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Mycolic Acids Constitute a Scaffold for Mycobacterial Lipid Antigens Stimulating CD1-Restricted T Cells

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SUMMARY

CD1-restricted lipid-specific T lymphocytes are primed during infection with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Here we describe the antigenicity of glycerol monomycolate (GroMM), which stimulates CD1b-restricted $CD4^+$ T cell clones. Chemical characterization of this antigen showed that it exists as two stereoisomers, one synthetic isomer being more stimulatory than the other. The hydroxyl groups of glycerol and the mycolic acid length are critical for triggering the T cell responses. GroMM was presented by *M. tuberculosis*-infected dendritic cells, demonstrating that the antigen is available for presentation during natural infection. Ex vivo experiments showed that GroMM stimulated T cells from vaccinated or latently infected healthy donors but not cells from patients with active tuberculosis, suggesting that GroMM-specific T cells are primed during infection and their detection correlates with lack of clinical active disease.

INTRODUCTION

T lymphocytes participate in host defense against microbial pathogens, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis. During *M. tuberculosis* infection, both conventional and unconventional T cells are stimulated. Conventional T cells use an $\alpha\beta$ T cell receptor (TCR) and recognize peptide antigens presented by major histocompatibility complex (MHC) II or MHC I antigen-presenting molecules on antigen-presenting cells (APCs), whereas unconventional T cells include $\gamma\delta$ TCR cells specific for mycobacterial phosphorylated ligands and CD1-restricted $\alpha\beta$ TCR cells recognizing mycobacterial lipid antigens (De Libero and Mori, 2005; Moody et al., 2005; Skold and Behar, 2005). Five CD1 proteins (CD1a, b, c, d, and e) are expressed by human cells. CD1a to CD1d have antigen presenting functions, whereas CD1e facilitates glycolipidic antigen processing (de la Salle et al., 2005).

CD1-restricted T cells are activated in the course of *M. tuberculosis* infections (Gilleron et al., 2004; Moody et al., 2000b; Ulrichs et al., 2003). Although in vivo data in animal models are only preliminary (Dascher et al., 2003; Hiromatsu et al., 2002), CD1-restricted T cells have been indicated to contribute to protection during infection because they kill intracellular pathogens and secrete proinflammatory cytokines, which in turn promote macrophage bactericidal activity (Gilleron et al., 2004). These studies have highlighted lipids of the mycobacterial envelope as key determinants of host immune response. Thus, mycobacterial lipids are now considered to be a novel class of antigens that could be used in subunit vaccine formulations. There is much evidence in support of this: (i) lipid antigens prime specific T cells during infection (Gilleron et al., 2004; Moody et al., 2000b; Ulrichs et al., 2003); (ii) because lipids are products of multistep biosynthetic pathways, in contrast to microbial protein antigens, their selection-induced structural variation is difficult; and (iii) CD1 proteins are less polymorphic than MHC proteins and, therefore, stimulatory lipids are probably immunogenic in the entire population. Two studies have shown that *M. tuberculosis* lipids used to vaccinate guinea pigs improve the pulmonary pathology (Dascher et al., 2003; Hiromatsu et al., 2002). These promising initial vaccination experiments also support the development of novel subunit vaccines priming immunity against mycobacterial lipid antigens (Martin, 2005; Skeiky and Sadoff, 2006).

The envelope of *M. tuberculosis* has an extraordinarily high lipidic content and a large number of structurally diverse lipids (Brennan and Nikaido, 1995). Only a few of these molecules have been shown to stimulate CD1-restricted T cells and the repertoire of mycobacterial lipid antigens is large. Immunogenic lipids are mostly presented by CD1b (Moody et al., 2005; Skold and Behar, 2005) and include molecules with very diverse structures like free mycolic acids (Beckman et al., 1994), glucose-6-O-monomycolate (Moody et al., 1997), diacylated sulfoglycolipids (Gilleron et al., 2004), or mannosyl-phosphatidylinositol-based glycolipids such as lipoarabinomannans (LAM) (Sieling et al., 1995), lipomannans (LM) (Sieling et al., 1995), and PIM (de la Salle et al., 2005; Ernst et al., 1998). CD1a presents the lipopeptide dideoxymycobactin (Moody et al., 2004), whereas CD1c presents mannosyl-phosphomycoketides

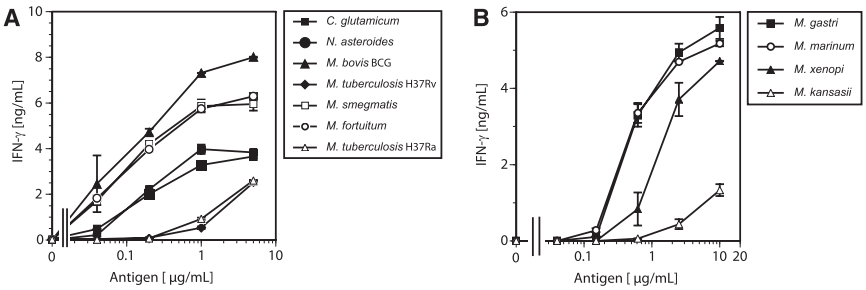


Figure 1. Immunogenicity of Lipidic Fractions from Various Bacterial Species of the CMN Group

(A and B) IFN-γ release was used to quantify T cell activation and expressed in nanograms per milliliter (mean ± SD; n = 3). The data are representative of three independent experiments.

(Moody et al., 2000b). To further characterize the repertoire of mycobacterial lipid antigens, we generated a panel of T cell clones using partially purified lipids (Gilleron et al., 2004). These T cells were used to identify immunogenic lipids. We now describe a new lipid antigen, glycerol monomycolate (GroMM), found among different bacteria of the Corynebacterium, Mycobacterium, and Nocardia (CMN) group. GroMM is presented by CD1b in a CD1e-independent manner, by DCs infected with *M. tuberculosis*. GroMM-specific T cells are detected in the circulating blood of purified protein derivative (PPD)-positive healthy donors but not in that of patients with active tuberculosis, raising the question of their role in the course of this disease.

RESULTS

Characterization of the Z5B71 T Cell Clone

To characterize the repertoire of CD1-restricted *M. tuberculosis* lipid antigens, we generated T cell clones using three crude mycobacterial lipid fractions differing in polarity and charge (Gilleron et al., 2004). Here we describe the immunological characteristics and the lipid antigen stimulating the Z5B71 T cell clone that was isolated after stimulation with a *M. bovis* bacillus Calmette-Guérin (BCG) lipid fraction enriched in phosphatidyl-*myo*-inositol dimannosides (PIM_2) (Gilleron et al., 2008). Z5B71 T cells use an αβ TCR and are CD4 positive. They reacted to lipid extracts from all the mycobacterial species tested, including *M. bovis* BCG, *M. smegmatis*, *M. gastri*, *M. marinum*, *M. fortuitum*, *M. xenopi*, *M. kansasii*, and *M. tuberculosis* H37Ra and H37Rv, and also to lipids isolated from *Corynebacterium glutamicum* and *Nocardia asteroides* (Figures 1A and 1B). Thus, the stimulatory lipid antigen is probably ubiquitous in bacteria of the CMN group. *M. bovis* BCG extracts always appeared to be the most stimulatory, suggesting that the antigen is more abundant in this species. This finding, together with the fact that the diversity of glycolipids is lower in *M. bovis* BCG than in *M. tuberculosis*, prompted us to use an *M. bovis* BCG extract to identify the stimulatory antigen.

When lipids were partitioned by acetone precipitation (see Figure S1A available online), the acetone-insoluble fraction showed the strongest activity (Figure S1B). This fraction was further precipitated with methanol and the methanol-insoluble fraction was the most stimulatory (Figure S1C). This latter fraction was then further fractionated on a silica gel column (Figure S1A). Z5B71 T cells were stimulated with fractions 5 to 9 (Figure 2B), which appeared to contain a homogenous product (Figure 2A) when analyzed by thin-layer chromatography (TLC). Hence, the active fractions were pooled and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and nuclear magnetic resonance (NMR).

Chemical Characterization of the Mycobacterial Lipid Antigen

The positive mode MALDI mass spectrum showed a complex pattern of peaks corresponding to cationized sodiated molecular ions $[M+Na]^+$ (Figure 3A). The main peaks were separated by 28 mass units, suggesting differences of two methylenic units between each molecular species, possibly as a result of heterogeneity in fatty acyl chain length. To confirm this hypothesis, the product was hydrolyzed under alkaline conditions and the fatty acids were analyzed by GC (data not shown) and MALDI-TOF MS (Figure 3B). The positive mode MALDI mass spectrum highlighted a series of peaks typical of mycolic acids (Laval et al., 2001). According to the presence of various chemical functions on the meromycolic chain, mycolates are subdivided into α-mycolates, methoxymycolates, and ketomycolates (Daffe and Draper, 1998). *M. tuberculosis* contains a ratio of α-mycolates/oxygenated mycolates (i.e., methoxymycolates and ketomycolates) of 1/1, whereas *M. bovis* BCG contains predominantly ketomycolates (Watanabe et al., 2001). In agreement with these data, peaks at *m/z* 1232, 1260, 1274, 1288, and 1302 (Figure 3B) were assigned to $[M+Na]^+$ forms of ketomycolates (Figure 3C). The mass difference (Δ74 amu) with respect to the native product suggested that the latter consisted of one mycolic acid esterifying a glycerol residue. Consistent with this notion, MALDI-MS analysis of the peracetylated molecule showed a displacement of 126 mass units (data not shown), indicating the presence of three alcohol functions.

The identification of this novel antigen as GroMM was further supported by 1D ^{1}H NMR analysis (Figures 4E and 4D). The resonances typical of mycolic acids were assigned according to published studies (Quemard et al., 1997; Schroeder and Barry, 2001; Watanabe et al., 2001). Protons *d* (δ 2.31) and *e* (δ 3.53, multiplet), which correlated together on the 2D ^{1}H-^{1}H correlated spectroscopy (COSY) spectrum (data not shown), were assigned to the protons of the carbons carrying the α chain and the β-hydroxyl group, respectively (Figure 4A). The protons *j* (δ 2.41, multiplet) and *k* (δ 0.92, doublet), which were also correlated on the COSY spectrum, were assigned to the -$CHCH_3$- group in the distal portion of the meromycolic chain. The signal *l* (δ 0.75, triplet) was assigned to the protons of the terminal methyl groups of the α- and meromycolic chains. The multiplets *h* at δ −0.46, δ 0.44, and δ 0.52, which were correlated on the COSY spectrum, represented the characteristic signals of the cyclopropanic function protons. Signals around 5 ppm (*g* at δ 5.16 and *f* at δ 4.83) corresponded to olefinic protons,

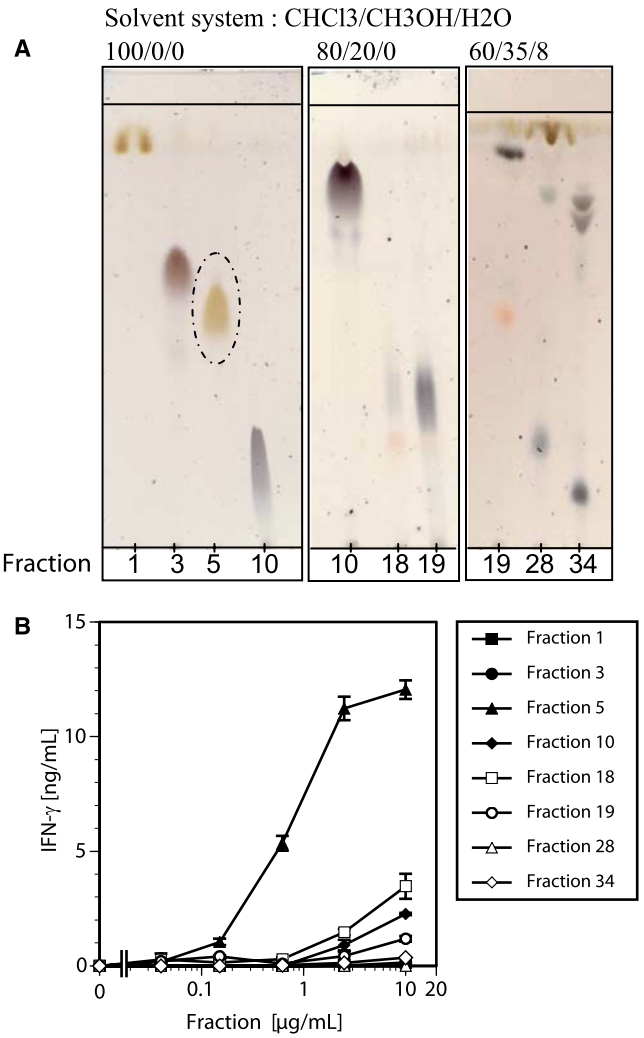
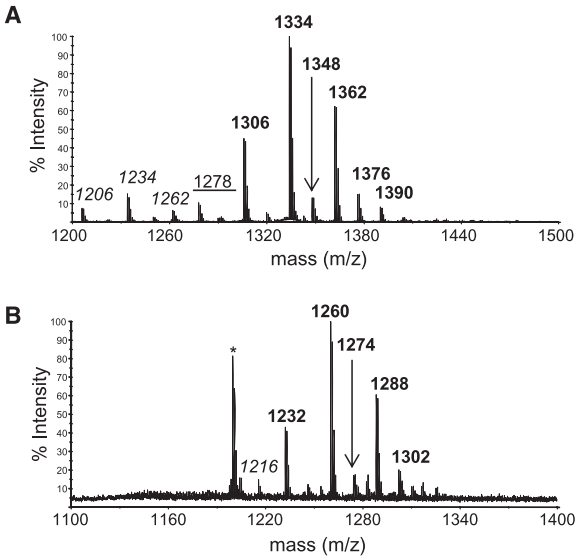


Figure 2. Fractionation of *M. bovis* BCG "Methanol-Insoluble" Lipid Pool and Immunogenicity of the Isolated Fractions

(A) TLC analysis of eight representative fractions (1, 3, 5, 10, 18, 19, 28, and 34) obtained by silica gel chromatography of "methanol-insoluble" lipids. The encircled spot corresponds to the active compound.

(B) The same fractions were tested for stimulation of Z5B71 T cells. IFN-γ release was used to quantify T cell activation and expressed in nanograms per milliliter (mean ± SD; n = 3). The data are representative of three independent experiments.



C

Mycolic acids		[GroMM, Na^+]$^+$
Type	[RCOOH, Na^+]$^+$	
α-	1132	*1206*
	1160	*1234*
	1188	*1262*
	1216	1290
Methoxy-	1204	1278
	1246	1320
	1248	1322
	1276	1350
	1304	1378
	1332	1406
Keto-	**1232**	**1306**
	1260	**1334**
	1274	**1348**
	1288	**1362**
	1302	**1376**
	1316	**1390**

Figure 3. Positive Mode MALDI-TOF MS Analysis of *M. bovis* BCG GroMM (A) and of the Mycolic Acids Obtained after Saponification of *M. bovis* BCG GroMM (B)

(A and B) The asterisk corresponds to a contaminant.

(C) Table showing the calculated masses of the sodium adduct ions of the expected mycolic acids and of the corresponding GroMM.

indicating that a small proportion of the mycoloyl chains contained ethylenic groups. Protons *a* (δ 4.11, 4.06 and 3.99), *b* (δ 3.75), and *c* (δ 3.47) (Figure 4D), which correlated on the 2D ^{1}H-^{1}H HOHAHA spectrum (data not shown), were assigned to glycerol protons (Figure 4A). However, the protons *a* were divided into two independent spin systems, *a1* (δ 4.11 and 3.99) and *a2* (δ 4.06) (Figure 4D), whose definitive assignment was achieved by NMR analysis of synthetic GroMM analogs.

A Hemisynthetic GroMM Stimulates Specific T Cells

In order to confirm the antigenicity of GroMM, analogs were synthesized (Figure S2) using mycolates isolated from *M. tuberculosis* H37Rv (Figure S3B). The chemical synthesis was initiated with (R)- or (S)-isopropylidene-glycerol, activated by p-toluenesulfonyl (Sowden and Fischer, 1942) (Figure S2). The esterification of glycerol by mycolic acids was realized by heating (R)- or (S)-isopropylidene-glycerol in the presence of potassium mycolates (Defaye and Lederer, 1956). The isopropylidene-mycoloylglycerol isomers were then deprotected under acidic conditions to give synthetic s(R)- or s(S)-GroMM. The MALDI mass spectra of both sGroMM isomers (Figure S3A) were in agreement with the expected mass distribution of GroMM-bearing *M. tuberculosis* H37Rv mycolic acids (Figure S3B). These synthetic molecules were then analyzed by 1D ^{1}H NMR (Figure 4B and C). The s(S)-GroMM showed *a* protons (designed *a1*) with chemical shifts at 4.00 and 4.12 ppm (Figure 4C), whereas the s(R)-GroMM showed *a* protons (designed *a2*) resonating at 4.05 ppm (Figure 4B). We therefore concluded that natural GroMM, which displayed *a1* and *a2* resonances of similar intensity (Figure 4D), is

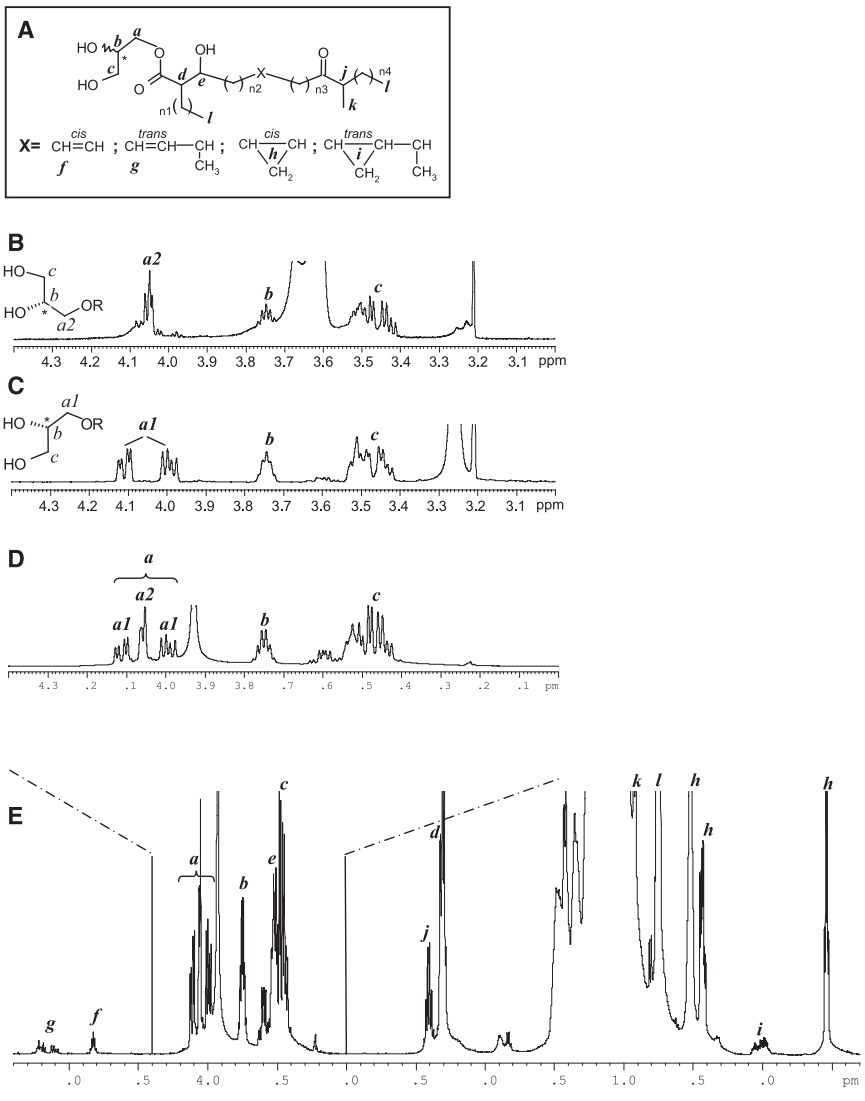


Figure 4. ^{1}H NMR Analysis of GroMM

The structure of *M. bovis* BCG GroMM is in presented in (A). Specific GroMM protons signals are annotated *a* to *k*, and are assigned on the structure of the major forms of *M. bovis* BCG GroMM, esterified by ketomycolic acids. n1 = 19, 21, or 23; n2 and n4 = 15, 17, or 19; n3 = 12 to 17.

(B and C) ^{1}H NMR analysis of synthetic (R)-1-O-mycoloyl-glycerol (B) and (S)-1-O-mycoloyl-glycerol (C). R corresponds to mycolic acid.

(D and E) ^{1}H NMR analysis of *M. bovis* BCG GroMM.

composed of the two stereoisomers in equal proportions. Using s(R)- or s(S)-GroMM to stimulate Z5B71 T cells, we found that the R isomer was more active than the S one, whereas the natural compound had an intermediate activity (Figure 5A). These data confirmed the nature and structure of the natural antigen and showed that one stereoisomer was preferentially recognized by the TCR of the specific T cell clone.

Structural Requirements of GroMM for T Cell Stimulation

Free mycolates have been reported to stimulate CD1b-restricted T cells, indicating that this structure on its own might form stimulatory complexes with CD1b (Beckman et al., 1994; Moody et al., 1997). However, when mycolates and corynomycolates were tested in our assay, they did not activate Z5B71 T cells (data not shown), thus revealing the importance of the glyceryl moiety in TCR recognition. To evaluate the importance of the alcohol functions, we acetylated the hydroxyl groups of glycerol. Substitution of an acetyl moiety at position *c* of Gro (Figure 4A) did not abolish T cell reactivity, whereas substitution of two acetyl moieties (at positions *b* and *c*) abolished it (data not shown), suggesting that the hydroxyl group in position *b* is involved in T cell recognition of this lipid. GroMM was also submitted to periodic acid cleavage and reduction to generate glycol-monomycolate. This compound displayed a drastically reduced ability to stimulate specific T cells (data not shown), demonstrating that the position *c*, absent in this molecule, is also important for T cell recognition.

We next investigated whether other natural mycolate derivatives stimulate Z5B71 T cells. Glucose-6-O-monomycolate (GlcMM), one of the first described CD1b-presented antigens (Moody et al., 1997), but neither trehalose-6-O-monomycolate (TMM) nor trehalose-6,6′-O-dimycolate (TDM), showed activity comparable to that of GroMM (Figure 5B).

The importance of the number and the structure, in particular the length, of the acyl chain(s) on the glycerol unit was first explored by testing the immunogenicity of different commercial glycerides. Mono-, di-, and triglycerides bearing palmitic acids did not stimulate Z5B71 T cells (data not shown). Hemisynthetic GroMMs were then prepared using corynomycolates (i.e., C_{32} mycolic acid derivatives) isolated from *C. glutamicum*. The R isomer stimulated T cells very inefficiently, even at high doses, whereas the S isomer was nonstimulatory (Figure 5A). However, GlcMM isolated from *C. glutamicum* (i.e., with corynomycolates) showed about the same activity as *M. phlei* GlcMM (i.e., with a C_{80} mycolic acid) (Figure 5C). Collectively, these results show that both the fatty acid chain length and the hydrophilic head influence the stimulation of Z5B71 T cells.

GroMM Presentation Is Restricted by CD1b and Is Not CD1e Dependent

When CD1-transfected APCs were used to investigate the restricting molecule, only those expressing CD1b stimulated Z5B71 T cells (Figure 6A) and the response was inhibited by anti-CD1b but not by other anti-CD1 monoclonal antibodies (mAbs) (Figure 6B). Because CD1e can facilitate the presentation of complex mycobacterial lipids (de la Salle et al., 2005), we also investigated whether the antigenicity of GroMM is influenced by

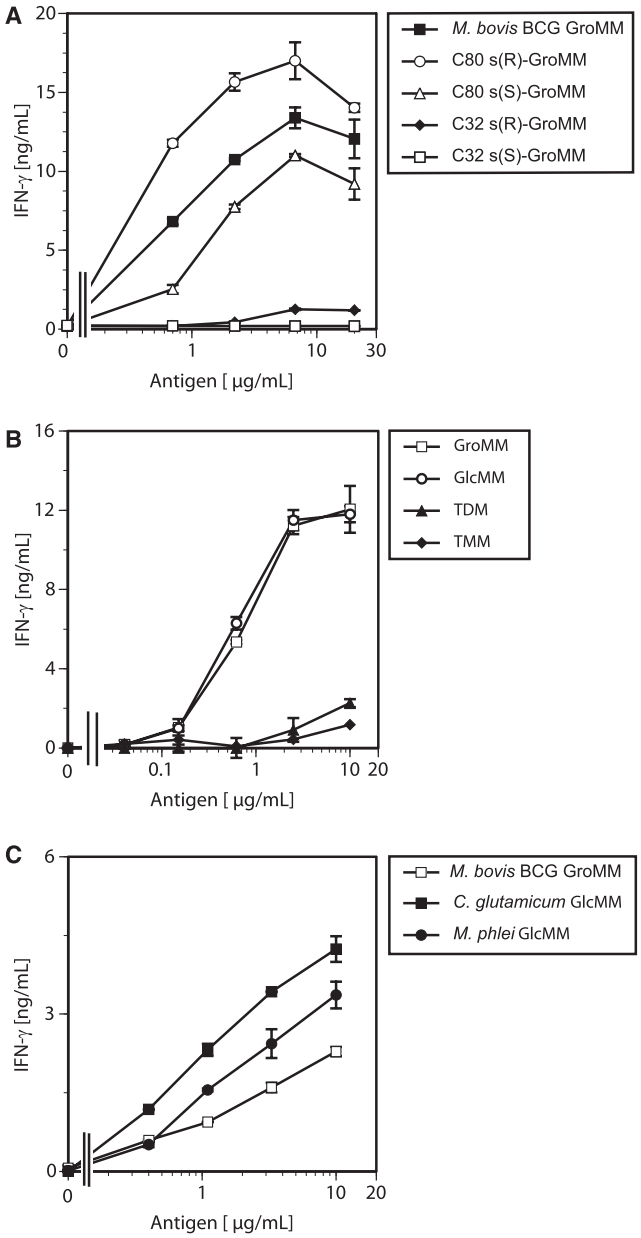


Figure 5. Biological Activities of Hemisynthetic GroMM, *M. bovis* BCG Mycolate Derivatives, and *C. glutamicum* and *M. phlei* GlcMM

(A–C) In C32 or C80 s(S)- or s(R)- GroMM, C32 corresponds to corynomycolic acids and C80 to mycolic acids whereas (S) corresponds to (S)-1-O-mycoloyl-glycerol and (R) to (R)-1-O-mycoloyl-glycerol. IFN-γ release was used to quantify activation of the T cell clone Z5B71 and expressed in nanograms per milliliter (mean ± SD; n = 3). The data are representative of three independent experiments.

CD1e. Similar T cell responses were observed using APCs expressing only CD1b or both CD1b and CD1e (Figure 6C), showing that CD1e is not required for GroMM presentation.

M. tuberculosis-Infected Dendritic Cells Stimulate Z5B71 T Cells

An important issue is whether mycobacterial lipid antigens also form immunogenic complexes with CD1 molecules during infection. This was explored using DCs pulsed with heat-killed *M. tuberculosis* or infected with live bacilli. In both cases a strong response of GroMM-specific T cells was detected (Figures 6D and 6E), confirming that this lipid is immunogenic when inserted into the bacterial cell envelope.

GroMM-Specific T Cells Are Not Detected in Patients with Active Tuberculosis

To assess whether GroMM-specific T cells participate in the immune response to *M. tuberculosis*, we recruited patients with active tuberculosis (n = 11), latently infected (n = 19), BCG vaccinated (n = 22), and PPD-negative healthy individuals (n = 10). As a correlate of T cell activation, we determined interferon (IFN)-γ release 6 days after stimulation with GroMM. Compared with naive donors, where GroMM-stimulated IFN-γ release did not exceed medium background (data not shown), significant responses were only observed in vaccinated or latently infected individuals. Similar to naive donors, no GroMM reactivity was observed in peripheral blood mononuclear cells (PBMCs) of tuberculosis patients (Figure 6F). This lack of reactivity was specific for GroMM, because patients responded readily to PPD (data not shown). To further corroborate the specificity of GroMM induced IFN-γ release, we tested the ex vivo reactivity to a limited set of unrelated mycobacterial lipids: out of five donors, only three responded to GroMM; one responded to phosphatidyl-*myo*inositol-di-mannoside (PIM_2), and only two responded to di-acyl-trehalose (DAT) (Figure S4). These differential responses are in agreement with the notion that they represent a recall of antigen specific T cell memory. GroMM-induced T cell activation was CD1 restricted, because a cocktail of blocking antibodies against CD1a, b, and c, (Figure 6G) but not MHC class I antibodies (data not shown), inhibited IFN-γ release. To define the specific CD1 isotypes restricting ex vivo T cell responses, we blocked T cell activation using CD1a, CD1b, or CD1c antibodies. In three donors, IFN-γ release was inhibited by CD1a and CD1b or CD1a alone. Neutralizing antibodies to CD1c or IgG1 isotype controls had no effect (Figure S5). In conclusion, these results demonstrate that GroMM triggers CD1 restricted T cells in mycobacteria-primed individuals, but this T cell subset is absent or highly reduced in tuberculosis patients.

DISCUSSION

The mycobacterial envelope contains a large number of diverse lipid molecules whose immunogenic potential is not completely understood. In the present study, we characterized a novel lipid antigen presented by CD1b, GroMM, which belongs to the mycoloyl-based class of lipids. GroMM is inserted in the outer layer of the outer membrane and is present in most of the mycobacterial species. The existence of GroMM in *M. bovis* BCG (Tsumita, 1956) and in some *M. tuberculosis* strains was reported in the 1950s (Bloch et al., 1957; Noll, 1957; Noll and Jackim, 1958). However, the esterification position of the mycoloyl residue on the glycerol primary OH group was only established 30 years later (Batrakov, 1985).

Using hemisynthetic molecules, we confirmed the immunogenicity of GroMM and investigated the importance of different structural elements of GroMM. With *M. tuberculosis* mycolic

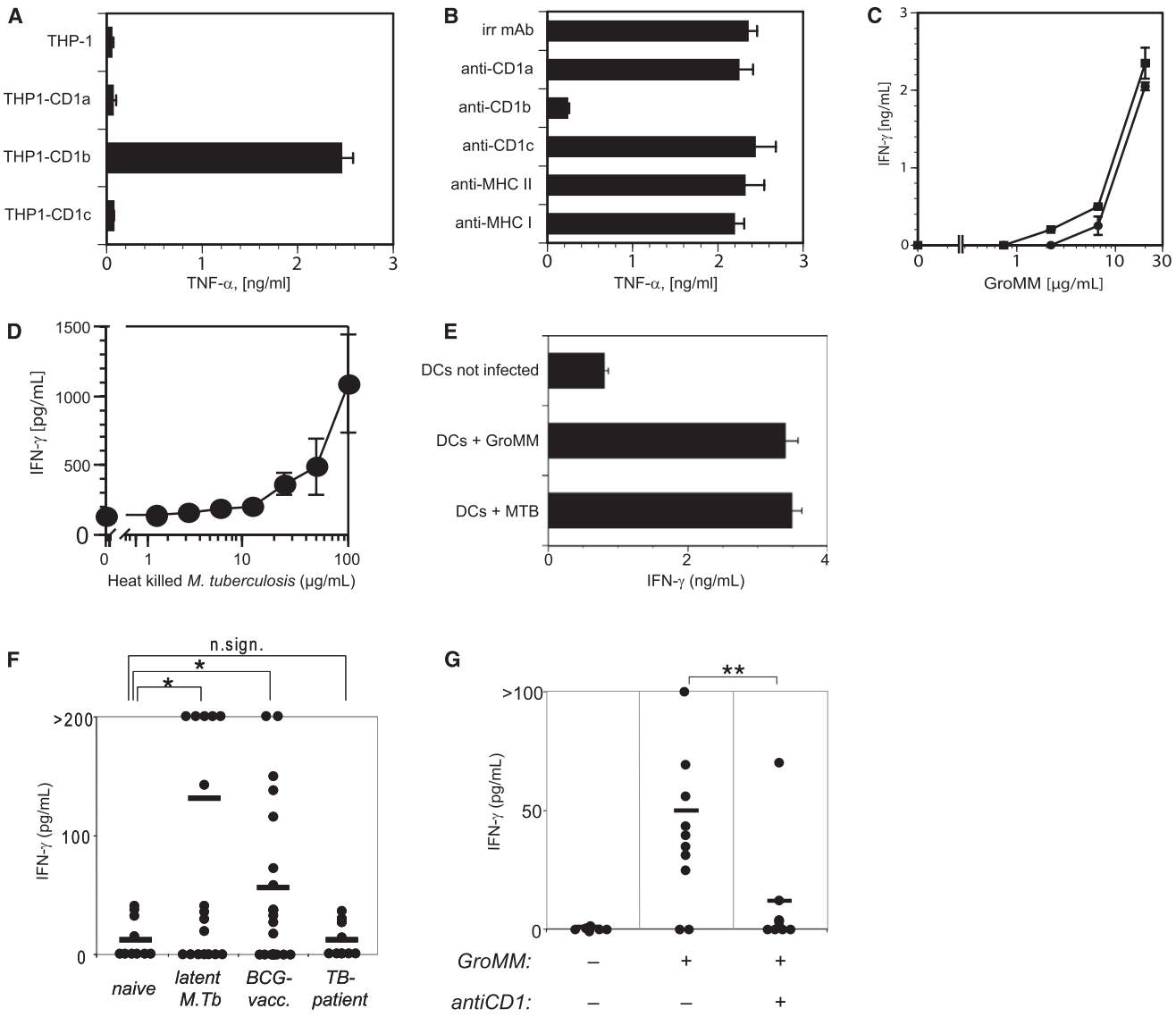


Figure 6. Functional In Vitro and Ex Vivo Activities of the GroMM-Specific T Cells

CD1b-restricted and CD1e-independent presentation of GroMM using THP-1 transfected cells (A) and mAbs-mediated inhibition of GroMM-induced T cell activation (B) using a GroMM-containing fraction. In (C), THP-1-CD1b (■) and THP-1-CD1b-CD1e (●) transfected cells were used. Activation of Z5B71 T cells by DCs pulsed with heat-killed *M. tuberculosis* (D) or infected with living *M. tuberculosis* (E). Release of TNF-α (A and B) or IFN-γ (C–G) was used to quantify activation of Z5B71 T cells and expressed in picograms or nanograms per milliliter (mean ± SD; n = 3). The data are representative of three independent experiments. Ex vivo reactivity to GroMM: PBMCs from naive (n = 10), BCG-vaccinated (n = 22), or latently infected healthy donors (n = 19) and tuberculosis patients (n = 11) were stimulated with GroMM in the presence of autologous DCs. After 6 days culture, supernatant was collected and IFN-γ concentration determined by ELISA (F). Medium controls (not shown) were similar for the different donor cohorts and on average below 3 pg/ml. In ten PPD-positive, healthy donors, the restriction was determined by GroMM stimulation in the presence or absence of a cocktail of CD1 type 1 blocking antibodies (G). Black circles show individual donors, horizontal bars represent the average for each group. One asterisk indicates a confidence interval of >95%; two asterisks indicate a confidence interval of >99%; and a confidence interval of <95% was considered to be nonsignificant.

acids, two diastereoisomers were synthesized, the (R)-1-*O*-mycoloyl-glycerol [s(R)-GroMM] diastereoisomer being more stimulatory than the (S)-1-*O*-mycoloyl-glycerol [s(S)-GroMM] one. Shortening the mycolic acid chain almost abrogated T cell response, as indicated by the poor activity of the s(R)-GroMM-containing corynomycolates and the total absence of activity of the s(S)-GroMM. This finding showing that the mycolic acid length influences the T cell response is in accordance with studies performed with GlcMM (Moody et al., 2002). These seminal investigations showed that GlcMM with long (C_{80}) acyl chains required internalization into late endosomes for efficient presentation, whereas GlcMM with short (C_{32}) acyl chains was rapidly loaded onto CD1b also on the cell surface. In addition, C_{80} GlcMM was more active than the C_{32} GlcMM, probably due to formation of stable and/or more immunogenic CD1b complexes when long fatty acid chains are involved. Whether

GroMM with long mycolic acid is more stimulatory because it forms more immunogenic and/or more stable complexes with CD1b remains to be investigated.

The finding that both GlcMM and GroMM activate Z5B71 T cells indicates that they share a common epitope. Chemical degradation showed that it corresponds to the minimal stimulatory structure, thus raising the question of how cross-reactivity between these two lipids occurs. The crystal structure of CD1b-GlcMM complexes (Batuwangala et al., 2004) shows that arginine 79 (R79) on the CD1b α1 helix forms a hydrogen bond with the oxygen of the alcohol function on the C4 of the glucosyl unit (Figure 7A), possibly stabilizing a specific orientation of the sugar. Superposition of the GroMM and GlcMM structures shows that the hydroxyl in position *c* of GroMM might correspond to GlcMM OH-4, whereas the hydroxyl in position *b*, possibly positioned like the oxygen atom of the GlcMM glucose ring, might be available for cognate interaction with the TCR (Figures 7B and 7C). We also found that the mycolic acid length was critical when Z5B71 T cells react to GroMM, but not to GlcMM. The current paradigm is that the length of fatty acyl chain affects the polar head positioning and its interaction with the TCR (Batuwangala et al., 2004; McCarthy et al., 2007; Moody et al., 2000a, 2002). It is tempting to speculate that the exact positioning of the polar antigen head dictated by the lipid tail is more critical for GroMM than for GlcMM because the number of possible interactions with the TCR is more limited with glycerol than with glucose.

Collectively, these data suggest that mycolic acids may constitute a scaffold for lipid antigens, which enables their binding to CD1b and controls the interaction of different polar heads with TCR. This resembles the scaffold nature of the ceramide and diacylglycerol moieties of other lipid antigens (Kinjo et al., 2006; Tsuji, 2006) and thus reveals a more general rule, whereby scaffold molecules that are well suited to CD1 binding confer immunogenicity on a large number of lipid molecules.

The observation that GroMM, GlcMM, and free mycolic acid are presented by CD1b molecules is probably facilitated by the unique presence of intricate and interconnected hydrophobic pockets in CD1b. Crystallographic data reveal that the meromycolate chain of GlcMM fills the A′, T′, and F′ pockets of CD1b, protrudes out of the F′ pocket, possibly interacting with the TCR, whereas the α chain occupies the C′ groove (Batuwangala et al., 2004). Another reason is that CD1b traffics through acidic compartments where lipid antigens are loaded with pH-dependent mechanisms, in some cases after processing (de la Salle et al., 2005). Although we cannot formally exclude that the GroMM meromycolate chain is partially degraded before binding to CD1b, it is more probable that it is stimulatory in its native form. This is supported by the finding that GroMM analogs with short fatty acid chains are poorly antigenic and that GroMM is presented in the absence of CD1e, a CD1 molecule that facilitates the processing of complex glycolipid antigens (de la Salle et al., 2005).

GroMM-specific T cells were detected in the peripheral blood of BCG-immunized or latently infected donors. Activation of these T cells was abrogated by CD1a and CD1b blocking antibodies, suggesting that in vivo GroMM can be presented by these CD1 molecules. This raises the question how GroMM fits into CD1a pockets. It might be possible towing to the unique

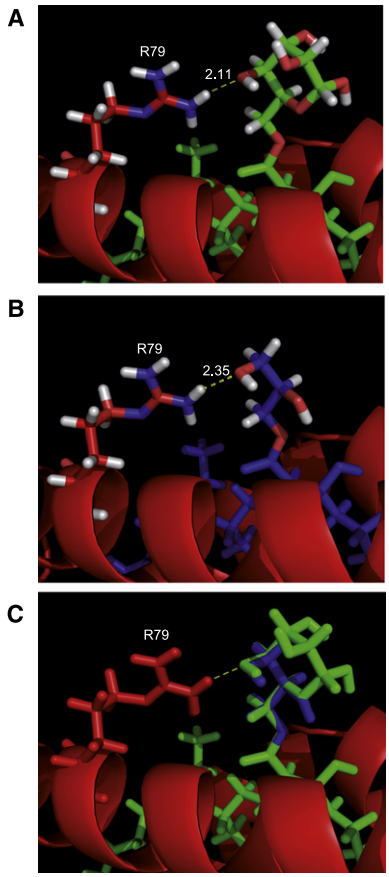


Figure 7. Structural Models Explaining the Cross-Reactivity between GlcMM and GroMM

The glycosyl and glyceryl units were both in close contact with R79.

(A) Model of CD1b-GlcMM complex.

(B) Model of CD1b-GroMM complex.

(C) Superimposition of the two structures, with the glyceryl unit well positioned on part of the glucosyl ring.

open conformation of the CD1a F′ pocket, in which mycolic acid might be partially accommodated, with the remaining part lying outside, as reported for the sulfatide tail in the CD1a crystal (Zajonc et al., 2003).

An unexpected finding was that patients with active tuberculosis did not respond to GroMM, though they react to Ac_2SGL (Gilleron et al., 2004). Several possible reasons might explain why tuberculosis patients failed to respond to GroMM. One is that in the patients tested, a general and antigen-nonspecific immune suppression could have prevented an in vitro response, a phenomenon that has been described for the initial phase of acute disease (Hirsch et al., 1996). However, this explanation is unlikely because in contrast to GroMM, T cell responses to mycobacterial protein antigens were readily detectable in the peripheral blood of the same individuals. Alternatively, GroMM-specific T cells might be sequestered in inflamed tissues, as suggested for Ag85-specific $CD8^+$ T cells (Caccamo et al., 2006). A third explanation is that GroMM-specific T cells might participate in protection against disease and limit the growth of the bacilli. The absence of these T cells could then directly contribute to the appearance of clinically active disease. The findings that

GroMM-specific T cells recognize mycobacteria-infected cells and release proinflammatory cytokines are in accordance with this hypothesis. Future studies will aim at investigating the potential protective value of GroMM in subunit vaccine formulations against tuberculosis.

SIGNIFICANCE

The role of CD1-restricted lipid-specific T lymphocytes during *Mycobacterium tuberculosis* infection is an important issue, because these cells might participate in protection. *M. tuberculosis* envelope has an extraordinarily high lipid content with wide molecular diversity. Although mycobacterial lipids with different structures stimulate CD1-restricted T cells, their relative contribution to specific immunity during infection is unknown.

Here we describe a novel mycobacterial lipid antigen, glycerol monomycolate (GroMM), which is presented by CD1b and stimulates CD4+ T cells. The following findings describe the rules of GroMM immunogenicity and its contribution to anti-*M. tuberculosis* repertoire in infected patients.

GroMM was found as two stereoisomers, (S)- and (R)-1-O-mycoloyl-glycerol, present in similar proportions in bacilli. Using hemisynthetic analogs, the R synthetic isomer was more stimulatory than the S isomer. Both the fatty acid and the hydrophilic head influence stimulation of GroMM-specific T cells, as long fatty acid and free hydroxyls of glycerol are essential. GroMM-specific T cells also recognize glucose-monomycolate (GlcMM), but not TMM or TDM. A model is proposed to explain this cross-reactivity.

GroMM is presented by *M. tuberculosis*-infected dendritic cells, thereby confirming its immunogenicity during infection. Ex vivo studies using peripheral blood of infected patients showed that GroMM efficiently stimulates T cells from PPD-positive healthy donors and not cells from patients with active tuberculosis, or from noninfected donors, suggesting that GroMM-specific T cells are primed during infection and are differently represented in donors with latent and active tuberculosis.

Thus, the overall data suggest that mycolic acids constitute a scaffold for lipid antigens, which supports their binding to CD1b and facilitates the interaction of different polar heads with the TCR.

EXPERIMENTAL PROCEDURES

Reagents

(R)-isopropylidene-glycerol and (S)-isopropylidene-glycerol, glyceryl tripalmitate (tripalmitin), 1,2 dipalmitoyl-*sn*-glycerol [(S)-glycerol 1,2-dipalmitate] and monopalmitoyl glycerol (1-O-palmityl-*rac*-glycerol) were purchased from Sigma (Saint Quentin Fallavier, France).

Bacterial Strain and Culture Conditions

Nocardia asteroides and *Corynebacterium glutamicum* were grown in suspension for 2 to 3 days at 32°C on Luria Broth and Brain Heart Infusion medium, respectively. Mycobacteria were grown for 4 to 8 weeks at 37°C on Sauton's medium as surface biofilm. Cells were harvested, separated from the culture media, and left in chloroform/methanol (2:1, v/v) at room temperature to kill bacteria.

Cell Culture

Human promyelocytic THP-1 cells were transfected with cDNA of human CD1A, or CD1B, or CD1C genes, or double-transfected with CD1B and CD1E using the BCMGS-Neo and -Hygro vectors by electroporation. Cells were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 2 mM UltraGlutamine II, MEM nonessential amino acids, 1 mM Na-pyruvate, and 100 μg/ml kanamycin (all from Invitrogen, Basel, Switzerland). DCs were isolated from PBMCs of healthy donors by culturing in the presence of recombinant IL-4 and GM-CSF, as described (Porcelli et al., 1992). T cell clones were generated as described previously (De Libero, 1997), and grown in RPMI-1640 supplemented with 5% AB human serum (Swiss Red Cross), 10 mM HEPES, 2 mM UltraGlutamine II, MEM nonessential amino acids, 1 mM Na-pyruvate, 100 μg/ml kanamycin, and 100 U/ml human recombinant IL-2.

T Cell Activation Assays

DCs (3×10^4/well) or CD1-transfected THP-1 cells (3×10^4/well) were incubated for 2 hr at 37°C with different concentrations of sonicated antigens before addition of T cells (10^5/well in triplicate). Supernatants were harvested after 36 hr of incubation, and cytokine release was measured by using enzyme-linked immunosorbent assay (ELISA) kits (IFN-γ from Instrumentation Laboratory, Schlieren, Switzerland; TNF-α from BD PharMingen, Basel, Switzerland). Data are expressed as mean pg/ml or ng/ml ± standard deviation (SD) of triplicates.

Analysis of CD1 Restriction

CD1 restriction was investigated by evaluating the inhibition of T cell response in the presence of the following mAb at 10 μg/ml added 30 min before T cells: OKT6 (anti-CD1a, American Type Culture Collection ATCC CRL-8019), WM-25 (anti-CD1b; Immunokontakt, Lugano, Switzerland), L161 (anti-CD1c; Instrumentation Laboratory), W6-32 (anti-MHC class I, ATCC) and L243 (anti-MHC class II, ATCC). Anti-TCRVγ9 (B3) mAb was used as irrelevant mAb.

Pulsing and Infection of DCs with *M.* tuberculosis

M. tuberculosis was killed at 80°C for 20 min and incubated in RPMI-1640 medium containing 10% FCS for 2 hr at 37°C with DCs (3×10^4/well) before addition of T cells (10^5/well in triplicate). In other experiments, DCs were infected as previously described (De Libero et al., 2005) and IFN-γ release was monitored by ELISA.

Lipidic Fractions Used for Generation of T Cell Clones

The TCRαβ CD4+ T cell clone Z5B71 was generated as described (Gilleron et al., 2004) by stimulating PBMCs of a PPD+ healthy donor with a *M. bovis* BCG PIM_2-enriched fraction (Gilleron et al., 2001). Briefly, *M. bovis* BCG cells were suspended in chloroform/methanol (1:1, v/v) and filtered four times. The chloroform/methanol extract was concentrated and constituted the lipidic faction, which was further partitioned between water and chloroform. The chloroform phase was evaporated and suspended in a minimum volume of chloroform. The addition of acetone overnight at 4°C led to formation of a precipitate, which was centrifuged (3000 *g* at 4°C for 15 min) to generate an "acetone-soluble" phase and an "acetone-insoluble" phase. The "acetone-insoluble" phase was applied to a QMA-Spherosil M (BioSepra SA, Villeneuve-la-Garonne, France) column eluted successively with chloroform, chloroform/methanol (1:1, v/v), methanol to elute neutral compounds and then with chloroform/methanol (1/2, v/v) containing 0.1 M ammonium acetate to elute negatively charged compounds. This last fraction corresponds to the "PIM_2-enriched fraction."

Z5B71 T cells are CD4+ and express only the TCRαV25S1J52 and TCRβV9S1J2S5 TCR chains, confirming their clonality.

Lipidic Fractions from Several Actinomycetes

Different lipidic fractions were prepared (as described above) from several actinomycetes species and tested with Z5B71 T cell clone. Lipidic fractions from the following bacteria were tested: *M. smegmatis*, *M. tuberculosis* H37Ra, *M. fortuitum*, *M. xenopi*, *N. asteroides*, *C. glutamicum*, the "acetone-soluble" phase from *M. smegmatis*, *M. tuberculosis* H37Rv, *M. gastri*, *M. kansasii*, *M. marinum*, and *M. bovis* BCG. The "acetone-insoluble" lipids from

M. tuberculosis H37Rv, *M. gastri*, *M. kansasii*, *M. marinum*, and *M. bovis* BCG were also tested.

Purification of the GroMM from *M. bovis* BCG

The acetone-insoluble fraction from *M. bovis* BCG, obtained as described above, was subjected to methanol precipitation giving "methanol soluble" and "methanol insoluble" fractions. The methanol-insoluble fraction (150 mg) was fractionated on a silica acid column (1.3 × 23 cm) eluted with 7 × 175 ml chloroform (fractions 1–7), 8x200 ml of chloroform - methanol (95:5, v/v) (fractions 8-15), 9x225 ml of chloroform - methanol (9:1, v/v) (fractions 16-24), 7 × 175 ml chloroform-methanol (85:15, v/v) (fractions 25–31), and 7 × 175 ml chloroform-methanol (7:3, v/v) (fractions 32–38). Fractions 5 to 8 were found to contain GroMM and were pooled.

Purifications were checked by TLC on aluminum-backed silica gel plates (Alugram Sil G; Macherey-Nagel) using as migration solvent chloroform/methanol (95:5, v/v). Anthrone (9,10-dihydro-9-oxo-anthracene; Sigma) at 0.2% dissolved in H_2SO_4 at 85% was used to detect glycolipids.

GroMM Acetylation

Peracetylation was performed on 200 μg GroMM, which was dissolved in acetic anhydride/anhydrous pyridine (1:1, v/v) and heated at 80°C for 30 min. The reaction mixture was dried under a stream of nitrogen and dissolved in chloroform for MALDI-TOF MS analysis. For selective acetylation of GroMM hydroxyl functions, 16 μl acetic acid was added to 10 mg GroMM dissolved in 150 μl pyridine and kept for 3 hr at 0°C. Mono- and diacetylated GroMM were extracted by $CHCl_3$ and purified on silica column eluted successively by $CHCl_3$ and $CHCl_3/CH_3OH$ 95/5 (v/v). The number of acetyl substituents and their location on GroMM were determined by MALDI-TOF MS and NMR analysis.

GroMM Saponification

A total of 800 μl methoxyethanol/KOH 20% (7:1, v/v) was added to 500 μg GroMM. The mixture was then heated at 100°C for 3 h. After cooling and acidification by aqueous sulfuric acid at pH 3.0, mycolic acids were extracted three times by diethyl ether (v/v). Ether phases were pooled, washed five times with water, and dried under a stream of nitrogen. Mycolic acids were redissolved in 100 μl chloroform for MALDI-TOF MS analysis.

GroMM Synthesis

Reactions were performed under argon in anhydrous solvents.

(S)-1-O-(4-Methylbenzene sulfonyl)-2,3-O,O-isopropylidene-glycerol

p-Toluene sulfonyl chloride (0.3 g, 1.57 mmol) was added at 0°C to (R)-isopropylidene-glycerol (230 mg, 1.74 mmol) dissolved in anhydrous pyridine (0.8 ml). After 24 h at room temperature, diethyl ether was added to the reaction mixture and the organic phase was washed sequentially with cold, 1 M hydrochloric acid, water, saturated sodium bicarbonate solution, and water. The ether extract was dried on magnesium sulfate and concentrated. Chromatography on silica gel (elution with petroleum ether/ethyl acetate 4/1, v/v) gave the product (around 100 mg, 0.35 mmol). In the same manner, (R)-1-*O*-(4 methylbenzene sulfonyl)-2,3-*O,O*-isopropylidene-glycerol was obtained from (S)-isopropylidene-glycerol.

Potassium Mycolates

Mycolic acids (gift of Dr. M.A. Lanéelle, IPBS, Toulouse) were released from *M. tuberculosis* H37Rv cells as described previously (Laval et al., 2001). Potassium mycolates were obtained by two successive washings of mycolic acids dissolved in chloroform with 10 mM hydrochloric acid and 5 M potassium hydroxide. After extraction with chloroform, potassium mycolates were dried on magnesium sulfate and concentrated.

(S)-1-O-Mycoloyl-2,3-O,O-isopropylidene-glycerol

Potassium mycolates (8 mg, ~6 μmol) were added to (S)-1-*O*-(4-methylbenzenesulfonyl)-2,3-*O,O*-isopropylidene-glycerol (2 mg, 7 μmol) in anhydrous dimethylformamide (0.150 ml) and stirred for 3 days at 135°C. The reaction mixture was acidified by 1 M hydrochloric acid and extracted by diethyl ether. Ether extract was then washed with water, dried on magnesium sulfate, and concentrated. Chromatography on silica gel (elution with petroleum ether/diethyl ether 5/1, v/v) gave the product (around 4 mg, 2.9 μmol). In the same way, (R)-1-*O*-mycoloyl-2,3-*O,O*-isopropylidene-glycerol was obtained from (R)-1-*O*-(4 methylbenzene sulfonyl)-2,3-*O,O*-isopropylidene-glycerol.

(S)-1-O-Mycoloyl-glycerol (GroMM)

A total of 100 μl concentrated hydrochloric acid (d = 1.017) was added to (S)-1-*O*-mycoloyl-2,3-*O,O*-isopropylidene-glycerol (4 mg, 2.9 μmol) dissolved in diethyl ether (0.1 ml). After 10 min of stirring at room temperature, water was added to the reaction mixture and the product (3 mg, 2.3 μmol) was extracted with diethyl ether. The ether extract was dried on magnesium sulfate and concentrated. Supplemental purification was not necessary. (R)-1-*O*-mycoloyl-glycerol was also obtained using (R)-1-*O*-mycoloyl-2,3-*O,O*-isopropylidene-glycerol.

MALDI-TOF MS

MALDI-TOF MS analysis was performed on a 4700 Proteomics Analyzer (with Tof-Tof Optics, Applied Biosystems) using the reflectron mode. Ionization was effected by irradiation with pulsed ultraviolet irradiation (UV) light (355 nm) from an Nd:YAG laser. GroMM samples were analyzed by the instrument operating at 20 kV in the positive ion mode using an extraction delay time set at 20 ns. Typically, spectra from 100 to 250 laser shots were summed to obtain the final spectrum. The HABA (2-[4-hydroxy-phenylazo]-benzoic acid) matrix (Sigma-Aldrich) was used at a concentration of ~10 mg/ml in ethanol/water (1:1, v/v). Then, 0.5 μl sample solution and 0.5 μl matrix solution 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.

NMR Analysis

NMR spectra were recorded with an Avance DMX500 spectrometer (Bruker) equipped with an Origin 200 SGI using Xwinnmx 2.6. GroMM was dissolved in $CDCl_3$-CD_3OD, (9:1, v/v) and analyzed in 200 × 5 mm 535-PP NMR tubes at 298 K. Proton chemical shifts are expressed in parts per million downfield from the signal of the chloroform (δ_H/TMS 7.27). All details concerning correlation spectroscopy and homonuclear Hartmann-Hahn spectroscopy sequences used and experimental procedures were as previously described (Gilleron et al., 2003).

Recognition of GroMM by Lymphocytes from Tuberculosis Patients and Healthy Donors

PBMCs were purified from blood of healthy donors and tuberculosis patients after informed consent was given. All patients included in this study had culture-proven pulmonary tuberculosis and were undergoing treatment with antituberculosis drugs for 2 to 8 weeks at the time of blood donation. PBMCs (10^5/well) were cultured with autologous DCs (2 × 10^4/well) in the presence or absence of 10 μg/ml natural GroMM. After 6 days 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 2 days of autologous monocytes differentiation to DCs. In parallel, PBMCs were stimulated with 10 μg/ml PPD (Chiron) and recombinant ESAT6 (Lionex). Healthy donors that responded by releasing at least 250 pg/ml IFN-γ in response to PPD were scored as BCG immunized. Donors that responded to both antigens were considered as latently *M. tuberculosis* infected. Those that did not respond to PPD were scored as PPD negative.

CD1 restriction was tested using a cocktail of CD1a-, CD1b-, and CD1c-blocking antibodies (10H3, BCD1b3.1 and F10 at 10 μg/ml each, resulting in a final concentration of 30 μg/ml) added 30 min before GroMM to prevent CD1 presentation. For individual experiments, the three antibodies were tested separately at a final concentration of 20 μg/ml. An isotype matched antibody served as control.

Molecular Modeling

CD1b in complex with GroMM was obtained from GlcMM by replacing the glucose moiety in the glycolipid head of the molecule by glycerol. The starting point for this work was the crystal structure of the CD1b-GlcMM complex (Batuwangala et al., 2004). The Builder and Biopolymer modules of InsightII (BIOSYM/MSI, San Diego) were used to replace the glucose moiety by glycerol. The bonds and partial charges were corrected and adjusted to the CVFF force field. In order to position the new fragment optimally with respect to the neighboring residues, we minimized the energy of the molecule with the Discover module, following the standard simulated annealing protocol. During the procedure, only the glycerol atoms were allowed to move, along with the

side chains of Phe75 and Arg79, the closest spatial neighbors. Molecular dynamics was simulated during 20 ps with the time step of 1 fs, with temperature initially raised to 1000 K and gradually lowered to 300 K. The minimization was performed with the Conjugate Gradient algorithm, and was terminated upon reaching the threshold value for the root-mean-square gradients of 0.001 kcal/mol. To be able to compare the results directly with the original complex, we subjected the CD1b-GlcMM complex to the identical procedure.

SUPPLEMENTAL DATA

The Supplemental Data include five figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00455-9.

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