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Structural reorganization of the antigen-binding groove of human CD1b for presentation of mycobacterial sulfoglycolipids

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The mechanisms permitting nonpolymorphic CD1 molecules to present lipid antigens that differ considerably in polar head and aliphatic tails remain elusive. It is also unclear why hydrophobic motifs in the aliphatic tails of some antigens, which presumably embed inside CD1 pockets, contribute to determinants for T-cell recognition. The 1.9-Å crystal structure of an active complex of CD1b and a mycobacterial diacylsulfoglycolipid presented here provides some clues. Upon antigen binding, endogenous spacers of CD1b, which consist of a mixture of diradylglycerols, moved considerably within the lipid-binding groove. Spacer displacement was accompanied by F' pocket closure and an extensive rearrangement of residues exposed to T-cell receptors. Such structural reorganization resulted in reduction of the A' pocket capacity and led to incomplete embedding of the methyl-ramified portion of the phthioceranol chain of the antigen, explaining why such hydrophobic motifs are critical for T-cell receptor recognition. Mutagenesis experiments supported the functional importance of the observed structural alterations for T-cell stimulation. Overall, our data delineate a complex molecular mechanism combining spacer repositioning and ligand-induced conformational changes that, together with pocket intricacy, endows CD1b with the required molecular plasticity to present a broad range of structurally diverse antigens.

three-dimensional structure | groove shrinking | diacylglycerol endogenous ligand | T lymphocyte activation | CD1b mutant transfectant

T lymphocytes have developed the capacity to recognize as antigens a large variety of molecules including peptides, (glyco)lipids, and phosphorylated metabolites (1). Specific recognition of peptides or lipids by T-cell receptors (TCR) occurs when these molecules form antigenic complexes with dedicated antigen-presenting molecules belonging to MHC or CD1 families, respectively. Diversity has forced the immune system to develop appropriate strategies to present antigens in immunogenic form. Polymorphic MHC molecules cope with the peptide repertoire by constraining the ligand conformational space (2). Less clear is how the immune system adapts to the large glycolipid antigenic range and forms antigenic complexes using the functionally nonpolymorphic CD1 molecules.

Human antigen-presenting cells (APC) display the CD1a, CD1b, CD1c, and CD1d proteins on their plasma membranes (1, 3). CD1 ectodomains consist of a heavy chain, which folds into three extracellular domains ($\alpha 1$ – $\alpha 3$) noncovalently associated with $\beta 2$ -microglobulin (4). Antigen-binding grooves nestle between the $\alpha 1$ and $\alpha 2$ domains and are mostly lined by hydrophobic residues. This allows the antigenic lipids to be anchored

via their hydrophobic chains, so that polar motifs protrude toward the aqueous milieu. Consequently, polar heads but not hydrophobic tails are assumed to establish stimulatory contacts with TCRs. Nevertheless, modifications in the lipid chains may also indirectly impact on TCR recognition (5).

The number, shape, and connectivity of pockets vary among CD1 molecules. CD1b has the most voluminous and intricate groove, being composed of A', C', and F' pockets and a T' tunnel that interconnects the deep ends of A' and F' pockets (6). In agreement with groove structural complexity, human CD1b presents the greatest diversity of antigenic structures among CD1 proteins, including mycobacterial lipoarabinomannans and phosphatidylinositol mannosides (7–9), diacylsulfoglycolipids (Ac₂SGL) (10), mycolic acids (MA) (11), glucose- and glycerolmonomycolates (GMM and GroMM, respectively) (12, 13), and self-lipids such as GM1 gangliosides (14) and sulfatides (15). The polar heads of these antigens vary in size from the small carboxylic group of MA to the pentasaccharide of GM1 or potentially larger structures in lipoarabinomannans. Hydrophobic tails, which decisively govern CD1-restricted presentation of some antigens (16, 17), also greatly differ in length and structural complexity. For example, up to C₈₀ long tails, which are not trimmed before presentation (18), compose MA and GMM, whereas short 18:1/16:0 tails are found in GM1.

Mycobacterium tuberculosis (Mtb) Ac₂SGL lipids are known to stimulate specific T cells from Mtb-infected donors (10) and therefore are good candidate for novel lipid-based vaccines. Herein, we investigated how CD1b generates T-cell-stimulatory complexes with a synthetic sulfoglycolipid analog of Ac₂SGL. Our study clarifies how CD1b copes with antigens of variable tail length and complexity and why in some cases T cells are strongly sensitive to the structure of antigen hydrophobic tails. These findings are relevant for the development of new-generation subunit vaccines.

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The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors reported in this paper have been deposited in the Protein Data Bank, <http://www.rcsb.org> (PDB ID 3T8X).

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Results

Stimulation of Specific T Cells by shCD1b Loaded in Vitro with Ac₂SGL or SGL12. In the present study we investigated the structural and functional characteristics of native recombinant soluble human CD1b (shCD1b, expressed from mammalian cells) in complex with the Ac₂SGL antigen or the synthetic analog SGL12 (Fig. S1A). Complex formation was confirmed by isoelectric focusing (IEF) (Fig. S1B and ref. 17) and found to be optimal at pH 4.0 (Fig. S1C), in agreement with previous findings proving that presentation requires CD1b and Ac₂SGL to encounter in acidic compartments of living APC (10). Importantly, plate-bound shCD1b–SGL12 elicited strong cytokine release from Ac₂SGL-specific T cells (Fig. S1D). These experiments also confirmed the antigen specificity, the CD1b restriction of the T-cell response, and ruled out potential effects of the CD1b-expression system (Fig. S1D–F).

Stimulatory shCD1b–SGL12 Complex Contains an Endogenous Spacer. Immunogenic shCD1b–antigen complexes were characterized by native mass spectrometry (n-MS) (19), an approach that permitted identification of phosphatidylcholine (PC) and a spacer molecule (UL) as major endogenous ligands associated to shCD1b (20). We focused on complexes with SGL12, because naturally occurring Ac₂SGL is molecularly heterogeneous in fatty acids linked at positions 2 and 3 of the sulfotrehalose (Fig. S1A).

To further facilitate the interpretation of MS data, shCD1b–SGL12 was enriched by chromatofocusing.

In positive-ion mode electrospray ionization (ESI) n-MS, the mass of the major species observed for fully glycosylated shCD1b–SGL12 (Fig. 1A, black line) supported the association of SGL12 (1,064 Da) with ligand-free shCD1b (major species with unloaded shCD1b; Fig. 1B, black line). The shCD1b–SGL12 species nearly disappeared when the desolvation energy was reduced, and a peak that suggested simultaneous UL presence emerged (Fig. 1A, gray line). The presence of shCD1b–SGL12–UL in solution was supported by the negative-ion mode ESI data. Thus, whereas ligand-free shCD1b was detected as major species (Fig. 1C, black line), a broad peak was again observed at lower desolvation energy (Fig. 1C, gray line). The higher than expected weight of this species is likely due to unspecific binding of water and buffer molecules onto the gas phase ions at low energies (21).

Tandem MS–MS studies helped to corroborate the identity of the shCD1b–SGL12–UL species. Subjecting the presumed shCD1b–SGL12–UL¹⁴⁺ pseudomolecular ions to collision-induced dissociation led to the precursor ion (indicated by an asterisk in Fig. 1D) and to two peaks indicative of dissociation of 600- to 640-Da ligands, which were directly detected in the low *m/z* region (Fig. 1D, *Inset*). In these experiments, dissociation of the anionic SGL12 was likely prevented by the tight interaction

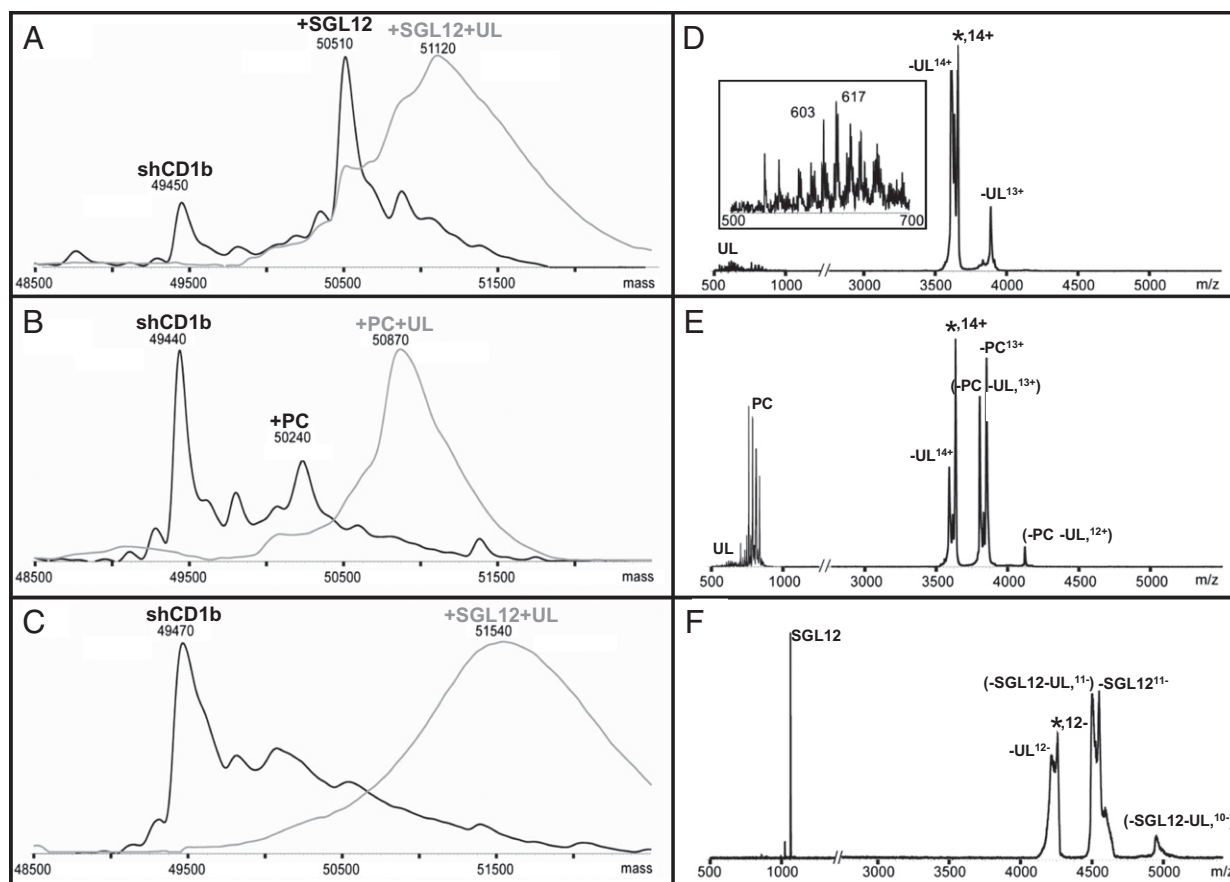


Fig. 1. Presence of combined SGL12 and endogenous spacer in stimulatory complexes. Neutral mass spectra obtained after averaging over all charged states the positive- (A and B) and negative-ion mode (C) ESI-MS native data. Spectra are from fully glycosylated shCD1b after incubation with SGL12 (A and C) or before addition of any lipid (B). Spectra recorded using harsh or low desolvation energies are drawn with black or gray lines, respectively. (D–F) Tandem MS–MS data recorded after selection of 14+ (D and E) or 12- (F) precursor ions corresponding to the most abundant species detected at low desolvation energies in A and B or C, respectively. Ligand dissociation from precursor ions (indicated with an asterisk) lead to appearance of additional protein peaks in the high-mass range. In addition, these ligands could be directly detected in the low-mass range, provided that the ligand charge is compatible with the ion mode detection. The 500- to 1,000-Da mass region of D and E is 20-fold enlarged on the y axis and 200-fold enlarged for *Inset* in D. The faint PC signals observed in D are attributed to <5% contaminant shCD1b–PC–UL.

with the positively charged protein surface. Definitive proof for the simultaneous presence of SGL12 was attained when the shCD1b-SGL12-UL¹²⁻ ions were subjected to tandem MS-MS. The precursor ion decomposed by loss of approximately 1,070 Da and a negative charge (-SGL12; Fig. 1*F*). Additional signals arose from neutral loss of *ca.* 550 Da (-UL) or a simultaneous loss of approximately 1,600 Da (-SGL12 -UL). Further supporting the presence of SGL12, its molecular ion was observed in the low *m/z* region of the spectrum, whereas no signal attributable to UL was detected.

Identification of the Spacer Lipid. Weak signal intensities and the difficulties to fragment UL species dissociating from shCD1b impeded their characterization by n-MS. We then attempted the characterization of protein extracts by Fourier transform ion cyclotron resonance (FT-ICR) MS, a technique that could permit direct attribution of molecular formula from mass values. UL species could be recovered in petroleum ether (PE) after complete digestion of shCD1b with proteinase K, whereas PC was mostly found in subsequent extractions with chloroform. UL signals were detected in positive-ion mode ESI FT-ICR MS (Fig. S2*A*), at *m/z* values matching well those observed for species that dissociate from shCD1b in n-MS experiments (Fig. 1*D* and Fig. S2*B*). Relative intensities varied notably between the two spectra. This was attributed to instrumentation effects (see below), because data obtained by n-MS on the PE extract resembled closely the spectra recorded from shCD1b (compare Fig. S2 *B* and *C*).

Elemental composition analysis permitted grouping of UL species detected by FT-ICR MS as belonging to three families. First-group masses invariantly furnished as best hit C₃₅₋₄₃H₆₇₋₇₃O₃ molecular formula (Table S1, in plain black letters). For the second family, masses matched within the instrument accuracy (<3 ppm) to C₃₅₋₄₃H₆₅₋₇₅O₄ formula (Table S1, bold black letters). Some of these masses also fitted to C_xH_yO₄Na⁺ or C_xH_yO₃Li⁺ adducts. Conversely, hits with four or six nitrogen atoms (e.g., C₃₈H₆₈N₄Na for *m/z* 603.5341 species) were ruled out because unprecedented in the literature and in public lipid databases. Finally, two peaks were assigned to C₃₇₋₃₉H₇₀₋₇₂O₅Na (Table S1 in gray), suggesting diacylglycerol sodium adducts (diAcGro).

With the exception of a few C_xH_yO₃Li⁺ hits, all species in Table S1 furnished formula with odd carbon atom number. We interpreted this as indicative of the presence of glycerol-based moieties (C₃). Extracted UL species continued to be reluctant to fragmentation in tandem MS experiments. Exploitable data could only be obtained for the species at *m/z* 603.5 (Fig. S2*D*), which furnished almost identical spectra to those obtained after selection of the *m/z* 603.5 signal present in commercial triolein, which arises from elimination of a single oleic acid (Fig. S2*E*).

Major UL species migrated on silica TLC plates comparably to synthetic diradylglycerols and were more retained than wax esters (i.e., stearyl-stearate) (Fig. S2*F*). The retention factor (*R_f*) of the most abundant species revealed with a CuSO₄ universal reagent was compatible with alkylacylglycerols (AkAcGro) and diAcGro. Although FT-ICR MS species intensities suggested that diAcGro (C_xH_yO₅Na⁺ adducts) could be less abundant, experiments with synthetic AkAcGro and diAcGro indicated that [M+H-H₂O]⁺ dehydration peaks could become prominent under FT-ICR MS conditions but not in n-MS experiments. Accordingly, C_xH_yO₃ and C_xH_yO₄ species of Fig. S2*A* could arise from C_xH_{y+2}O₄ (AkAcGro) and C_xH_{y+2}O₅ (diAcGro) species, as proposed in Table S1. To clarify this point, a ¹H NMR spectrum was recorded overnight on a PE-extracted UL sample and compared with a control sample (Fig. S2*G*, *Top* vs. *Middle*). Signals in the 2.2- to 5.2-ppm interval supported that UL mostly consisted of a mixture of 1,2- and 1,3-regioisomers of AkAcGro and diAcGro. Indeed, the UL spectrum closely resembled the ¹H-NMR data recorded for a mixture of synthetic AkAcGro and diAcGro. Of note, such diradylglycerol structures are fully

compatible with the linear electron densities attributed to UL in high-resolution structures of shCD1b (ref. 20 and below).

Determination of the Structure of the CD1b-SGL12-Spacer Complex.

MS experiments identified shCD1b-SGL12-UL with 1:1:1 stoichiometry as the T-cell stimulatory complex. To clarify how diacylsulfoglycolipid presentation to T cells is compatible with the presence of UL, a strongly stimulatory sample containing fully glycosylated shCD1b loaded to approximately 75% occupancy with SGL12 was crystallized, and the structure was solved at a resolution of 1.90 Å (Table S2). Two molecules (copy A and B) were found per crystallographic asymmetric unit. All main structural features were similar for both molecules (rmsd of 0.40 Å on backbone atoms) and were common to published CD1b structures (6, 20, 22). The C' portal was closed in both copies.

SGL12 was unequivocally identified in copy A (Fig. 2*A*). Electron density consistent with the sulfotrehalose polar head and disconnected stretches of the lipid tails was present in this copy from the earliest electron density maps. Conversely, weak electron density was found at the same relative location in the second copy (Fig. 2*B*), suggesting low SGL12 pocket occupancy. For that reason, the polar head for this second copy was omitted from the final structure. Extra electron density indicative of spacers presence was also found filling the T' channel and the F' pocket in both copies (Fig. 2*A*). The species and length heterogeneity of spacers and the impossibility of predicting its insertion orientation within the groove led us to build into such electron density a C₄₀-long carbon chain representative for the average length of diradylGro species.

The electron density of the SGL12 sulfotrehalose head in copy A connected during refinement to the electron density tube present in the C' pocket but not with those extending downward to the A' or F' pockets. Nonetheless, such lack of connectivity was not a source of structural ambiguity concerning the lipid insertion mode, because the structural constraints imposed by the well-defined sulfotrehalose head dictated an unequivocal arrangement with the palmitic acid in the C' pocket and the phthioceranoyl-like tail in the A' pocket. The gap of electron density between the A' entrance and the polar head corresponded to the tetramethyl-branched portion of the fatty acid tail that esterifies position 3 of SGL12 (Fig. S1*A*). This hydrophobic patch is located in an unfavorable water-exposed environment that must prevent the establishment of a privileged conformation. In addition, its emplacement at the entrance of the A' pocket must fluctuate depending on the length heterogeneity of accompanying spacer molecules. These two factors might explain the poor definition in the electron density map for this patch.

Polar Head Exposure and Positioning Above the Groove. Hydrogen bonds between the guanidinium group of Arg79 and the 3'- and 4'-OH groups of the sulfated glucose stabilized in copy A the SGL12 polar head in a conformation that may favor the interaction of the sulfate with the TCR (Fig. 2*C*), in agreement with its essential role in T-cell activation (10). Arg79 is conserved in CD1b and CD1d of other species and plays a key ligand-binding role in CD1d structures (23-25). However, unlike CD1d, which often holds lipid polar heads by establishing hydrogen bond interactions with several residues (23-28), no further direct polar head-anchoring residues were found in the SGL12-loaded CD1b structure. The interaction between the SGL12 sulfotrehalose and Arg79 was solely reinforced by indirect hydrogen bonds, mediated by water molecules, with Gly76 and Gln152.

Polar heads are usually weakly defined in 3D structures of CD1b-lipid complexes (6, 20, 22). In relation to this, the SGL12 sulfotrehalose is *ca.* 3 and 10 Å more elevated above the pocket main portal than, for instance, the galactosyl rings of αGalCer and sulfatides bound to CD1d (27) and CD1a (29), respectively. Taken together, these data indicate that CD1b weakly interacts with

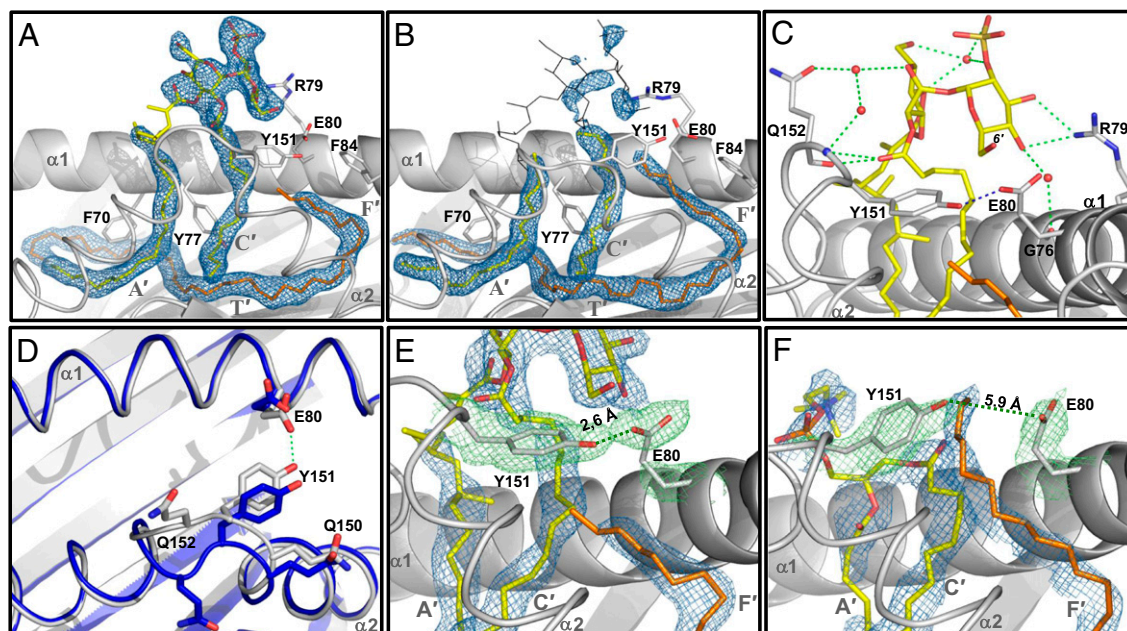


Fig. 2. Ligand binding and pocket alterations in shCD1b-SGL12-UL. (A) Electron density for the SGL12 and spacers in copy A (final $2F_o - F_c$ map contoured at 1.0σ , blue mesh). Modeled lipids are drawn as sticks. SGL12 is colored by atom type (carbon yellow, oxygen red, sulfur orange), spacer in orange. The $\alpha 2$ domain helices are represented as thin ribbons. Gray letters indicate the groove channels occupied by nearby portions of electron density. (B) Electron density in the lipid-binding groove for copy B. Only ligand portions shown as yellow or orange sticks were modeled. For comparison, a full SGL12 molecule in the conformation observed in copy A (black lines) is superimposed onto the groove of copy B. (C) Hydrogen bonds established between SGL12 and CD1b. Hydrogen bonds between the lipid and Arg79 or Gln152, as well as indirect interactions mediated by water molecules (red spheres) with Gln152 and Gly76, are depicted as green dashed lines. (D) Comparison of the conformation adopted by residues from the $\alpha 1$ - $\alpha 2$ domains of shCD1b-SGL12-UL (copy A, in gray) with those from shCD1b-PC-UL (20) (Protein Data Bank entry 2H26, in blue), after superimposition of backbone atoms of residues 6–180 from each structure. For clarity, lipid ligands are omitted from this top-view representation. (E) A Tyr151-Glu80 hydrogen bond prevents spacer protrusion in the SGL12-loaded structure. The $2F_o - F_c$ map (1.0σ contour) is represented as a green mesh for side-chain atoms of Tyr151 and Glu80, in blue for ligand atoms. The interatomic distance between the two closest oxygens from Tyr151 and Glu80 is indicated. (F) Similar representation to that of E prepared using data from the crystal structure of shCD1b-PC-UL. PC atoms are colored exactly as SGL12 atoms in B, and with phosphorus in orange.

polar groups of anchored ligands, which might be required to facilitate the presentation of antigens with a range of polar heads.

Antigen Binding Induces Conformational Changes in CD1b. Remarkably, the presence of SGL12 was accompanied by an important shift toward the $\alpha 1$ -helix of backbone and side-chain atoms of residues 149–152 that connect two helical segments of the $\alpha 2$ -domain (Fig. 2D). The rmsd of backbone atoms from these four residues in copy A compared with their position in natively folded shCD1b was 2.4 \AA (20). In contrast, the conformation of these residues in copy B, in shCD1b and in other published CD1b structures (6, 22), was essentially identical (rmsd below 0.6 \AA). The displacement in the SGL12-loaded copy resulted in the establishment of a hydrogen bond between the Glu80 side-chain carboxylate and the Tyr151 hydroxyl group (Fig. 2D and E). The interatomic distance between the two closest oxygen atoms from each residue decreased from 5.9 \AA in natively folded shCD1b (Fig. 2F) to 2.6 \AA in the SGL12-loaded copy. Accompanying these movements, the side-chain of Gln152 oriented toward the TCR-contacting interface, compared with other published structures (6, 20, 22), suggesting that this residue might play a role in modulating the interaction with TCR.

The conformational change of residues 149–152 had important structural consequences. It occluded the F' pocket entrance, preventing spacer egress toward the TCR contacting surface. Compared with unloaded shCD1b, spacers repositioned by the length of at least 6 methylene units toward the A' pocket in copy A, clearing above the F' pocket the necessary space to accommodate the SGL12 sulfated glucose (compare Fig. 2E and F). The groove volume decreased from approximately $2,400 \text{ \AA}^3$ in natively folded shCD1b to $<2,200 \text{ \AA}^3$ in the presence of SGL12. Overall, this conformational rearrangement appears incompat-

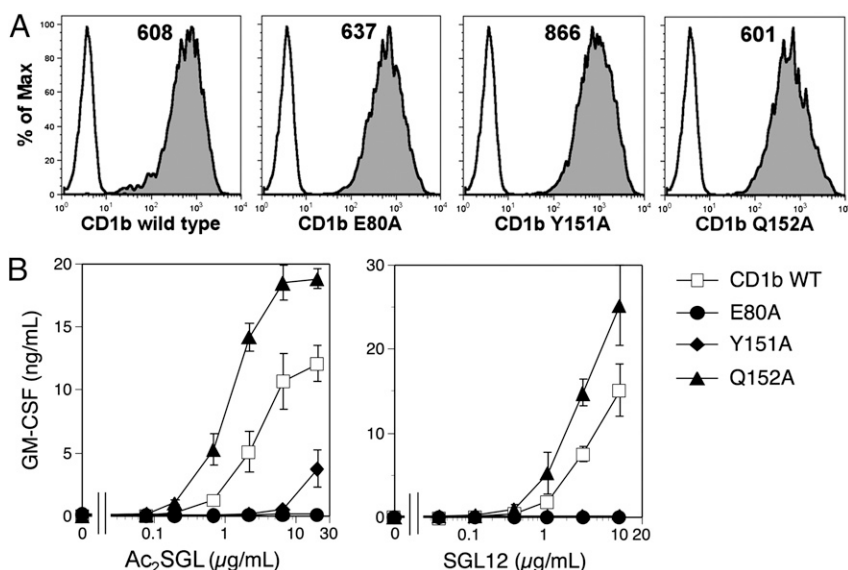
ible with full embedding of the SGL12 phthioceranol-like tail inside the A' pocket and thus forces its methyl-branched motif to remain exposed above the CD1b surface.

Influence of CD1b Conformational Changes on T-Cell Stimulation. To investigate whether such groove structural reorganization is important for Ac₂SGL/SGL12 presentation to T cells, we evaluated T-cell responses using CD1b with Ala point mutations at residues Glu80 or Tyr151 (implicated in F' pocket closure) or at Gln152 (residue that flipped toward the TCR in copy A). Stably transfected C1R cells expressing matched surface levels of wild-type and mutant CD1b were selected (Fig. 3A), and their capacities to activate the Ac₂SGL-specific T cells were evaluated (Fig. 3B).

Replacement of Glu80 or Tyr151 with Ala fully abrogated T-cell responses to Ac₂SGL and SGL12, supporting the importance of the conformational changes observed in the CD1b-SGL12-UL structure for productive Ac₂SGL presentation. Differently from E80A and Y151A, the Q152A mutation resulted in increased T-cell responses to Ac₂SGL and SGL12 at all antigen doses. Enhanced cytokine secretion at saturating Ac₂SGL doses argued against differences in the number of antigenic complexes displayed by Q152A CD1b at the APC surface, compared with wild-type CD1b, and suggested that complexes with stronger stimulatory capacity were formed. These results collectively indicate that the conformational changes of CD1b residues 149–152 control T-cell responses to Ac₂SGL antigens.

Discussion

CD1b presents a remarkable variety of (glyco)lipids to T cells (1, 3). Previous structural studies described the intricacy of the



flexibility in adapting to foreign antigens, thus facilitating lipid antigen recognition and antimicrobial immune responses.

Methods

Reagents, cell lines, the preparation of APC transfectants, T-cell activation assays, and other experimental details are described in *SI Methods*.

Native Nano ESI Mass Spectrometry. Recombinant shCD1b loaded with SGL12 was purified by chromatofocusing on a MonoP column (GE Healthcare) using a pH gradient between 6.5 and 4.0. Enriched CD1b–SGL12 complex was recovered as second eluting peak, concentrated, and buffer-exchanged against 10 mM Na acetate, pH 5.0. The last 18 residues belonging to the BirA extension tag were then removed by incubation of CD1b–SGL12 with LysC endoproteinase (Roche) (200:1 weight ratio) at 30 °C for 6 h, in 25 mM Tris/5 mM EDTA, pH 8.5. Details concerning the analysis by n-MS are given in *SI Methods*.

Extraction of the Endogenous Spacer from shCD1b. Recombinant shCD1b (2 mg) was immunopurified as previously described (20), with modifications. After PBS, column wash was continued with deionized water (30 mL), and the protein was eluted with aqueous acetic acid (25 mL, 0.2 M, pH 2.6) and collected in glass tubes. After pH neutralization with ammonium hydroxide (30%), pooled fractions were concentrated under vacuum to 1 to 2 mL and treated overnight at 30 °C with proteinase K (20 U, from *Tritirachium album*; Sigma). Lipids were extracted by three successive steps with PE (HPLC grade, 2 mL per step). Upper organic phases were pooled and dried. The aqueous phase was flowed with N₂ to remove remaining PE, and extraction was pursued with 2 × 2 mL of CHCl₃ (HPLC grade). The bottom phases were pooled and dried.

Analysis by FT-ICR MS. Protein ligands extracted with PE or CHCl₃ were analyzed by ESI FT-ICR MS, using a hybrid APEX Qe Instrument (Bruker Daltonics) equipped with an actively shielded 7-T magnet and a dual ESI source. Mass spectra were acquired in the positive-ion mode using standard experimental sequences as provided by the manufacturer. Samples were dissolved in a 50:50:0.03 (vol/vol/vol) mixture of 2-propanol/water/AA (30 mM) at pH 4.5 and sprayed at a flow rate of 2 μ L/min. Capillary entrance voltage was set to 3.8 kV and drying gas temperature to 200 °C. Mass scale cali-

bration was performed externally and controlled internally with compounds of known structure assuring a mass accuracy below 3 ppm.

Thin-Layer Chromatography. Silica gel TLC plates (SIL G/UV₂₅₄ 0.20 mm, 10 cm high; Macherey-Nagel) were prewashed by running successively to 9 cm first with CHCl₃/MeOH (9:1, vol/vol) and second with PE/Et₂O (15:5, vol/vol). After the plate dried, samples (1–4 μ g) were loaded and developed with PE/Et₂O/acetic acid (15:5:0.2, vol/vol/vol) to 8 cm. For visualization, the plates were dipped into 10% cupric sulfate in MeOH containing 4% phosphoric acid and 4% sulfuric acid, before being charred at 180 °C for 10 min.

Crystallization of the Glycosylated shCD1b–SGL12–UL Complex. Mouse-expressed shCD1b was loaded with SGL12 (*SI Methods*). After pH neutralization, the protein was purified by anion-exchange chromatography (BioScale Q2 column; Bio-Rad) using a linear gradient from buffer A (10 mM Bis-Tris/1 mM EDTA/pH 6.5) to buffer B (500 mM NaCl in buffer A). The best crystals were grown after 1–3 d at 20 °C, using the hanging-drop vapor-diffusion method, from drops containing 1 μ L shCD1b–SGL12 (5 mg/mL in 10 mM Bis-Tris/30–50 mM NaCl/1 mM EDTA/pH 6.5) and 1.0 μ L precipitant [1.9 M (NH₄)₂SO₄, 0.2 M NH₄Ac, pH 6.6]. Experimental details concerning X-ray data collection, structure determination, analysis and presentation are given in *SI Methods*.

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Supporting Information

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SI Methods

Reagents and Cell Lines. Bovine brain sulfatides, gangliosides type II, 1,2-dipalmitoyl-*sn*-glycerol, 1,3-diolein, 1,2-di-*O*-hexadecyl-*rac*-glycerol and stearyl stearate were purchased from Sigma, 1-*O*-hexadecyl-2-*O*-arachidonoyl-*sn*-glycerol from Enzo Life Sciences, 1,3-di-*O*-hexadecyl-*rac*-glycerol and 1-hexadecyl-3-*O*-stearoyl-*rac*-glycerol were respectively provided by Dr. Dominique Lafont (Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, Centre National de la Recherche Scientifique, Villeurbanne, France) and Prof. Gudmundur G. Haraldsson (University of Iceland), and other lipids were from Avanti Polar Lipids. *Mtb* Ac₂SGL was purified as previously reported (1). SGL12 was synthesized as previously reported (2).

Recombinant CD1b (shCD1b), expressed from either mouse J558 cells or S2 *Drosophila* cells, and soluble CD1e were purified as previously described (3, 4). Recombinant soluble CD1d and CD1a were expressed from mouse cells as described for shCD1b.

Isoelectric-Focusing (IEF) Electrophoresis. The basic protocol to measure lipid loading onto CD1 molecules has been described (4). Briefly, *N*-glycosylated shCD1b (10–20 μ M) and 100 μ M lipid were shaken in glassware tubes buffered at pH 4.0, at 37 °C and 600 rpm. After 6 h, aliquots (2 μ L) were loaded onto the IEF 4–6.5 gel (Amersham Biosciences) and electrophoresed in a PhastGel system (Amersham Biosciences).

Native Nano Electrospray Ionization (ESI) Mass Spectrometry. Recombinant shCD1b loaded with SGL12 was purified by chromatofocusing on a MonoP column (GE Healthcare) using a pH gradient between 6.5 and 4.0. Enriched CD1b–SGL12 complex was recovered as second eluting peak, concentrated, and buffer-exchanged against 10 mM Na acetate, pH 5.0. The last 18 residues belonging to the BirA extension tag were then removed by incubation of CD1b–SGL12 with LysC endoproteinase (Roche) (200:1 weight ratio) at 30 °C for 6 h, in 25 mM Tris/5 mM EDTA, pH 8.5.

Before mass analysis, protein samples were buffer exchanged extensively into 50 mM aqueous ammonium acetate, pH 6.7, using 5-kDa Millipore centrifuge filters. Other details on instrumentation and measurement conditions are described in ref. 4.

Structure Determination, Analysis, and Presentation. Crystals were briefly immersed into the crystallization solution supplemented with 20% glycerol before being transferred into a gaseous nitrogen flux at 100 K. Diffracted intensities were collected from a single crystal at European Synchrotron Radiation Facility (Grenoble, France) on beamline ID23 EH2, tuned at 0.8726 Å, using a MarCCD detector. Diffraction images were processed using MOSFLM (5), scaled with SCALA (6), and further processed with the CCP4 programs package (7). Data processing statistics are summarized in Table S2.

The structure was solved by the molecular replacement method, with the program PHASER (8), using both polypeptidic chains from the crystal structure of native shCD1b [Protein Data Bank

entry 2H26 (4)]. A single unambiguous solution was identified with a Z-score of 64.2 for data between 35.0- and 3.0-Å resolution. This solution was refined using data between 20.0- and 1.9-Å resolution with REFMAC (9). The F_o-F_c electron density maps allowed modeling of portions of the GlcNAc(β 1–2)Man(α 1–6)[GalNAc(β 1–4)GlcNAc(β 1–2)Man(α 1–3)]Man(β 1–4)GlcNAc(β 1–4)[Fuc(α 1–6)]GlcNAc oligosaccharide onto Asn20 and Asn57 on each heavy chain. Two lipid molecules were included in the model for copy A and copy B, to account for electron density visible in the Fo-Fc map, at a contour level of 3.0 σ . Final model includes 750 residues, 14 sugar moieties, four lipids [complete and partial SGL12, and two a spacer molecules (ULs)], four sulfates, one chloride and one acetate anions, three glycerol molecules, and 524 water molecules. Statistics for the final refined structure are given in Table S2. Ramachandran analysis shows 712 residues in preferred regions (97.4%), 18 in allowed regions (2.46%), and one outlier (Asp33, chain C) (0.14%). Protein stereochemistry was validated using PROCHECK (10).

Molecular surface areas and volumes were calculated using the CASTp server (11). A solvent probe of 1.4 Å was used to map internal cavities and pockets of the proteins. Figure 3 was generated with PyMOL (12).

Generation of APC Expressing CD1b Mutants. Three CD1b mutant molecules bearing a single amino acid substitution were generated by site-directed mutagenesis of CD1B cDNA: E80, Y151, and Q152 were mutated into alanine. Nested PCR were performed to introduce the mutations using CD1B cDNA as template, Advantage-HF 2 PCR kit (Clontech), and the primers 5' *SalI*/CD1b-tatagtcgacatgctgctgctgcc-atttcaac; 3' *Bam*/HICD1b-agg-gatcctcatgggatattctgatgacc; 5' CD1bE80A-cgagcagtagaagatttgcgggtgatttc; 3' CD1bE80A-gtactgctcgagcgaatccaagatgtgactc; 5' CD1bY151A-acaagctcaaggt-atcatggaaactgtgag; 3' CD1bY151A-cttgagcttgatgattagtgacacagaatttc; 5' CD1bQ152A-aatatgc-aggtatcatggaaactgtgagaa; and 3' CD1bQ152A-tacctgcatattgtatgattagtcacagaatttc. Mutant constructs were cloned in pCR2.1-TOPO (Invitrogen), sequenced, subcloned into BCMGS *hph*, and used for transfection of C1R cells. Stable transfectants were selected and cell surface expression of CD1b analyzed by flow cytometry using a CyAn ADP flow cytometer (DakoCytomation). Data were analyzed using FlowJo software (Tree Star).

T-Cell Activation Assays. Transfectants with CD1b expression levels closely matching those of wild-type CD1b were used to activate Ac₂SGL-specific and CD1b-restricted Z4B27 T cells (1). Briefly, APC (3×10^4 per well) were preincubated for 2 h at 37 °C with different concentrations of Ac₂SGL or synthetic SGL12 analog before addition of T cells (10^5 per well in duplicate). Supernatants were harvested after 36 h, and GM-CSF release was measured by ELISA (R&D Systems). Data are expressed as mean ng/mL \pm SD. All transfectants were analyzed by flow cytometry within 24 h of each assay to confirm CD1b surface expression levels. Plate-bound activation assays were performed as described in ref. 2.

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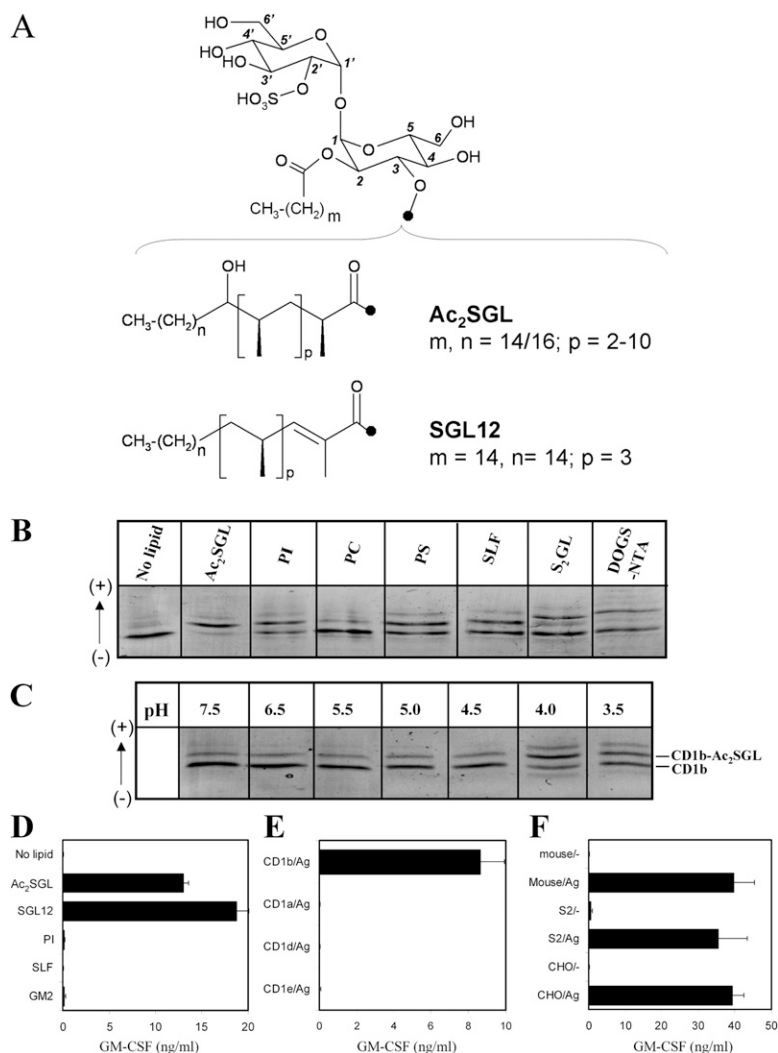
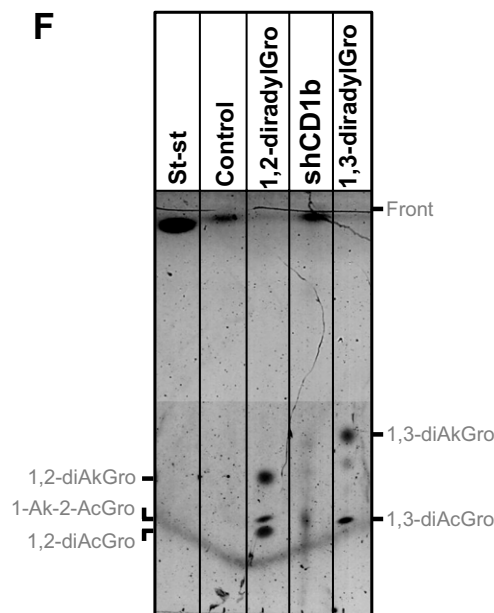
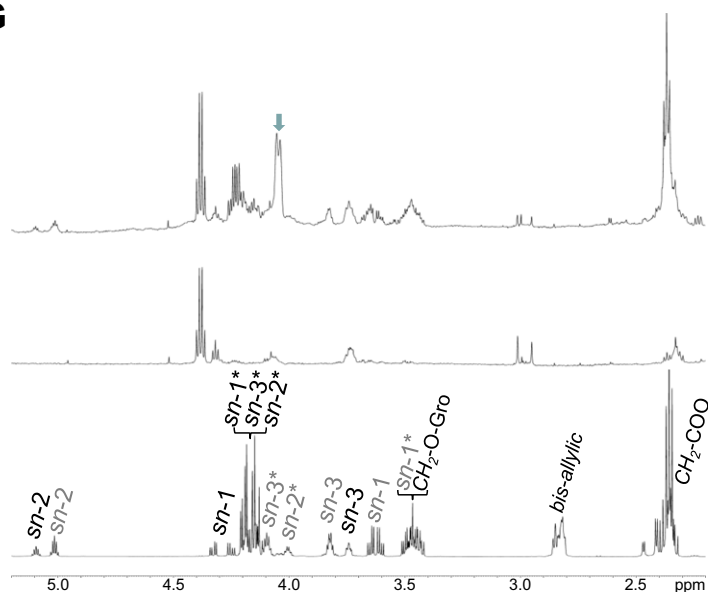


Fig. S1. Activation of specific T cells by CD1b–Ac₂SGL and CD1b–SGL12 complexes. (A) Chemical structure of *Mtb* Ac₂SGL and SGL12. Palmitic or stearic fatty acids esterify the sulfotetraose position 2 in Ac₂SGL, only palmitic acid in the synthetic analog SGL12. The heterogeneous C₂₀ to C₄₀ hydroxyphthioceranyl appendage at position 3 is replaced by a tetra-methyl branched α,β -unsaturated chain in SGL12. (B) Lipid binding to shCD1b monitored by IEF. (C) Efficient binding of Ac₂SGL to CD1b requires pH 4.0. (D–F) Stimulation of Ac₂SGL-specific T cells by immobilized CD1b–Ac₂SGL and CD1b–SGL12 complexes. Various shCD1 preparations were immobilized on plastic wells after incubation with or without indicated lipids. After addition of Z4B27 T cells, GM-CSF release was measured and expressed as mean of duplicates + SD. (D) Antigen specificity of stimulation of Z4B27 T cells with shCD1b. (E) Stimulation with Ac₂SGL (Ag) is CD1b-restricted. (F) Stimulation of Z4B27 T cells is independent of the CD1b-expression system. Purified shCD1b expressed from mouse cells, S2 *Drosophila* cells, or CHO cells were tested in the absence (–) or presence (Ag) of Ac₂SGL. All data illustrated are representative of three independent experiments.



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Table S1. Characterization of UL by positive mode Fourier transform ion cyclotron resonance MS

Experimental* <i>m/z</i>	Formula [†]	Theoretical [‡] <i>m/z</i>	Proposed [§]
535.5084	C ₃₅ H ₆₇ O ₃	535.5085	AkAcGro _{32:1} -H ₂ O
537.5241	C ₃₅ H ₆₉ O ₃	537.5241	AkAcGro _{32:0} -H ₂ O
549.4876	C₃₅H₆₅O₄	549.4877	diAcGro_{32:1}-H₂O
561.5239	C ₃₇ H ₆₉ O ₃	561.5241	AkAcGro _{34:2} -H ₂ O
563.5395	C ₃₇ H ₇₁ O ₃	563.5398	AkAcGro _{34:1} -H ₂ O
565.5552	C ₃₇ H ₇₃ O ₃	565.5554	AkAcGro _{34:0} -H ₂ O
575.5028	C₃₇H₆₇O₄	575.5034	diAcGro_{34:2}-H₂O[¶]
577.5189	C₃₇H₆₉O₄	577.5190	diAcGro_{34:1}-H₂O[¶]
589.5550	C ₃₉ H ₇₃ O ₃	589.5554	AkAcGro _{36:2} -H ₂ O
591.5708	C ₃₉ H ₇₅ O ₃	591.5711	AkAcGro _{36:1} -H ₂ O
601.5184	C₃₉H₆₉O₄	601.5190	diAcGro_{36:3}-H₂O[¶]
	C₃₇H₇₀O₄Na	601.5166	AkAcGro_{34:2} + Na
603.5341	C₃₉H₇₁O₄	603.5347	diAcGro_{36:2}-H₂O[¶]
	C₃₇H₇₂O₄Na	603.5323	AkAcGro_{34:1} + Na
605.5500	C₃₉H₇₃O₄	605.5503	diAcGro_{36:1}-H₂O[¶]
611.5394	C ₄₁ H ₇₁ O ₃	611.5398	AkAcGro _{38:5} -H ₂ O
613.5549	C ₄₁ H ₇₃ O ₃	613.5554	AkAcGro _{38:4} -H ₂ O
617.5113	C ₃₇ H ₇₀ O ₅ Na	617.5115	diAcGro_{34:1} + Na
617.5865	C ₄₁ H ₇₇ O ₃	617.5867	AkAcGro _{38:2} -H ₂ O
619.6021	C ₄₁ H ₇₉ O ₃	619.6024	AkAcGro _{38:1} -H ₂ O
627.5343	C₄₁H₇₁O₄	627.5347	diAcGro_{38:4}-H₂O[¶]
629.5493	C₄₁H₇₃O₄	629.5503	diAcGro_{38:3}-H₂O[¶]
	C₃₉H₇₄O₄Na	629.5479	AkAcGro_{36:2} + Na
631.5655	C₄₁H₇₅O₄	631.5660	diAcGro_{38:2}-H₂O[¶]
	C₃₉H₇₆O₄Na	631.5636	AkAcGro_{36:1} + Na
637.5551	C ₄₃ H ₇₃ O ₃	637.5554	AkAcGro _{40:6} -H ₂ O
643.5268	C ₃₉ H ₇₂ O ₅ Na	643.5272	diAcGro_{36:2} + Na
653.5500	C₄₃H₇₃O₄	653.5503	diAcGro_{40:5}-H₂O[¶]
655.5657	C₄₃H₇₅O₄	655.5660	diAcGro_{40:4}-H₂O[¶]

*Experimental masses of most intense peaks detected by positive-ion mode Fourier transform ion cyclotron resonance MS in a PE extract obtained from proteinase K-treated shCD1b. Only those peaks confirmed in two independent experiments are listed.

[†]Molecular formula fitting to the given experimental mass within instrument accuracy (below 3 ppm). The next combinations of elements (maximal number) were considered: C (50), H (100), N (6), O (8), S (4), Li (1), Na (1), and K (1).

[‡]Calculated monoisotopic ion mass.

[§]Proposed chemical identity: AkAcGro: alkylacylglycerol; AkAcGro-H₂O: AkAcGro dehydration product; diAcGro: diacylglycerol. Numbers indicate the cumulated length and number of insaturations in the two fatty acid chains (i.e., excluding those from carbonyls and dehydration). The proposition takes into account TLC and ¹H NMR data discussed in the text.

[¶]AkAcGro [M+H]⁺ species might also contribute to the indicated isobar.

^{||}C_xH_yO₃Li formula also fits within the instrument accuracy to this experimental mass.

Table S2. Data collection and refinement statistics for CD1b–SGL12–UL

Data collection

Space group	C2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	203.25, 67.58, 88.19
α , β , γ (°)	90.00, 112.26, 90.00
Resolution (Å)	36.54–1.90 (2.00–1.90)*
<i>R</i> _{sym}	0.070 (0.362)
<i>I</i> / σ	11.2 (1.8)
Completeness (%)	92.8 (71.1)
Redundancy	2.9 (1.6)
Refinement	
Resolution (Å)	20.00–1.90
No. reflections	80,866
<i>R</i> _{work} / <i>R</i> _{free}	0.1700/0.2208
No. atoms	
Protein	5884
Glycan	174
Ligand	183
Solvent (water, ions, glycerol)	571
<i>B</i> -factors (Å ²)	
Protein	34.6
Glycan	66.8
Ligand	57.1
SGL copy A (73 atoms) UL copy A (40 atoms)	68.1 44.4
SGL copy B (31 atoms) UL copy B (39 atoms)	53.3 52.8
Solvent (water, ions, glycerol)	46.1
rms deviations	
Bond lengths (Å)	0.024
Bond angles (°)	2.039

*Values in parentheses are for the highest-resolution shell.