a stable footprint for self-reactive TCR binding.

Although the galactosyl headgroup in ACGal had no measurable influence on CD1c self-reactive NM4-TCR binding in our studies, it is possible that other TCRs can differentiate between either ACGal or CE, between different ASG, or even between different chemically modified CE. CD1c may be involved in immune regulation by different bacterial steryl glycosides, including ACGal from B. burgdorferi, the causative agent of Lyme disease (16), and α-cholesteryl

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**Fig. 5.** Molecular docking into CD1c-SL binding cavity. (A) Surface representations of the binding cavities of CD1c-SL (Top) and CD1c-PM (Bottom; PDB ID code 3OV6) shown in different orientations. (B) Docking simulations for binding of cholesteryl 6-O-oleoyl β-D-galactopyranoside (ACGal), cholesteryl 6-O-tetradecanoyl α-D-glucopyranoside (α-ACGlu), and cholesteryl olate (CE) into the cavity of CD1c-SL. Acyl chains and cholesterol moieties are accommodated within the A′ and the F′ channel, respectively. Ligand chemical structures are shown above the corresponding docking pose.
CD1c constructs. Two CD1c constructs were generated for these studies: (i) wild-type CD1c (CD1cwt), encoding the extracellular α1–α3 domains of human CD1c, and (ii) a CD1c/CD1b hybrid construct (CD1c∆α2), encoding α1 and α2 domains of human CD1c and the α3 domain of human CD1b. Primers for CD1cwt: forward, 5′-ATGGGCAAAGCGGATGCTCCCAG-3′; reverse, 5′-AGCTTAATGCCATTCGATTTTCTGAGCTT-3′. CD1c∆α2 was generated by constructing a wild-type CD1c (containing residues 18–296) splice variant by introducing an ApaI site at V200 (GTA to GTG) by site-directed mutagenesis and subsequently cloned CD1b α3 into this construct using the primers CD1b α3 forward, 5′–TTCTGTGCATATCAATCATCAATATCAAGG-3′ and CD1b α3 reverse, 5′–CACCGGATCCGGCAAGGATGTAAGTCC-3′.

CD1 protein production and in vitro refolding. Plasminids encoding the extracellular domains of human CD1b, human CD1d (3), CD1cwt, CD1c∆α2, and human j2m were separately cloned into the prokaryotic expression vector pET23d (Novagen), and recombinant proteins were generated separately as inclusion bodies in Escherichia coli Rosetta strain (Novagen). Inclusion bodies were thoroughly washed and fully denatured and reduced in 6 M guanidine-HCl and 20 mM DTT before in vitro refolding. Refolding of CD1cwt/(j2m, CD1c∆α2)/j2m, human CD1b/j2m, and human CD1d/j2m was carried out by oxidative in vitro refolding as previously described (33, 34), in the presence of the following detergents and lipids: sorbitan stearate (SPAN60), CHAPS hydrate (both Sigma), and α-galactosylceramide KRN7000 (Avanti Polar Lipids). Glucose monomycolate (GMM) (35) and ACGal were synthesized as previously described (15). Correctly folded proteins were purified by repeated FPLC (Pharmacia) size-exclusion chromatography using preparatory grade SD57 26/60 and analytical grade SD75 GL10/300 gel filtration columns (GE Healthcare).

CD1 tetramers. Refolded CD1 complexes (CD1b, CD1c, and CD1d) were bio- tinylated via an engineered BirA motif at the C terminus, repurified by size exclusion chromatography, and used to generate fluorescent-labeled CD1 tetramers (34) by conjugating them to phycoerythrin (PE)-streptavidin (Sigma). Soluble TCR and TCR tetramers. TCR heterodimers were generated as described previously (33, 34, 37). Briefly, the extracellular region of each TCR chain was produced as inclusion bodies from E. coli Rosetta following cloning into the bacterial expression vector pGEM7. To produce stable, disulfide-linked het-
erodimers, cysteines were incorporated into the TCR α- and β-chain constant regions, replacing residues Thr48 and Ser57, respectively. Expression, refolding, and purification of the disulfide-linked NM4-TCR αβ heterodimers were carried out as previously described (38). Purified refolded TCR proteins were assessed by both reducing and nonreducing SDS/PAGE analysis (Fig. 5).

**Molecular Docking.** Three acylated steryl ligands, including two ASG (ACGal and β-sitosteryl glucoside (ASGlu), or the cholesteryl ester 5-cholestene 3-palmitate (CE)). Numbers shown in top right quadrant of dot plots in B and C show the mean fluorescence intensity (MFI) of NM4-TCR tetramer staining. Data in B and C are representative of at least three independent experiments.

**Protein Crystallography.** Proteins (in 20 mM Tris HCl, pH 7.5, and 50 mM NaCl) at 8 mg/mL concentration were crystallized using sitting-drop vapor diffusion in 96-well plates at 20 °C, with 0.2 M magnesium chloride, 0.1 M Tris, pH 8, and 10% PEG 8000 as precipitant (1:1 protein to precipitant ratio), using an ARI Gryphon nanodrop dispenser (Art Robbins Instruments). Crystals were examined at the Southampton Diffraction Centre and data collected at the Diamond Light Source beamline I04 at the cryogenic temperature of 100 °C and a wavelength of 0.9795 Å. Data reduction, molecular replacement, and refinement were carried out with CCP4 (39). The structure was solved by molecular replacement with CD1c-MPM (PDB ID code 3OV6) and iteratively built with Coot (40), using automated water structure building in ArpWarp (41) and refinement in Refmac5, resulting in good Ramachandran statistics (96.3% favored, 3.7% allowed, no outliers). Residues of the F’ channel roof are highly ordered in the structure, with His87 being modeled in double conformation. Pymol (42) was used to create the figures shown in this paper.

**ChemPLP** has been shown to be the most effective scoring function implemented in GOLD for both pose prediction and virtual screening (45). For each ligand, 25 independent docking runs were conducted with 10 GA runs allowed, no outliers). Starting from SMILES string representations of the ligands, protonation states were assigned and low-energy 3D conformations were generated with CORINA (43). Ligands were docked into the CD1c-SL crystal structure using GOLD 5.0 (44). The binding cavity was defined as follows: Based on the ligands presented in the binding pocket of CD1c-SL, all protein residues with heavy atoms within a radius of 6 Å were selected and the atom numbers were saved in a cavity file using the graphical visualization and analysis program Hermes from the GOLD suite. This cavity file was defined in the GOLD configuration file as the area into which the ligands are docked. Additionally, the “do_cavity” option in GOLD was set to 1 to allow the restriction of the binding site to concave, solvent-accessible surfaces. Ligands were treated as flexible whereas the protein structure was kept rigid. For the genetic algorithm (GA), automatic settings were used. The search efficiency was set to 1 using the autoscale flag to allow GOLD to apply optimal settings for each ligand. Predicted poses were assessed using the ChemPLP, an empirical scoring function optimized for binding pose prediction. ChemPLP has been shown to be the most effective scoring function implemented in GOLD for both pose prediction and virtual screening (45). For each ligand, 25 independent docking runs were conducted with 10 GA runs each, resulting in a total of 250 poses per ligand and snapshot, keeping the...
top-scoring pose for analysis to ensure sufficient sampling and reproduc-
ibility of the docking runs.

MD Simulations. To prepare the CD1c-SL 3D structure, hydrogen atoms were added using the PROTANATE3D module of the MOE software package (46), and all water molecules were retained. The structure was further solvated with the TIP3P water model in a box with a minimum distance from protein of 8 Å. Sodium cations were added to neutralize the overall charge of the system, giving a total system size of 50,000 atoms. Three different simulation setups were created: (i) the deposited crystallographic model with stearic acid bound in the A′ channel and two lauric acid molecules bound to the F′ channel; (ii) CD1c with all ligand molecules removed; and (iii) CD1c with stearic acid in the A′ channel, and three further polyethylene glycol short chain moieties. Of these one was located within the A′ channel and two more within the F′ channels.

Simulations were carried out using the AMBER 12 package (47, 48) with the ff99SB forcefield (49) and applied using the ANTECHAMBER module. Partial charges were assigned using the AM1-BCC method (50). The equilibration protocol included a series of successive minimizations, gradually releasing restraints on the heavy atoms of the system. Heavy atom of the protein were then restrained with a force constant of 1,000 kcal mol\(^{-1}\) Å\(^{-2}\) and the system was gradually heated to 300 K over 200 ps and equilibrated to 1 atm pressure for 200 ps under the isothermal-isobaric ensemble. The system was then cooled and the procedure repeated with protein restraints removed. Production runs were carried out under constant volume and temperature dynamics. Temperature control was achieved using the Langevin thermostat, with a collision constant of 3.0 ps\(^{-1}\). Pressure regulation used the Berendsen barostat with a relaxation time of 2.0 ps. Dynamics were carried out using a 2-fs timestep for integration of the equations of motion and a cutoff distance of 8 Å for nonbonded interactions. PME was used to calculate reaction-field electrostatics. The trajectories were saved on the Emerald GPU cluster, using the CUDA implementation of the PME MD module (51). Three repeats of each simulation setup were carried out to give a total of nine molecular dynamics trajectories, each 200 ns in length. Pocket volumes during MD trajectories were calculated using the softwares tool PocketAnalyzer (52).

DCs, T Cells, and T-Cell Assays. DC generation. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare). CD1c-expressing monocyte-derived dendritic cells (mo-DC) were differentiated from CD14+ monocytes that were purified by positive selection using the CD14+ MACSbeads kit from Miltenyi Biotec and cultured in RPMI in the presence of 25 ng/mL IL-4 and 10 ng/mL GM-CSF (ImmunoTools) for 5 d. CD1c expression was confirmed by FACS analysis using APC-conjugated anti-CD1c antibody (clone ADS-87; Milteny). T-cell lines and clones. To generate CD1c restricted T-cell lines, CD14- T-cell clones were sorted into 96-well round-bottom plates by FACSAria (BD Biosciences) and cultured in RPMI 1640, 2% human AB serum (Sigma), 10% FBS, 0.1 mg/mL penicillin, 100 IU penicillin, and 100 μg/mL streptomycin.

Flow Cytometry. The following fluorescent reagents were used: PE-conjugated tetrarmers (tet) PE-CD1c+/-; ACGal-tet; PE-CD1c+/-SL or -ACGal-tet; PE-CD1b-GMM-tet; PE-CD1d+/-; GCTet; PE-NM4-Tet; FITC-conjugated anti-human CD4, and APC-conjugated anti-human CD3 (ImmunoTools); and PE-conjugated anti-human TCR alpha (Miltenyi). After staining reagents, cells were incubated at 4 °C for 45 min, washed twice in ice-cold PBS/1% BSA, and acquired on a one-color FACSCalibur flow cytometer (Becton Dickinson). Propidium iodide (Sigma) was used to exclude dead cells. Data were processed using CellQuest Pro software (BD Biosciences).

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