

Diacylated Sulfoglycolipids Are Novel Mycobacterial Antigens Stimulating CD1-restricted T Cells during Infection with *Mycobacterium tuberculosis*

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Abstract

Mycobacterial lipids comprise a heterogeneous group of molecules capable of inducing T cell responses in humans. To identify novel antigenic lipids and increase our understanding of lipid-mediated immune responses, we established a panel of T cell clones with different lipid specificities. Using this approach we characterized a novel lipid antigen belonging to the group of diacylated sulfoglycolipids purified from *Mycobacterium tuberculosis*. The structure of this sulfoglycolipid was identified as 2-palmitoyl or 2-stearoyl-3-hydroxyphthioceranoyl-2'-sulfate- α - α' -D-trehalose (Ac₂SGL). Its immunogenicity is dependent on the presence of the sulfate group and of the two fatty acids. Ac₂SGL is mainly presented by CD1b molecules after internalization in a cellular compartment with low pH. Ac₂SGL-specific T cells release interferon γ , efficiently recognize *M. tuberculosis*-infected cells, and kill intracellular bacteria. The presence of Ac₂SGL-responsive T cells in vivo is strictly dependent on previous contact with *M. tuberculosis*, but independent from the development of clinically overt disease. These properties identify Ac₂SGL as a promising candidate to be tested in novel vaccines against tuberculosis.

Key words: vaccination • intracellular bacteria • protection • cytotoxic CD8⁺ T cells • lipids

Introduction

Tuberculosis remains a major health problem in the world, despite significant progress in antibiotic therapy and health care measures. It is unlikely that the global burden of tuberculosis will be eradicated without the use of effective vaccines, which, however, are not available yet. The intracellular location of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, shields the bacilli from detection by antibody-

ies. Therefore, a protective response requires activation of antigen-specific T cells, which may recognize different types of ligands such as microbial peptides or lipids (1–3).

Conventional T cell responses directed against short peptides presented by MHC molecules are implicated in protection during mycobacterial infection in several animal models (4) and in human disease (5). Individuals with defective CD4⁺ T cell responses and genetic deficiencies in mounting Th1-dominated immune responses have an increased susceptibility to *M. tuberculosis* infection (6). In addition, evidence for an important role of CD8⁺ T lymphocytes is accumulating (7–12).

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Abbreviations used in this paper: ¹H NMR, proton nuclear magnetic resonance; Ac₂SGL, 2-palmitoyl or 2-stearoyl-3-hydroxyphthioceranoyl-2'-sulfate- α - α' -D-trehalose; BCG, bacillus Calmette Guérin; MALDI-ToF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectrometry; m/z, mass/charge; NMR, nuclear magnetic resonance; PIM, phosphatidyl-myo-inositol mannosides; PPD, purified protein derivative of *M. tuberculosis*; SGL, sulfoglycolipids.

Another population of T cells, capable of recognizing microbial lipids or self-glycosphingolipids, has been identified (13, 14). These antigens are amphipatic molecules comprised of a hydrophilic cap bound to aliphatic hydrocarbon chains. Specific T cells recognize complexes formed by individual glycolipids associated with dedicated antigen-presenting molecules belonging to the CD1 family. CD1 molecules resemble MHC class I antigen-presenting molecules as they associate with β 2-microglobulin and assume an MHC class I-like tertiary conformation. They have evolved unique features enabling binding of glycolipids. In the CD1-glycolipid complex the alkyl chains of glycolipids are embedded inside deep hydrophobic pockets, whereas the hydrophilic part of glycolipids is flanked by the two α helices of CD1, which delimit the CD1 antigen-binding groove. The TCR likely interacts with individual polar residues contributed by the glycolipid and with amino acids provided by the two α helices of CD1 (15, 16).

Only few bacterial CD1 ligands have been identified to date. Most notably, all of them were derived from mycobacterial species, possibly reflecting a special role for CD1-restricted T cells in protection against tuberculosis. Specifically, free mycolic acids (13), glucose monomycolate (17), mannophosphoisoprenoids (18), and lipoglycans such as mannosylated lipoarabinomannan (19), lipomannan (19), and phosphatidyl-*myo*-inositol mannosides (PIM; 20) are capable of eliciting CD1-mediated T cell responses. With the exception of mannophosphoisoprenoids, which stimulate CD1c-restricted T cells, all known antigens are presented by the CD1b molecule. This apparent bias to CD1b restriction might derive from the unique capacity of CD1b to recycle in the late endosomal or lysosomal compartments (21, 22) and from the organization of its antigen-binding pockets (23).

Mouse CD1d (24) and human CD1a (25) have glycolipid-binding capacities different from CD1b and it has been proposed that each CD1 molecule has evolved the capacity to present unique types of lipid antigens (18). The structures of human CD1c and CD1d are not available yet and, therefore, it is difficult to predict the presence of biased binding modes. However, all CD1 molecules can bind and present to T cells the same glycolipids with short acyl chains (26), thus pointing to overlapping presentation capacities.

To understand the role of lipid-specific T cells in protection against tuberculosis it is important to clarify whether the repertoire of immunogenic lipids synthesized by *M. tuberculosis* is limited to a few molecules or rather comprises a wide spectrum of structurally distinct molecules. Identification of the most immunogenic lipids would facilitate selection of candidate molecules for a lipid-based vaccine against tuberculosis.

A significant issue concerns the relative contribution of lipid-specific CD4⁺ and CD8⁺ T cell subsets to the control of mycobacterial infection. In tuberculosis patients the majority of lipid-specific T cells circulating in the peripheral blood appear to be CD4⁺ (27). Notably, recent findings indicate that most of CD8⁺ T cells activated by bacillus Cal-

mette Guérin (BCG)-infected APCs recognize mycobacterial lipid antigens, whose nature remains unknown (28). As CD8⁺ T cells participate in protective responses (9, 29), it is important to identify the nature of lipid antigens activating this T cell subset.

Here we identify and structurally characterize a novel mycobacterial ligand, expressed by virulent bacilli, which activates CD8⁺, CD1b-restricted T cells. T cells specific for this new antigen show a strong bactericidal capacity and are present in the large majority of tuberculosis patients and purified protein derivative of *M. tuberculosis* (PPD)⁺ healthy donors, but not in PPD⁻ donors.

Materials and Methods

Bacterial Strain and Culture Conditions. *M. tuberculosis* H37Rv (American Type Culture Collection [ATCC] 27294) was grown at 37°C on Sauton's medium as surface biofilm. Cells were harvested after 4 wk, separated from the culture media, and left in chloroform/methanol (2:1, vol/vol) for 2 d at room temperature to kill bacteria.

Lipid Fractions Used for Generation of T Cell Clones. Bacterial cells were suspended in chloroform/methanol (1:1, vol/vol) and filtered four times. The chloroform/methanol extract was concentrated and partitioned between water and chloroform. The chloroform phase was applied to an anion exchange Sep-pak[®] cartridge (Waters Accell[™] Plus QMA; Waters Corporation) eluted successively by 5 ml chloroform/methanol/water (60:35:8, vol/vol/vol) to elute neutral compounds and then with 5 ml chloroform/methanol/water (60:35:8, vol/vol/vol) containing 0.1 M ammonium acetate to elute negatively charged compounds (as phospholipids). PIM₂-enriched fraction was prepared as previously described (30).

Purification of the Novel Lipidic *M. tuberculosis* Antigen. Bacterial cells were suspended in chloroform/methanol (1:1, vol/vol) and filtered four times. The chloroform/methanol extract was concentrated and constituted the whole lipid extract (22.7 g), which was further partitioned between water and chloroform. The chloroform phase was evaporated and constituted the lipidic fraction (5.3 g), which was redissolved in a minimum volume of chloroform. The addition of acetone overnight at 4°C led to formation of a precipitate, which was centrifuged (3,000 g at 4°C for 15 m) to generate an acetone-soluble phase (fraction 1; 2.8 g) and an acetone-insoluble phase (fraction 2; 2.2 g). The "acetone-soluble" phase (fraction 1; 1 g) was then fractionated on a silicic acid column (19 × 2 cm) irrigated by chloroform (fraction 4; 120 ml), chloroform containing 10% (fraction 5 and 6; 100 ml), 20% (fraction 7; 100 ml), and 30% (fraction 8; 200 ml) methanol, and finally by chloroform/methanol/water (6:4:0.4, vol/vol/vol; fraction 9; 100 ml). Fractions 5 and 6 were found to contain Ac₄ sulfoglycolipids (SGL) and Ac₃SGL, respectively, and fraction 7 was found to contain 2-stearoyl-3-hydroxyphthioceranyl-2'-sulfate- α - α' -D-trehalose (Ac₂SGL).

Fraction 7 was further purified by reverse phase chromatography. This purification was realized on a Sep-pak[®] cartridge (Sep-pak[®] Light C18 cartridge; Waters Corporation) eluted two times with 2 ml methanol/water (9:1, vol/vol; fractions 7.1 and 7.2), two times with 2 ml methanol (fractions 7.3 and 7.4), and finally with 5 ml chloroform (fraction 7.5).

Purifications were checked by TLC on aluminum-backed silica gel plates (Alugram Sil G; Macherey-Nagel) using as migra-

tion solvent chloroform/methanol (9:1, vol/vol). Orcinol (3,5-dihydroxytoluene [Sigma-Aldrich] at 0.3% dissolved in H₂SO₄ at 10%) and anthrone (9,10-dihydro-9-oxo-anthracene [Sigma-Aldrich] at 0.2% dissolved in H₂SO₄ at 85%) were used to detect carbohydrate-containing lipids.

Generation of Glycolipid-specific Bulk T Cell Lines and Clones. All experiments were performed using RPMI 1640 medium containing 2 mM glutamine, 1 mM Na pyruvate, 1% nonessential amino acids (all from Cambrex), 100 µg/ml kanamycin (GIBCO BRL), and 5% AB human serum (Swiss Red Cross). T cell lines specific for mycobacterial lipids were generated by stimulating PBMCs (10⁶/well) from a PPD⁺ healthy donor that had been in contact with tuberculosis patients for several years and vaccinated with BCG 27 yr before blood was drawn for these experiments. PBMCs were plated in 24-well plates with autologous CD1⁺ APCs (10⁵/well), generated as previously described (14), and pulsed with different mycobacterial fractions (see Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20031097/DC1>). After 5 d of culture, 10 U/ml recombinant human IL-2 was added in each well and cells were split using the same medium according to their proliferation rate. T cell clones were generated as previously described (31).

Analysis of CD1 Restriction. CD1 restriction was investigated by inhibiting T cell activation using the following anti-CD1 mAbs at 10 µg/ml 30 min before the addition of T cells: WM-25 (anti-CD1b; Immunokontakt), OKT6 (anti-CD1a), and L161 (anti-CD1c; Instrumentation Laboratory). W6-32 (anti-MHC class I) and L243 (anti-MHC class II) were used as control antibodies. In some experiments CD1b-transfected THP-1 cells (32) were used as APCs.

Antigen Presentation Assays. CD1⁺ APCs, CD1b-transfected THP-1 cells, HL-60, U-937, or MonoMac6 (ATCC) were preincubated at 5 × 10⁴ cells/well for 2 h at 37°C with 1–10 µg/ml sonicated antigen before the addition of T cells (5 × 10⁴/well in triplicate). After 36 h, released TNF-α and IFN-γ were measured using sandwich ELISA kits (Instrumentation Laboratory). Data are expressed as mean nanogram/milliliter or picogram/milliliter ± SD of triplicates. All experiments were repeated at least two times. Sulfatide was purchased from Fluka.

In some experiments CD1⁺ APCs were fixed as previously described (32) and used as APCs. To investigate the requirements for antigen internalization, 10⁶ CD1⁺ APCs were preincubated for 1 h at 4°C or at 37°C in the presence or absence of 80 µM chloroquine or 20 µM monensin and then pulsed with 10 µg/ml Ac₂SGL for an additional 3 h. After washing, CD1⁺ APCs were fixed and used to stimulate T cells. The sulfatide-specific DS1C9b T cell clone has been described (26).

Desulfatation of Ac₂SGL. 1 mg Ac₂SGL was dissolved in 1 ml of a 50-mM solution of HCl in methanol and kept at room temperature for 16 h. 3 ml chloroform, 0.5 ml methanol, and 1 ml of 0.2% sodium acetate were then added. The lower phase was washed twice with 2 ml chloroform/methanol/water (3:48:47, vol/vol/vol), dried, and tested on silica gel high performance thin layer chromatography plates using chloroform/methanol (9:1) as migration solvent to confirm loss of the sulfate group. The glycolipids were visualized by orcinol staining.

SGL Peracetylation. 150 µg SGL were dissolved in acetic anhydride/anhydrous pyridine (1:1, vol/vol) at 80°C for 2 h. The reaction mixture was dried under stream of nitrogen. The reaction was checked by 1D and 2D proton nuclear magnetic resonance (¹H NMR) and by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-Tof-MS).

MALDI-Tof-MS. Analysis by MALDI-Tof-MS was performed on a Voyager DE-STR (PerSeptive Biosystems) using the

reflectron mode. Ionization was effected by irradiation with pulsed UV light (337 nm) from an N₂ laser. SGL samples were analyzed by the instrument operating at 20 kV in the negative ion mode using an extraction delay time set at 200 ns. Typically, spectra from 100 to 250 laser shots were summed to obtain the final spectrum. All of the samples were prepared for MALDI analysis using the on-probe sample cleanup procedure with cation exchange resin (33). The HABA matrix (2-[4-hydroxy-phenylazo]-benzoic acid; Sigma-Aldrich) was used at a concentration of ~10 mg/ml in ethanol/water (1:1, vol/vol). Typically, 0.5 µl SGL sample (10 µg) in a CHCl₃-CH₃OH solution and 0.5 µl of the matrix solution, containing ~5–10 cation exchange beads, were deposited on the target, mixed with a micropipette, and dried under a gentle stream of warm air. The measurements were externally calibrated at two points with mycobacterial PIM.

MALDI-Tof-MS/MS Analysis. Positive MALDI mass spectra of Ac₂SGL, using α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) as matrix, were recorded using a QSTARPULSAR mass spectrometer equipped with an oMALDI ionization source (Applied Biosystems). Pseudomolecular ions were observed at mass/charge (m/z) 1,323 and 1,295, which were used as precursor ions for MS/MS studies.

Nuclear Magnetic Resonance (NMR) Analysis. NMR spectra were recorded with an Avance DMX500 spectrometer (Bruker GmbH) equipped with an Origin 200 SGI using Xwinmr 2.6. Native Ac₂SGL and peracetylated Ac₂SGL were dissolved in CDCl₃-CD₃OD, (4:1, vol/vol) and CDCl₃, respectively, and analyzed in 200 × 5 mm 535-PP NMR tubes at 295 K. Proton chemical shifts are expressed in parts per million downfield from the signal of the chloroform (δ_H/TMS 7.27 and δ_C/TMS 77.7). All the details concerning correlation spectroscopy and homonuclear Hartmann-Hahn spectroscopy sequences used and experimental procedures were as previously described (30).

Activation of T Cells by Infected CD1⁺ APCs. Monocytes were purified from freshly isolated PBMCs by adherence on plastic for 1 h. Cells were treated with 1,000 U/ml GM-CSF (Novartis) and 1,000 U/ml IL-4 (PBH). After 3 d all cells expressed high levels of CD1a, CD1b, and CD1c, and are referred to as CD1⁺ APCs (13). These cells were infected with *M. tuberculosis* H37Rv (from the collection of the Microbiology Unit in Erlangen) at a multiplicity of infection of 5 as previously described (34). Infected cells were detached (with 1 mM EDTA; Sigma-Aldrich) and plated in a 96-well plate at a density of 2 × 10⁴ cells together with 10⁵ T cells in each well. Supernatants were collected after 48 h, filtered (0.2 µm), and tested for presence of IFN-γ by sandwich ELISA (Endogen) with a sensitivity of 15 pg/ml.

Killing of Intracellular *M. tuberculosis* by T Cells. CD1⁺ APCs were infected with the virulent strain *M. tuberculosis* H37Rv at a multiplicity of infection of 5. The efficiency of infection was 43 ± 6% as determined by auramine rhodamine stain. T cells were added at different effector target ratios as indicated. All conditions were set up as duplicates. After 72 h cells were lysed with 0.3% saponin (Sigma-Aldrich) and sonicated in a water bath to disperse bacterial aggregates. Lysates were then plated in 10-fold dilutions on 7H11-agar plates (Becton Dickinson). After 21 d of culture CFU were enumerated.

Recognition of Ac₂SGL by Lymphocytes from Tuberculosis Patients. PBMCs were purified from the blood of healthy donors and tuberculosis patients after informed consent was given. All suffered from pulmonary tuberculosis (culture positive). 21 patients were male, 10 female, and the age ranged from 30–62 (median 39). Blood was drawn within the first 2 wk after initiation of antituberculosis therapy. 22 patients were PPD⁺ and in 9 patients test-

ing was not performed because the diagnosis of tuberculosis was obvious. Healthy donors with an induration of 5 mm after standard tuberculin skin testing without any clinical signs of disease were scored as PPD⁺ healthy. PBMCs (10⁵/well) were cultured with autologous CD1⁺ APC (2 × 10⁴/well) in the presence or absence of 10 μg/ml Ac₂SGL. After 24 h supernatants were collected and tested for the presence of IFN-γ by sandwich ELISA. PBMCs were kept at 37°C in supplemented RPMI 1640 medium (see above) during the 3 d of autologous monocytes differentiation to DCs.

Online Supplemental Material. The purification scheme of Ac₂SGL is depicted in Fig. S1. The Ac₂SGL-specific CD8⁺ T cell clones do not react against Ac₃SGL and Ac₄SGL, which are present in fractions 6 and 5, respectively. This is illustrated in Fig. S2. Restriction by CD1b was also confirmed by inhibiting the response to CD1b-transfected THP-1 APCs using anti-CD1b mAbs. WM-25 anti-CD1b antibodies were added at 10 μg/ml 30 min before the addition of T cells (Fig. S3). The dose response curve of synthetic SGL analogue is compared with that of the natural Ac₂SGL in Fig. S4.

In Table S1 the mass spectra peaks were assigned to the different acyl forms of Ac_xSGL, which differ by the hydrocarbon chain lengths. In Table S2 the ¹H chemical shifts of the native and peracetylated Ac₂SGL are presented. Figs. S1–S4 and Tables S1 and S2 are available at <http://www.jem.org/cgi/content/full/jem.20031097/DC1>.

Results

T Cell Clones as Tools to Identify Relevant *M. tuberculosis* Lipid Antigens. To assess the immunogenicity of mycobacterial lipids we used the strategy of purifying lipid fractions of *M. tuberculosis* H37Rv strain according to their polarity and charge. Three fractions were used: one enriched in negatively charged compounds, one essentially made of neutral compounds, and a third fraction enriched in phosphatidyl-*myo*-inositol dimannosides (PIM₂). Bulk lines of T cells were established using each of these preparations and showed specific responses against the same lipid fractions used for the initial stimulation (unpublished data). These lines were derived from PBMCs of one healthy donor highly reactive to PPD. From these lines 10 individual T cell clones were established by limiting dilution. All the clones were CD1 restricted, expressed the TCR αβ, and belonged to two distinct subsets: eight were CD4⁺, whereas two were CD8⁺. Because only few mycobacterial antigens stimulating CD8⁺ T cells are known (29), CD8⁺ cells provide unique effector functions in mycobacterial disease (35, 36), and most of CD8⁺ cells reacting to BCG-infected APCs are CD1 restricted (28), we chose to focus on defining the antigen specificity of CD8⁺ clones Z4B27 and Z4A26.

Purification and Chemical Characterization of a Novel *M. tuberculosis* Lipid Antigen. To identify the antigenic lipids, a more sophisticated purification was used. First, a pool of lipids was generated by extraction of mycobacterial H37Rv cells with a mixture of chloroform/methanol (1:1, vol/vol). Hydrophilic compounds were removed from this pool by water/chloroform partition and the lipids were then allowed to precipitate in acetone, a solvent known to precipitate phospholipids. An acetone-soluble phase and an ace-

tone-insoluble phase were obtained, indicated as fractions 1 and 2, respectively (Fig. S1). CD8⁺ αβ T cell clones reacted to the acetone-soluble phase (unpublished data). To gain insights into the molecules present, this latter fraction (fraction 1) was analyzed by MALDI-*Tof*-MS in negative mode.

The mass spectrum revealed peaks assigned to deprotonated molecular ions [M-H]⁻ typical of phosphatidyl-*myo*-inositol (m/z 851) and PIM (Ac₃PIM₂ at m/z 1,413 and Ac₃PIM₆ at m/z 2,061; Fig. 1 A). Furthermore, three families of SGL differing in their acylation degree and fatty acyl appendage structure were also detected. SGL is used to describe the global family of sulfoglycolipids, composed of α-α'-D-trehalose-2'-sulfate core acylated by two to four fatty acids (Fig. 1, inset). In Ac_xSGL, x refers to the total number of acyl groups, whatever the nature of the fatty acids, which could be either palmitic, stearic, hydroxyphthioceranoic, or phthioceranoic acids. SGL were initially described as sulfolipids (37) acylated by three or four fatty acids. In the high mass range, a set of peaks is dominated by the deprotonated molecular ions [M-H]⁻ at m/z 2,429 and 2,457, typifying Ac₄SGL with three hydroxyphthioceranoic acid residues and either one palmitic or one stearic acid residue. In the middle mass range, a set of peaks with the major ion species at m/z 1,867 correspond to Ac₃SGL containing two hydroxyphthioceranoic acids and one stearic or one palmitic acid residue. Finally, in the low mass range, a third set of peaks was observed to be dominated by

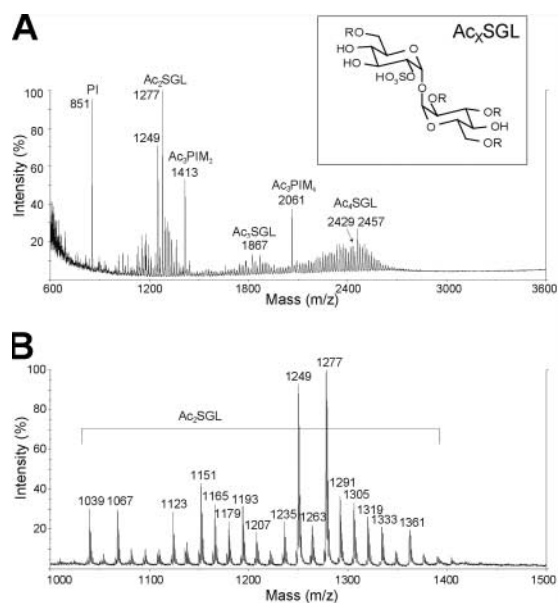


Figure 1. Characterization of the T cell antigen. Negative MALDI-*Tof* mass spectra of (A) the “acetone-soluble” fraction (fraction 1) and (B) fraction 7 obtained after silicic acid column chromatography containing the stimulatory activity. See Table S1 for peak interpretation. Inset: Structural model of the *M. tuberculosis* SGL with R corresponding to hydroxyphthioceranoic, palmitic, or stearic acid. Ac₄SGL contains three hydroxyphthioceranoic acids and one palmitic or stearic acid, Ac₃SGL contains two hydroxyphthioceranoic acids and one palmitic or stearic acid, and Ac₂SGL contains one hydroxyphthioceranoic acid and one palmitic or stearic acid. MS peaks are labeled with mass values calculated using C = 12, H = 1, O = 16, P = 31, and S = 32.

the deprotonated molecular ions at m/z 1,249 and 1,277 typifying Ac_2SGL with one hydroxyphthioceranoic acid and either one palmitic or stearic acid residue, respectively.

The acetone-soluble phase was further fractionated on silicic acid column irrigated by chloroform containing increasing amounts of methanol (Fig. S1). $CD8^+$ T cell clones were stimulated by fraction 7 and eluted with a mixture of chloroform containing 20% of methanol. The different fractions were analyzed by negative MALDI-*Tof*-MS. Mass spectrum of the stimulatory fraction 7 (Fig. 1 B) is complex and dominated by peaks at m/z 1,249 and 1,277 assigned to Ac_2SGL . The acyl appendages were unambiguously assigned to hydroxyphthioceranoic and palmitic or stearic acids by gas chromatography, gas chromatography-mass spectrometry, and electronic impact-mass spectrometry analysis (unpublished data). The acyl appendages of Ac_2SGL (mol wt 1,250) correspond to palmitic acid and hydroxyphthioceranoic acid with $m = 14$ and $n = 7$, whereas Ac_2SGL of mol wt 1,278 contains stearic acid and hydroxyphthioceranoic acid with $m = 14$ and $n = 7$ or palmitic acid residue and hydroxyphthioceranoic acid with $m = 16$ and $n = 7$ (see Table S1 for their assignment, available at <http://www.jem.org/cgi/content/full/jem.20031097/DC1>). Be-

sides these two major Ac_2SGL acyl forms, the mass spectrum shows other peaks distant of 14 mass units, assigned to Ac_2SGL acyl forms differing by the chain length of the hydroxyphthioceranoic acid (Table S1). In summary, all the peaks of the MALDI-*Tof* mass spectrum of fraction 7 were assigned to Ac_2SGL acyl forms. The two fractions eluted with 10% of methanol (fractions 5 and 6) were found by MALDI-*Tof*-MS to contain Ac_3SGL and Ac_4SGL , respectively (unpublished data). These fractions did not stimulate Z4B27 and Z4A26 T cell clones (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20031097/DC1>).

Fraction 7 was further purified by reverse phase chromatography and five new subfractions (7.1–7.5) were obtained. Their purity was controlled by silicic acid TLC (Fig. 2 A) and negative MALDI-*Tof*-MS (Fig. 2 B). Fraction 7.1 is a mixture of several compounds, none of them corresponding to Ac_2SGL . In contrast, TLC shows one spot for fraction 7.3 and 7.4 and two spots for fraction 7.5. MALDI-*Tof* mass spectra of the three fractions highlighted the presence of Ac_2SGL typified by the previously assigned $[M-H]^-$ ions at m/z 1,249 and 1,277. As expected, this reverse phase chromatography allowed the separation of the Ac_2SGL acyl forms according to the chain length of their hydroxyphthiocera-

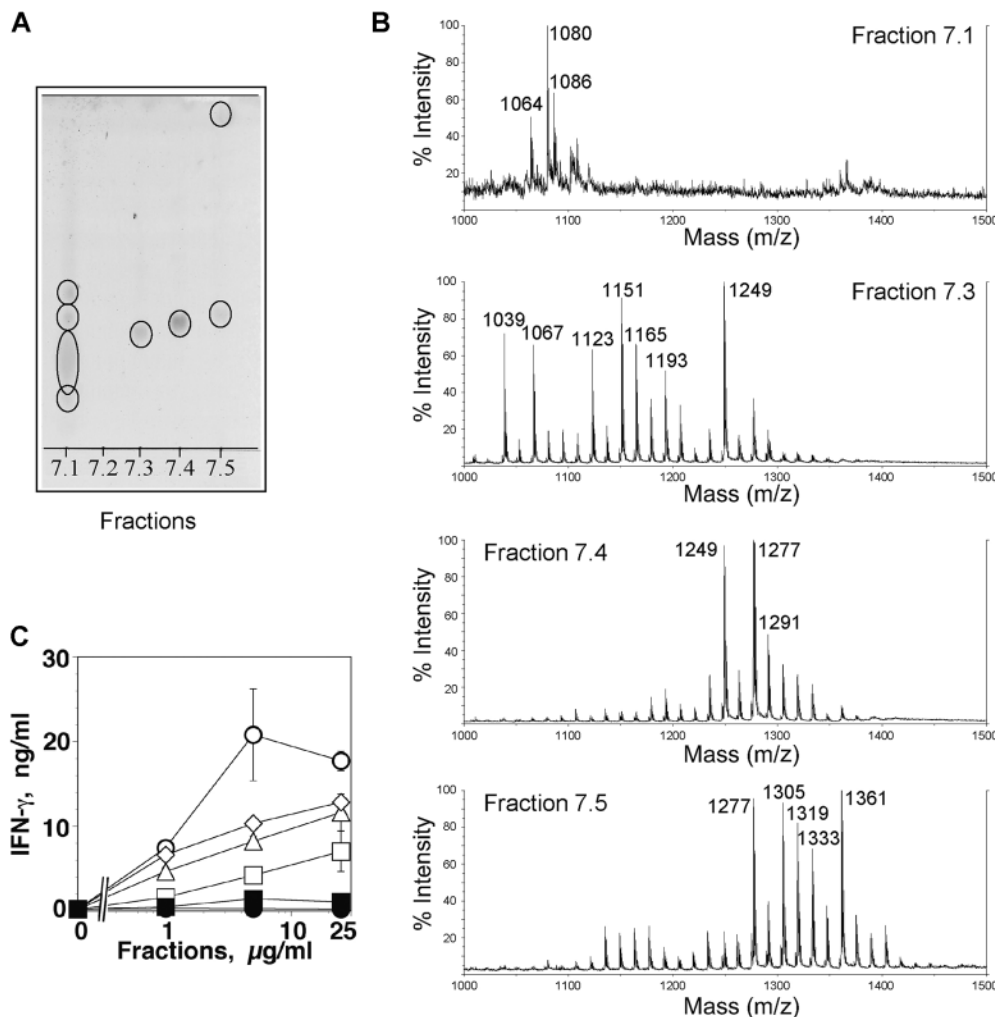


Figure 2. Identification of the active ligand present in fraction 7 as Ac_2SGL . (A) TLC analysis of the components present in fraction 7 after reverse chromatography purification revealed by orcinol. Numbers indicate the subfractions tested in the following panels. (B) Negative MALDI-*Tof* mass spectra of the subfractions 7.1, 7.3, 7.4, and 7.5. See Table S1 for peak interpretation. (C) Stimulation of the Z4B27 T cell clone by (●) subfraction 7.1 from A, (△) subfraction 7.3, (◇) subfraction 7.4, (□) subfraction 7.5, (■) desulfated Ac_2SGL , and (○) control active fraction 7 as measured by IFN- γ ELISA. Bars indicate SD.

noic acid. Indeed, fraction 7.3 is characterized by Ac₂SGL containing hydroxyphthioceranoic acid with shorter chains (m/z 1,039, 1,067, 1,123, 1,151, 1,165, 1,193, and 1,249) than those of Ac₂SGL fractions 7.4 (m/z 1,249, 1,277, and 1,291) and 7.5 (m/z 1,277, 1,305, 1,319, 1,333, and 1,361). In summary, the sub-fractions 7.3, 7.4, and 7.5 are composed of Ac₂SGL acyl forms that differ by the hydroxyphthioceranoic acid chain length (Table S1).

The capacity of the different subfractions to stimulate the CD8⁺ T cell clones was investigated by measuring INF- γ production. As shown in Fig. 2 C, the activity was restricted to the fractions 7.3, 7.4, and 7.5. Taken together, these data demonstrated that Ac₂SGL acyl forms are the immunogenic compounds of the CD8⁺ T cell clones.

To support the antigen structure and the contribution of the sulfate group to immunogenicity, the sulfate group was removed under mild acidic conditions. Desulfatation of Ac₂SGL completely abrogated IFN- γ production (Fig. 2 C), supporting the previous assignment of the antigenic activity to Ac₂SGL, but also revealing that immunogenic activity of Ac₂SGL requires the sulfate residue.

The immunological importance of the sulfate group prompted mass spectrometry and NMR studies aimed to precisely assign the position of sulfate as well as the acyl appendages. We performed a MALDI-MS analysis of Ac₂SGL in positive mode using a QSTARPULSAR mass spectrometer (unpublished data). The mass spectrum was dominated by two peaks at m/z 1,295 and 1,323, which were assigned to cationized molecular ions of the sodiated Ac₂SGL (M-H+2Na)⁺. MS/MS spectra of the precursor ions 1,295 and 1,323 showed loss of HOSO₃⁻ Na⁺ for both compounds, in agreement with the presence of a sulfate group. Furthermore, loss of sulfoanhydroGlc and sulfoGlc was detected from both precursor ions, revealing that the two fatty acyl appendages including hydroxyphthioceranoic and palmitic or stearic acids were located on the Glcp moiety, which did not bear the sulfate group (Glcp-I; unpublished data).

This result was in agreement with 1D and 2D ¹H NMR experiments. In the anomeric zone of the 1D ¹H spectrum (Fig. 3), four signals were observed, namely I₁, II₁, II₂, and II₃. The resonances I₁ and II₁ were assigned to the two anomeric protons of the α - α' -trehalose core. Starting from these protons, the complete spin systems of both Glcp units could be determined from ¹H-¹H correlation spectroscopy and homonuclear Hartmann-Hahn spectroscopy experiments (Table S2, available at <http://www.jem.org/cgi/content/full/jem.20031097/DC1>). These data allowed assignment of the resonances II₂ and II₃ to H2 and H3 of the Glcp-II, respectively. The downfield shifts of the H2 and H3 protons proved that the fatty acyl appendages (hydroxyphthioceranoic acid and palmitic or stearic acids) were located on C2 and C3 of Glcp-II. In addition, from the comparative analysis of the proton chemical shifts (Table S2, Δ) of the native and peracetylated SGL, the sulfate residue was located on the C2 of the Glcp-I. Indeed, variations in chemical shifts of >1 ppm for H3 and H4 indicated that these positions were not substituted in the native molecule. A very weak chemi-

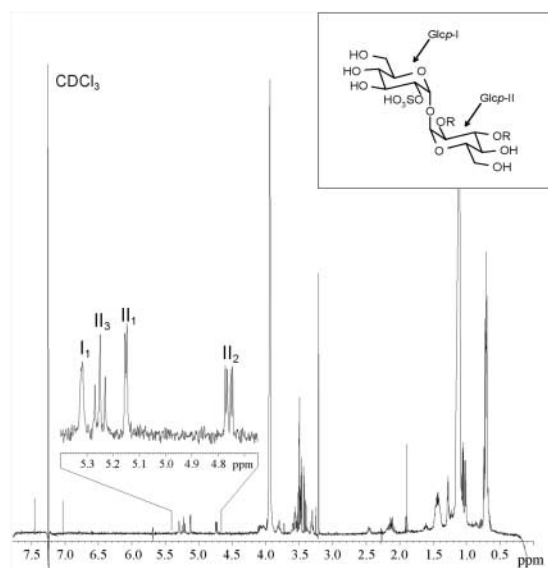


Figure 3. Structure determination of Ac₂SGL by 1D ¹H NMR spectrum analysis (δ ¹H: 0–7.8). The structure of Ac₂SGL is shown in the inset. The two glucose units are labeled I and II, as indicated in the inset. R corresponds to hydroxyphthioceranoic, palmitic, or stearic acid.

cal shift variation was observed for H2 and H3 of Glcp-II (0.27 and 0.45 ppm, respectively) in agreement with the fact that fatty acyl appendages were located on C2 and C3. A high chemical shift variation was observed for H3 and H4 of Glcp-I (1.54 and 1.71 ppm, respectively) and H4 of Glcp-II (2.03 ppm) in agreement with the fact that these positions were not substituted in the native molecule. The sulfate was located on the C2 of Glcp-I as a small variation (0.44 ppm) was observed (Fig. 3, inset). Moreover, the exactness of the proposed structure was definitely proven by comparison of proton chemical shifts of natural Ac₂SGL with a synthetic molecule, i.e., 2,3-dipalmitoyl-2'-sulfate- α - α' -D-trehalose (not depicted; the synthesis of this molecule will be reported elsewhere).

To confirm that Ac₂SGL is the recognized antigen, this synthetic molecule was also used to stimulate T cells. A significant stimulation of Ac₂SGL-specific T cells was observed (Fig. 4 A), which did not occur when sulfatide-specific T cells were used (Fig. 4 B). The synthetic molecule stimulated the specific T cells with a lower efficacy than the natural Ac₂SGL, likely due to the lack of the hydroxyphthioceranoic acyl chain.

In conclusion, 2-palmitoyl or 2-stearoyl-3-hydroxyphthioceranoyl-2'-sulfate- α - α' -D-trehalose (Ac₂SGL) is the antigen recognized by CD8⁺ T cell clones (Fig. 4 C).

Requirements for Presentation of Ac₂SGL to T Cells. To determine the restricting element of Ac₂SGL we used APCs from different sources to stimulate CD8⁺ cells. Autologous CD1⁺ APCs but not PBMCs nor EBV-transformed lymphoblastoid cell lines presented Ac₂SGL to specific T cells (Fig. 5 A). Heterologous CD1⁺ APCs from multiple donors were almost equally efficient in presenting this ligand, suggesting that the response was restricted by a nonpolymor-

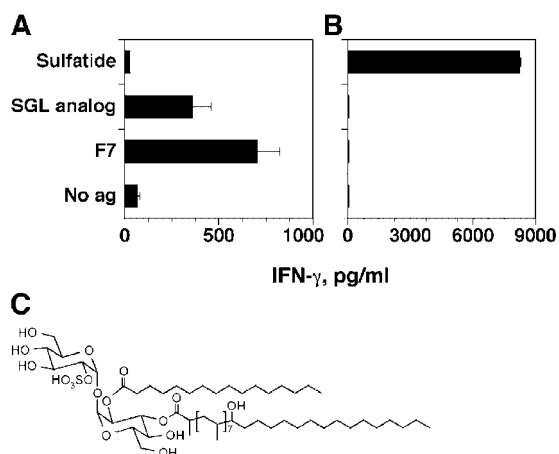


Figure 4. Biological activity of a synthetic analogue and structure of the natural active molecule. (A) Activation of the Ac_2SGL -specific T cell clone Z4B27 using synthetic 2,3-dipalmitoyl- α - α' -D-trehalose-2'-sulfate (SGL analogue). Purified Ac_2SGL and synthetic Ac_2SGL analogue were used at 10 μ g/ml. Release of IFN- γ was tested by ELISA. The results are representative of two experiments. Bars indicate SD. (B) Control with sulfatide-specific DS1C9b T cell clone. Sulfatide was used at 10 μ g/ml. Bars indicate SD. (C) Structure of *M. tuberculosis* Ac_2SGL . The presented structure corresponds to one of the major Ac_2SGL acyl forms (mol wt 1,250).

phic molecule (Fig. 5 B). Furthermore, the promyelocytic HL-60, the histiocytic U-937, and the macrophage MonoMac6 cell lines did not present Ac_2SGL . These findings exclude that presentation of this antigen is a property of all cells belonging to the monocytic lineage and suggest that a nonubiquitous presenting molecule is involved. Because CD1 molecules are nonpolymorphic and nonubiquitous we explored the possibility that Ac_2SGL was presented by these molecules. Ac_2SGL is presented by CD1b as shown by inhibition of T cell activation by anti-CD1 mAbs (Fig. 5 C) and efficient presentation by CD1b-transfected but not mock-transfected THP-1 cells (Fig. 5 D).

An important question is in which cellular compartment is the Ac_2SGL ligand loaded on CD1b? The current consensus is that lipids with long acyl chains are loaded in late endosomal compartments in which low pH and partial unfolding of CD1b molecules facilitate insertion of long fatty acids in the CD1 lipid-binding pockets (20). As Ac_2SGL contains the C32 long hydroxyphthioceranoic acid, we investigated whether intracellular internalization in APCs is necessary for presentation. Pulsing of THP-1 CD1b cells with Ac_2SGL at 4°C or in the presence of monensin, two conditions that prevent internalization of surface-bound molecules, prevented T cell activation (Fig. 6 A), suggesting that internalization is required. This was confirmed by lack of presentation when CD1⁺ APCs were first fixed and then pulsed with the antigen (Fig. 6 C). On the contrary, the same APCs were fully capable of activating specific T cells when they were first pulsed and then fixed. To further test the requirements for internalization, CD1⁺ APCs were pulsed in the presence of chloroquine, an agent that blocks endosomal acidification. In addition, this treatment prevented T cell activation (Fig. 6

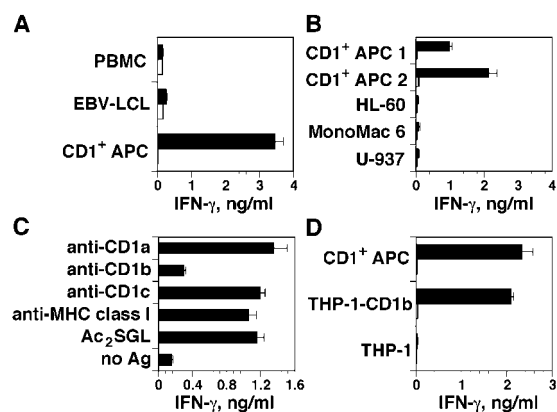


Figure 5. CD1b-restricted presentation of Ac_2SGL . (A) Autologous CD1⁺ APCs and not PBMCs or EBV-transformed lymphoblastoid cell lines present Ac_2SGL . (B) Presentation by CD1⁺ APCs isolated from different individuals and by cell lines of the monocytic lineage. (C) Inhibition of T cell activation by anti-CD1b mAbs. (D) Presentation by CD1b-transfected THP-1 cells. Release of IFN- γ by the Z4B27 T cell clone was measured by ELISA. White bars represent response in the absence of antigen. A second Ac_2SGL -specific and CD1b-restricted T cell clone gave similar results (unpublished data). Bars indicate SD.

A), further suggesting that presentation occurs only when CD1b and Ac_2SGL converge in an endosomal compartment undergoing acidification.

All of these treatments did not abolish presentation of another glycolipid, sulfatide, to specific T cells, thus excluding possible artifacts due to APC treatment (Fig. 6, B and D).

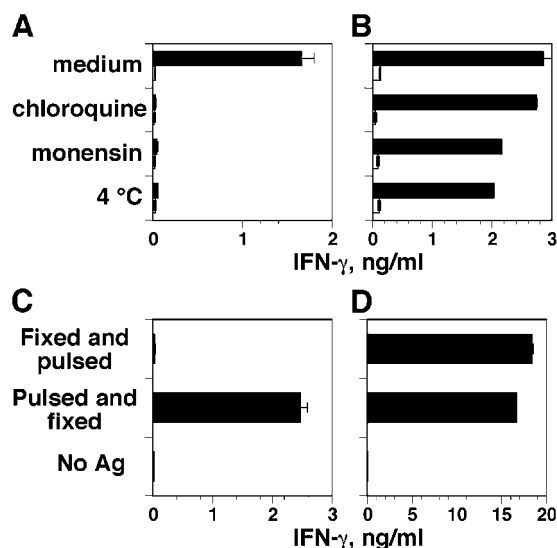


Figure 6. Requirements for presentation of Ac_2SGL . (A) Presentation of Ac_2SGL is inhibited when APCs are pulsed with Ac_2SGL (solid bars) at 4°C, or in the presence of monensin or chloroquine. White bars represent response in the absence of antigen. (C) APCs pulsed and then fixed present Ac_2SGL . (B and D) The same treatments did not affect presentation of sulfatide to the CD1b-restricted and sulfatide-specific DS1C9b T cell clone. (A and C) Release of IFN- γ by the Z4B27 T cell clone measured by ELISA. Similar results were obtained in three additional experiments and also with the Z4A26 T cell clone (unpublished data). Medium indicates response in the absence of treatment. Bars indicate SD.

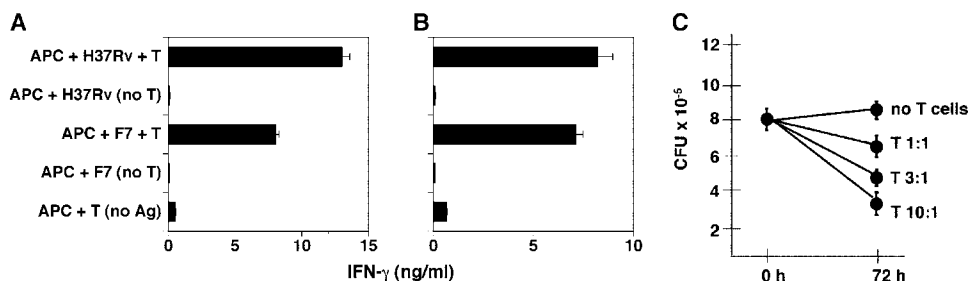


Figure 7. Ac₂SGL-specific T cells recognize and kill *M. tuberculosis* H37Rv-infected cells. CD1⁺ APCs were pulsed with Ac₂SGL or infected with virulent *M. tuberculosis* and used to stimulate the Z4B27 (A) or the Z4A26 (B) T cell clones. Released IFN- γ was detected by ELISA. The results are representative of four independent experiments. Bars indicate SD. (C) Ac₂SGL-specific T cells kill intracellular *M. tuberculosis*. CD1⁺

APCs were infected with virulent *M. tuberculosis*. Z4B27 T cells were added at different ratios as indicated. After 72 h of incubation, living intracellular bacteria were counted as described in Materials and Methods. Average CFU \pm SEM calculated from four dilutions, each performed in duplicate are shown. Similar results were obtained in two additional experiments.

Immunogenicity of Ac₂SGL. Important requirements for a promising vaccine compound are the capacity to prime T cells that recognize *M. tuberculosis*-infected cells and kill the intracellular pathogen. To address these key issues we incubated CD8⁺ T cells with CD1⁺ APCs that had been infected with virulent *M. tuberculosis* H37Rv (Fig. 7, A and B) and measured antigen-specific release of IFN- γ . Only infected but not uninfected CD1⁺ APCs stimulated Ac₂SGL-

specific cytokine release by T cells. Furthermore, IFN- γ was released only when T cells were present, suggesting that this cell population and not DCs is responsible for IFN- γ production. The amount of the cytokine measured was comparable when *M. tuberculosis*-infected or Ac₂SGL-pulsed CD1⁺ APCs were used, demonstrating that the presentation of this antigen during infection is highly efficient.

Because killing of intracellular pathogens is a key function of CD8⁺ T cells, we investigated the antimicrobial activity of Ac₂SGL-specific T cells. *M. tuberculosis*-infected cells were incubated with increasing numbers of Ac₂SGL-specific T cells and the number of surviving bacteria was determined after 72 h. The number of bacteria significantly decreased and correlated with the number of effector cells (Fig. 7 C). These findings confirm the efficient presentation of Ac₂SGL during infection and highlight the bactericidal potential of Ac₂SGL-specific T cells.

Recognition of Ac₂SGL by Lymphocytes from Tuberculosis Patients. To provide direct evidence for the activation of Ac₂SGL-specific T cells during tuberculosis we measured IFN- γ release by freshly isolated lymphocytes derived from human donors. We recruited a large cohort of healthy donors that were scored PPD⁻ ($n = 54$) or PPD⁺ ($n = 51$) according to tuberculin skin testing. Ac₂SGL selectively induced IFN- γ release in PPD⁺ donors with a median of 312 pg/ml (Fig. 8 A). Only a minority of healthy PPD⁻ donors ($n = 4$) responded to Ac₂SGL. Most importantly, Ac₂SGL also stimulated T lymphocytes from patients with active pulmonary tuberculosis ($n = 31$). The levels of IFN- γ secretion were independent of susceptibility to disease, but strictly dependent on previous contact with mycobacteria, suggesting participation of sulfoglycolipid-reactive cells in immune responses during infection with *M. tuberculosis*.

To confirm that these responses were dependent on CD1-restricted T cells, representative experiments were performed in the presence of CD1-blocking antibodies. IFN- γ release was largely dependent on CD1b (70%) and CD1c (35%), but independent of CD1a, MHC class I, and MHC class II (Fig. 8 B).

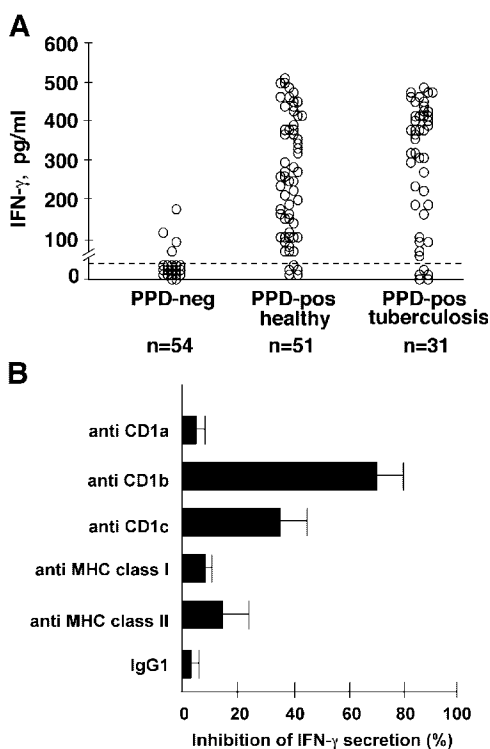


Figure 8. Response of PBMCs to Ac₂SGL ex vivo and inhibition with anti-CD1 and anti-MHC mAbs. (A) PBMCs from healthy donors (PPD⁺ or PPD⁻) and from patients with pulmonary tuberculosis were cultured in the presence of autologous CD1⁺ APCs overnight pulsed with Ac₂SGL. Supernatants were harvested and IFN- γ content was measured by ELISA. Each dot presents an individual donor and the dashed line depicts the detection threshold of 15 pg/ml of this assay. Unstimulated samples served as controls and IFN- γ was regularly below the level of detection. (B) This graph summarizes the results \pm SEM from three PPD⁺ healthy donors using fraction 7 (10 μ g/ml) as antigen. IFN- γ production in the individual experiments was 473, 357, and 349 pg/ml.

Discussion

Published studies indicate that mycobacterial lipid antigens may stimulate CD1-restricted T cells. However, the

pool of studied mycobacterial antigenic lipids is small compared with the diversity of the lipid components of the *M. tuberculosis* envelope. So far, only three classes of molecules were shown to stimulate CD1b-restricted T cells: free mycolic acids (13), mycoloyl glycolipids such as glucose monomycolate (17), and phosphoglycolipids represented by lipoarabinomannan (19), lipomannan (19), and PIM (20). These molecules are commonly present in the envelope of both virulent and nonvirulent mycobacteria. Their immunogenicity during infection with virulent or avirulent strains has not been compared.

In these studies we have characterized the structure of a novel mycobacterial glycolipid, a diacylated sulfoglycolipid (Ac₂SGL), presented by CD1b on the cell surface of *M. tuberculosis*-infected APCs. This antigen stimulates bactericidal CD8⁺ T cells and evokes strong responses in healthy PPD⁺ donors and tuberculosis patients.

Although *M. tuberculosis* sulfur-containing lipids have been known for a long time, the purification of Ac₂SGL and the structural determination and identification of its immunostimulatory capacity are novel. SGL were first described in virulent *M. tuberculosis* (38). Later studies allowed identification and characterization of a family of SGL (acylated trehalose 2'-sulfate) that were detectable only in *M. tuberculosis* and their amounts correlated with the relative virulence in guinea pigs (39). They were all identified as multi-acylated trehalose sulfates differing from each other in the number and type of acyl appendages and in the position on trehalose (40). Very recently, sulfated molecules have been detected in *M. tuberculosis* using Fourier transform-ion cyclotron resonance mass spectrometry (41). Among them, a molecule with a mass ascribed to Ac₂SGL was evidenced from mass spectra analysis. In other investigations, a sulfated compound with a mass of 1,278 was found to accumulate in a mycobacterial mutant (42). In both of these studies Ac₂SGL was not purified and its exact chemical structure was not determined. In our studies Ac₂SGL was purified, its immunological importance was recognized, and its structure was identified as 2-palmitoyl or 2-stearoyl-3-hydroxyphthioceranyl-2'-sulfate- α - α' -D-trehalose using MALDI-ToF-MS, MS/MS, and 2D NMR spectroscopy. The purification strategy developed also allowed the isolation of the previously described tetraacylated (SL-1, SL-I', SL-II) and tri-acylated (SL-III) SGL (Ac₄SGL and Ac₃SGL, respectively; 43). These compounds were unable to stimulate the Ac₂SGL-specific T cell clones, thus implying that immunogenic diacylated SGL are not generated inside the APC as a result of antigen processing.

Ac₂SGL is presented by CD1b to the isolated T cell clones. Likely, CD1b presents Ac₂SGL because of its unique antigen-binding capacities. Analysis of the CD1b crystal structure has revealed three hydrophobic pockets, denoted A', C', and F', tailored for alkyl binding (23). A fourth pocket, which resembles a tunnel and therefore is called "T channel," connects the A' and F' pockets. It has been proposed that this unique structure could allow accommodation of very long fatty acids such as the C₆₀ meromycolate chain (44). As the major Ac₂SGL acyl form is composed of a

C₄₀ hydroxyphthioceranoic fatty acid and a palmitate or stearate, it is tempting to speculate that the linear alkyl chain of 32 carbon atoms is inserted in the A' or F' pockets and protrudes into the T channel. This type of binding would require a partial unfolding of the CD1b molecule, which occurs in late endosomes (20). This hypothesis is indirectly supported by the observation that Ac₂SGL is presented only when it is internalized by APCs into an acidic intracellular compartment and is in agreement with similar findings obtained with other glycolipid antigens (20).

In addition to CD1b, CD1c may also present Ac₂SGL, as suggested by the findings that anti-CD1c mAbs blocked a minor but still relevant percentage (35%) of the response to Ac₂SGL in healthy PPD⁺ donors. CD1c was found to present mycobacterial lipids assigned to polyisoprenoid phosphoglycolipids (14). In this case, the aliphatic chain corresponds to a C₃₂, which is in agreement with the chain length of the major hydrophthioceranoic acid homologous. This result also suggests that CD1c is characterized by at least two lipid-binding pockets, as already inferred by the demonstration that it presents sulfatide to T cells (26).

Finally, CD1a does not appear to participate in the presentation of Ac₂SGL. Most likely the structure of CD1a does not allow binding of the long chain hydroxyphthioceranoic fatty acid (25) and therefore does not form immunogenic complexes with Ac₂SGL.

Ac₂SGL-specific T cells release proinflammatory cytokines such as IFN- γ and TNF- α , which may contribute to the antimicrobial inflammatory response during infection. Furthermore, these T cells are characterized by expression of granulysin (unpublished data) and efficient bactericidal capacities, as previously reported for other CD1- or MHC class I-restricted T cells recognizing microbial products (34, 45). Both properties endow these T cells with potential protective functions in vivo.

Ac₂SGL is also highly immunogenic when presented by APCs infected with *M. tuberculosis*. The response to these SGL as measured ex vivo in the PBMCs of tuberculosis patients and of PPD⁺ donors was much higher than that of PPD⁻ subjects. This applies not only to the average amounts of released IFN- γ , but also to the percentage of responding donors.

In conclusion, the powerful response in infected donors, together with the capacity to prime protective T cells, identifies Ac₂SGL as a novel glycolipid with strong immunogenicity during infection with *M. tuberculosis*. Therefore, this antigen is a new glycolipid candidate to be tested as a potential antimycobacterial subunit vaccine.

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