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*J Immunol* 2009; 182:424-432; doi: 10.4049/jimmunol.182.1.424

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A Simple Mycobacterial Monomycolated Glycerol Lipid Has Potent Immunostimulatory Activity

Claire S. Andersen,2* Else Marie Agger,2,3* Ida Rosenkrands,‡ Jessica M. Gomes,† Veemal Bhowruth,‡ Kevin J. C. Gibson,‡ Rune V. Petersen,* David E. Minnikin,† Gurdyal S. Besra,‡ and Peter Andersen* 

It is a long held belief that the strong immunostimulatory activity of the Mycobacterium bovis bacillus Calmette-Guérin vaccine and Freund’s complete adjuvant is due to specific mycobacterial cell envelope components, such as lipids and polysaccharides. Implicated mycobacterial lipids include, among others, the so-called cord factor or trehalose dimycolate, but limited information is available regarding the precise molecular nature of the stimulatory components responsible for the interaction with human APCs. In this regard, the majority of research aimed at identifying and characterizing individual immunostimulatory mycobacterial lipids has been performed in the murine system using bone marrow-derived dendritic cells. In this study, it is documented that potent immunostimulatory activity lies within the bacillus Calmette-Guérin nonpolar lipid class. This activity can be narrowed down to a remarkably simple monomycolyl glycerol (MMG) with the ability to stimulate human dendritic cells as assessed by enhanced expression of activation markers and the release of proinflammatory cytokines. A synthetic analog of MMG based on 32 carbons (C32) was found to exhibit comparable levels of immunostimulatory activities. Immunization of mice with the tuberculosis vaccine candidate, Ag85B-ESAT-6, in MMG or the synthetic analog using cationic liposomes as the delivery vehicle was found to give rise to a prominent Th1 response characterized by significant levels of IFN-γ. Together, this development opens up the possibility of producing a novel class of chemically defined lipid adjuvants to enhance the activity of new vaccine formulations, directed against infectious agents including tuberculosis. 

Dendritic cells (DCs)4 are professional APCs that play an essential role in directing the immune response upon infection with pathogens, such as Mycobacterium tuberculosis. The production of proinflammatory cytokines, e.g., IL-12, by activated DC represents a vital step in controlling M. tuberculosis infection because it is these cytokines that are of paramount importance in driving the production of IFN-γ by Th1 cells, which promotes the activation of macrophages (1). Among the strategies that have evolved in mycobacteria to enhance survival within the host are those that block IFN-γ-initiated activation of macrophages. This action inhibits phagosome-lysosome fusion and encourages resistance to killing by oxygenated metabolites thereby promoting their survival within macrophages that thus provide a safe haven in which the mycobacteria can replicate (2). The balance between the ability of the host to mount an effective immune response against M. tuberculosis and the ability of the bacteria to circumvent and develop mechanisms to counteract this response and persist in the host is therefore fundamental in determining the outcome of infection (3). Therefore, the interaction between M. tuberculosis and DC represents a crucial interface in the host-pathogen interaction.

An increasing number of studies have highlighted the vital role that mycobacterial lipids play in this process, acting as both immunostimulatory and immunosuppressive factors. In this regard, TDM (trehalose 6,6’-dimycolate or cord factor) was found half a century ago to be a highly immunostimulatory molecule and today constitutes the classical example of a mycobacterial lipid with various biological activities. The list of mycobacterial cell envelope components mediating immunopotentiating effects also includes waxes, lipoolarabinomannan, and phosphatidylglycerol; the latter being capable of activating murine DC when delivered as liposomes (4). Recently, immunosuppressive lipids expressed in highly virulent strains of M. tuberculosis, and a polyketide synthase-derived phenolic glycolipid (PGL) associated with the hyperlthality in murine models of a subset of isolates from the W-Beijing family (5), have served to indicate that the differential repertoire of lipids associated with different strains contributes to the variation in host immune response and long term disease progression observed upon infection (5–7). However, the large majority of all these studies have been performed using bone marrow-derived murine DC or various animal models, typically mice or guinea pigs. Hence, there is a lack of data pertaining to individual lipids with the ability to stimulate human cells and the involvement of human DC.

Published on 23 April 2009. Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00.

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In this study, we report the identification of the simple monomycyl glycerol (MMG) lipid, isolated from M. bovis bacillus Calmette-Guérin (BCG), with potent immunostimulatory activity characterized by the ability to activate human DC. This activity was mimicked by a synthetic analog of MMG with shorter fatty acids. Using cationic liposomes as the delivery vehicle, MMG and this synthetic analog were found to induce a very prominent TH1-biased response in mice to the co-delivered tuberculosis vaccine Ag, Ag85B-ESAT-6. The simple nature of MMG and the activity associated with its analog raise the possibility of the development of a novel class of synthetic lipids suitable for inclusion as adjuvants in vaccines for use in humans.

Materials and Methods

Extraction of lipids

The M. bovis BCG Danish 1331 was cultured in modified Sauton medium. The mycobacteria were harvested after 2–3 wk, suspended in PBS, and killed by incubating for 1.5 h at 60°C. Apolar and polar lipids were extracted according to standard protocols (8, 9). Lipid fractions were analyzed by two-dimensional TLC on aluminum-backed silica gel plates (5554; Merck). Lipids were detected by spraying with 5% ethanolic molybdophosphoric acid followed by charring with a heat gun. Glycolipids were detected by gentle charring after spraying with α-naphthol/sulfuric acid. Samples (10 μl) of rehydrated lipid extracts (1 mg/ml) were analyzed by SDS-PAGE and silver staining for residual protein content.

Purification of individual apolar lipids and mycolates

Phthiocerol dimycocerosate (PDFM) and triacylglycerol (TAG) were isolated using TLCs run in petroleum ether/acetone (98:2); only the major PDIM component, based on phthiocerol A, was recovered. Glycosylophosphatidyl inositols (PGI) and MMG were isolated together by TLCs run in toluene/acetone (95:5). PGI and MMG were separated on one-dimensional TLC in chloroform: methanol:ammonia (8:3:0.5). The PDIM (18 mg), TAG (12 mg), PGI (11 mg), and MMG (10 mg) were subjected to 500 MHz 1H and 13C nuclear magnetic resonance (NMR, d500; Bruker) and MALDI-TOF Mass Spectrometry (Biflex IV; Bruker).

Synthesis of C32 MMG

Synthetic C32 corynomycolic acid (3-hydroxy-2-tetradecyl-octadecanoic acid 2,3-dihydroxypropyl ester, 100 mg, 0.20 mmol, 1 Eq) and 4-pyridinoliponipidine (100 mg, 3 Eq) was placed in a 50 ml of round-bottom flask and a solution of 0.5 μl of 2,2-dimethyl-4-hydroxyxymethyl-1,3-dioxol (isosropic acid) in dichloromethane (500 μl) was added, along with 4 Å molecular sieves. The mixture was taken to complete dryness under high vacuum at room temperature and DCC N,N-dicyclohexylcarbodiimide (15 ml, 0.1 M in DCM, 5 Eq) was added and the reaction was left to stir at room temperature overnight. The molecular sieves were removed by filtration, the reaction mixture reduced to dryness in vacuo and the residue was purified using flash column chromatography (60:40 Silica Gel 60; Fluka), eluting with hexane to hexane:ethyl acetate (8:2) in 5% increments to give the pure isopropylidene-protected compound (3-hydroxy-2-tetradecyl-octadecanoic acid-2,2-dimethyl-(1,3)-dioxol-4-ylmethyl ester) in 56% yield (68 mg). 1H NMR (CDCl3, 300 MHz) δ 0.90 (t, 6H, CH3), 1.20 (s, 5H, CH2), 1.40 (s, 3H, CH2), 1.45 (s, 3H, CH2), 2.50 (m, 1H, CH), 4.05–4.40 (m, 5H, CH2, CH3); 13C NMR (CDCl3, 75 MHz) δ 15.0 (CH3), 22.1, 28.8, 28.9, 29.0, 31.4 (CH2), 39.1 (CH3), 52.1 (CH2(CH2)xCH3), 63.2 (CH2OCO), 69.3 (CH2O), 73.4 (CH(CH3)xCH2), 74.3 (C = O); m/z (EI) 633.55 (M+Na+)(100%); HRMS calcd for C38H74O5Na (M+Na+) 593.5536 found 593.5527.

3-Hydroxy-2-tetradecyl-octadecanoic acid-2,2-dimethyl-(1,3)-dioxol-4-ylmethyl ester (68 mg, 1 Eq) was dissolved in 6 ml of a trifluoroacetic acid: tetrahydrofuran:water (8:17:3, by volume) solution and stirred at room temperature overnight. The solution was neutralized with saturated aqueous sodium carbonate and the mixture extracted twice with chloroform. The organic extract was washed with water and brine, dried and reduced in vacuo to yield the crude product as a white solid, which was purified by flash column chromatography on a 10-g silica gel Varian Bond Elut 122506206 cartridge, eluting with hexane to hexane:ethyl acetate (7:3) in 5% increments, to give the title compound as a white solid in 49% yield (32 mg). Melting point 72–74°C. 1H NMR (CDCl3, 300 MHz) δ 0.90 (t, 6H, CH3), 1.25 (s, 5H, CH2), 2.50 (m, 1H, CH), 3.45–3.85 (m, 3H, CH2, CH3), 4.25 (m, 2H, CH2); 13C NMR (CDCl3, 75 MHz) δ 15.0 (CH3), 26.3, 30.9, 31.3, 33.5 (CH2), 47.5 (CH2(CH2)xCH3), 68.4 (CH2), 69.5 (CH2(CH2)xCH2), 72.5 (CH2O), 76.4 (CH2), 175.4 (C=O); m/z (EI) 593.50 (M+Na+)(100%); HRMS calcd for C36H68O5Na (M+Na+) 573.5122 found 573.5143.

DC assays

Human PBMC were obtained from the blood bank and screened for their reactivity toward purified protein derivative (PPD) as an indication of previous exposure to mycobacteria. Only PPD-negative donors were included in this study. PBMC-derived immature DC (iDC) were obtained according to a method modified from Romani et al. (11). Briefly, monocytes were isolated by Ficoll-Hypaque centrifugation (Lymphoprep 1077 density medium; Nycomed) followed by separation of CD14+ cells using anti-CD14- coated magnetic beads (MACS; Miltenyi Biotec). The monocytes were cultured in complete RPMI 1640 supplemented with 50 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (all Life Technologies) and human recombinant IL-4 (BDS Biosciences) for 7 days at 37°C, 5% CO2. The resulting iDC were cultured in RPMI 1640 supplemented with 50 μM 2-ME, 100 μM perilucin, 100 μg/ml streptomycin, 2 mM l-glutamine (all Life Technologies) and in the presence of 100 ng/ml of human recombinant CSF (Peprotech) and 50 ng/ml human recombinant IL-4 (BD Biosciences) for 7 days at 37°C, 5% CO2. The resulting DC were stained for surface markers by incubation first with the relevant primary human mAb. The stained cells were examined by flow cytometry immediately, using a FACScan flow cytometer (BD Biosciences) and analyzed with CellQuest software.

Human cytokine measurement

Flow cytometric analysis

DC were stained for surface markers by incubation first with the relevant mAb (30 min, 4°C; BD Pharmingen) followed by 1/20 diluted FITC-conjugated goat anti-mouse Ig (Jackson ImmunoResearch Laboratories) (30 min, 4°C). Nonspecific Ab binding was blocked with 10% FCS solution (15 min, 4°C) before addition of the relevant primary human mAb. The stained cells were examined by flow cytometry immediately, using a FACSscan flow cytometer (BD Biosciences) and analyzed with CellQuest software.

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Human cytokine measurement

DC culture supernatants were collected and stored at −20°C. Secreted IL-6 and TNF-α were measured by ELISA (BDS Biosciences), according to the manufacturer’s instructions.
The iDC for the MLR assay were generated from monocytes as outlined. The resultant cells were cultured for 24 h in the same medium or in the medium containing lipids (100 μg/ml). Titrations of DC from 0.125–2 × 10^5 were incubated at 37°C/5% CO_2 with allogeneic T cells (10^5 cells/well) from a PPD-negative donor in flat-bottom 96-well microtiter plates. T cells were isolated using a Pan-T cell isolation kit (Miltenyi Biotec), according to the manufacturer’s instructions. The DC allogeneic T cell cocultures were incubated for 6 days. The supernatant was harvested and stored at −20°C until secreted IFN-γ was measured by ELISA (BD Biosciences) according to the manufacturer’s instructions. Cells were subsequently pulsed with medium containing 1 μCi/well [3H]thymidine for the final 18 h of culture. Cells were harvested, and T cell proliferation was measured by liquid scintillation counting (Microbeta Systems). All assays were performed in triplicate.

Ag-specific T cell responses

The iDC were cultured in medium containing lipids (100 μg/ml). After 24 h of incubation, 1 × 10^5 cells were incubated with autologous T cells (10^5 cells/well) from a PPD-positive donor in flat-bottom 96-well microtiter plates with or without coincubation with PPD (5 μg/ml; Statens Serum Institut). The cultures were incubated for 6 days after which the supernatants were harvested for determination of secreted IFN-γ measured by ELISA.

Immunization

Female H-2b C57BL/6 mice 6- to 8-wk-old mice were bred at Harlan Scandinavia (Alleroed). Mice were s.c. immunized three times at 2-wk intervals at the base of their tails with vaccines containing 2 μg of Ag85B-ESAT-6 produced as previously described (12). The Ag were administered...
with or without the addition of MMG (10 g), C32 MMG (10 g), or TDB (trehalose 6,6'-dibenenate, 50 g; Avanti Polar Lipids) in a volume of 0.2 ml. The lipid-containing vaccine was prepared by mixing the Ag with lipid that was probe-sonicated into saline water and finally adding DDA by vortex mixing. The vaccine was left overnight to allow adsorption of the Ag.

Stimulation of murine PBMC

PBMC were purified on a density gradient of Mammal Lympholyte Cell Separation medium (Cedarlane Laboratories). PBMC were cultured in microtiter wells (96-well plates; Nunc) containing 2 × 10^5 cells in 200 μl of RPMI 1640 supplemented with 1% (v/v) premixed penicillin-streptomycin solution (Invitrogen Life Technologies), 1 mM glutamine, and 5% (v/v) FCS at 37°C/5% CO2. Culture supernatants were harvested after 72 h of incubation in the presence of 5 μg/ml Ag85B-ESAT-6 and the amount of IFN-γ determined by ELISA. Purified rat anti-mouse IFN-γ (BD Pharmingen) were used as the coating Ab, biotin-labeled rat anti-mouse IFN-γ (BD Pharmingen) as capture Ab and HRP-conjugated streptavidin (Zymed Laboratories) for detection.

Statistical analyses

Data were tested by ANOVA. When significant differences were indicated, differences between mean data were determined by Dunnett’s multiple comparison test.

Results

Apolar lipids derived from M. bovis BCG Copenhagen activate human DC

To study the interactions between mycobacterial lipids and DC, we extracted protein-free polar and apolar lipid fractions from M. bovis BCG Danish. These fractions were analyzed by two-dimensional TLC, according to established protocols (8, 9). In the polar fraction, the lipids that could be identified were phosphatidylinositol mannoses (1–4), phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol (Fig. 1) as previously reported (9). In the apolar fraction, the major lipids were PDIM, TAG, PGL, and MMG (Fig. 1, ii and iii), with no evidence of the presence of TDM.

The comparative immunostimulatory activity of the apolar and polar lipid fractions was subsequently examined using human PBMC-derived iDCs (Fig. 2). Treatment for 24 h with apolar lipids resulted in a dose-dependent elevation of the levels of the activation markers CD86, CD40, and HLA-DR as compared with untreated controls (Fig. 2A). Indeed, a dose of 100 μg/ml apolar lipids resulted in DC activation comparable to that observed with the potent immunostimulatory LPS (0.1 μg/ml). In contrast, very little activation was observed upon treatment with BCG polar lipids. Up-regulation of the maturation markers after stimulation with apolar lipids was accompanied by the secretion of the proinflammatory mediators TNF-α and IL-6. The levels of these proinflammatory cytokines in the supernatants of iDC treated with polar lipids were at a level comparable to that obtained with medium alone. Also stimulation with the immunomodulator M. tuberculosis TDM (100 μg/ml) resulted in negligible activation (Fig. 2B). Low levels of IL-12 were seen upon stimulation with the apolar lipids only (data not shown).

Subsequently we used the MLR assay with allogeneic T cells from a PPD-negative donor (Fig. 3) as an additional readout for DC activation. Further supporting the high activation state of iDC treated with the apolar lipids, we found high levels of proliferation...
FIGURE 5. MMG is a potent immunostimulatory lipid. A, iDC were incubated for 24 h in the presence of medium alone (gray-filled histogram) or MMG, PDIM, PGL, or TAG (10 μg/ml) (open histogram) and the levels of CD86, CD40, and HLA-DR surface markers assessed with MFI after lipid stimulation indicated in each experiment. The corresponding mean fluorescence intensity (MFI) levels for unstimulated DC were 15, 145, and 250 for CD86, CD40, and HLA-DR. B, Culture supernatants obtained following treatment with MMG, PDIM, PGL, or TAG (10 μg/ml) were analyzed by ELISA for the presence of the cytokines IL-6 and TNF-α. C, iDC (0.125–2 × 10^5) were stimulated with 100 μg/ml MMG, PDIM, PGL, TAG for 24 h. Allogeneic T cells from a PPD-negative donor were added and proliferation and IFN-γ release (1 × 10^6) measured after 6 days. Significantly different from unstimulated cells as assessed by ANOVA and Dunnett’s multiple comparison test. *, p < 0.05 and **, p < 0.01.

and IFN-γ release from allogeneic T cells whereas no MLR was induced by the polar lipid fraction.

Isolation of lipids from the immunostimulatory apolar fraction of M. bovis BCG

Lipids from the immunostimulatory apolar fraction were subsequently purified to give samples of PDIM A, TAG, PGL, and MMG. The structure and identity of the lipids were confirmed by 1H NMR and mass spectroscopy (data not shown). The MMG component (Fig. 4) displayed 1H and 13C NMR spectra characteristic of a 1-monocacyl glycerol (13). The 1H NMR spectrum revealed the presence of α-mycolate and cis- and trans-isomers of keto-mycolate; the approximate ratio of the main components was 1.00:0.29:0.24, respectively. MALDI-TOF mass spectroscopy of the total MMG fraction showed m/z (M+Na+) for C79, C81, C83 α-mycolate MMGs (1206, 1234, 1262), C84, C86, C88, C90 cis-keto-mycolate MMGs (1306, 1335, 1363, 1391) and C87, C89, C91 trans-keto-mycolate MMGs (1349, 1377, 1405) (main components underlined).

MMG is a potent inducer of DC activation

Purified MMG, PDIM A, PGL, and TAG were evaluated for their ability to activate human iDC. In these assays MMG was consistently found to be the most potent inducer of DC activation leading to a pronounced up-regulation of CD86, CD40, and HLA-DR (Fig. 5A). The release of TNF-α and IL-6 followed the same trend (Fig. 5B), confirming the pronounced activity of MMG. Mean values of a panel of seven to eight donors exhibited the same order of activation (MMG > PDIM A > PGL > TAGs) when assessing activation markers as well as cytokine release (Table I). MMG activated DC more efficiently than PDIM A, a lipid that has been clearly associated with pathogenicity (14), whereas PGL and, in particular, TAGs induced notably less activation. In addition, testing the four lipids in a MLR assay again identified MMG as the most active of the lipids giving rise to significant enhanced level of T cell proliferation and IFN-γ release compared with cells incubated with medium alone (Fig. 5C).

The lipid MMG contains mycolic acids, common components with TDM; a glycolipid that has previously been observed to have adjuvant activity in murine models (6, 15, 16) with the fine structure of the mycolate components being of importance for its proinflammatory activity (17). We therefore investigated whether the different active mycolate components of MMG responsible for its potent immunostimulatory capacity in humans could be identified. The α-MMG and keto-MMG (Fig. 4) were separated following the preparation of trimethylsilyl ethers of MMG, preparative TLC and subsequent hydrolysis of the trimethylsilyl ether-protecting groups to afford α-MMG and keto-MMG (containing equivalent amount of the keto-MMG with cis- and trans-configuration). When assessed for their ability to

<table>
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<tr>
<th>Lipid</th>
<th>CD40</th>
<th>CD86</th>
<th>HLA-DR</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMG</td>
<td>2.05 (1.3–3.0)</td>
<td>3.16 (1.2–7.5)</td>
<td>3.50 (1.0–9.2)</td>
<td>4597 (34–27256)</td>
<td>878 (&lt;10–3663)</td>
</tr>
<tr>
<td>PGL</td>
<td>1.88 (1.0–2.9)</td>
<td>2.66 (1.3–3.0)</td>
<td>1.45 (1.0–1.9)</td>
<td>289 (&lt;10–919)</td>
<td>96 (&lt;10–269)</td>
</tr>
<tr>
<td>PDIM</td>
<td>1.89 (1.3–3.6)</td>
<td>3.12 (1.0–1.7)</td>
<td>1.40 (1.0–1.7)</td>
<td>620 (&lt;10–3481)</td>
<td>348 (&lt;10–865)</td>
</tr>
<tr>
<td>TAG</td>
<td>1.30 (1.0–1.8)</td>
<td>1.80 (1.0–1.6)</td>
<td>1.29 (1.0–1.6)</td>
<td>135 (&lt;10–546)</td>
<td>50 (&lt;10–259)</td>
</tr>
</tbody>
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Cd/a iDC were stimulated with 10 μg/ml lipids, and the mean fluorescence intensity of stimulated compared with control wells was measured as stimulation index. Data are mean values obtained from eight donors with range shown in parentheses.

Table I. Activation of DC by purified lipids

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activate human iDC, the α-MMG and keto-MMG both stimulated ~2-fold increase in the levels of activation markers at a concentration of 100 μg/ml (Fig. 6A). As recent studies showed that keto-mycolates prefer a much more condensed confirmation than α-mycolates (18), this more open structure of α-MMG may facilitate its interaction with DC receptors potentially leading to a more rapid response. To investigate the kinetics of the response to the two types of MMG, iDCs were stimulated for 12, 24, and 48 h of stimulation, and up-regulation of activation markers as well as cytokine release were monitored. As shown in Fig. 6B, there was no evidence of different kinetics upon stimulation with the two lipid variants with peak values of activation markers reached after 24 h of activation for both.

The potent immunostimulatory activity can be mimicked by a simple synthetic MMG analog

To generate a simplified synthetic MMG analog, a 32 carbon mycolate analog was chosen; this choice corresponds to the mycolic acids of Corynebacterium diphtheriae whose synthesis has been previously described (19) (Fig. 4). Synthesis of the C32 mycolic acid was followed by its coupling to isopropylidene glycerol; removal of the isopropylidene protecting group provided the MMG analog. This compound was tested for its ability to stimulate iDC in comparison with that of natural MMG. As shown in Fig. 7A, synthetic C32 MMG exhibited a dose-dependent immunostimulatory activity as assessed by up-regulation of the maturation marker CD86. This activation was at a level comparable to that of natural MMG (Fig. 7A). In addition, stimulation with the synthetic analog also resulted in considerable levels of the proinflammatory cytokines (Fig. 7B) confirming that the activity seen upon stimulation with natural MMG was mimicked by the synthetic C32 MMG. The functional ability of MMG-stimulated DC was further assessed by incubation with autologous T cells obtained from PPD-positive donors. As shown in Fig. 7C, MMG as well as C32 MMG led to T cell stimulation as monitored by IFN-γ release in the presence of PPD, whereas no stimulation was seen in the absence of Ag.

FIGURE 6. The α-mycolate and keto-mycolate MMGs are immunostimulatory. The iDC were incubated for 24 h in the presence of α-mycolate or keto-mycolate MMGs (1–100 μg/ml) (A) or incubation for 12, 24, and 48 h with α-mycolate or keto-mycolate MMGs (100 μg/ml) (B). The geometric mean fluorescence intensity (MFI) of levels of surface markers on DC following treatment compared with untreated control wells as shown by stimulation index (SI). Culture supernatants obtained following treatment were analyzed by ELISA for the presence of the cytokines IL-6 and TNF-α. Significant difference from unstimulated cells as assessed by ANOVA and Dunnett’s multiple comparison test is shown. *, p < 0.05 and **, p < 0.01.

FIGURE 7. Stimulation of DC by synthetic C32 MMG. A. The iDC were incubated for 24 h in the presence of MMG and synthetic C32 MMG at 2, 10, or 25 μg/ml and levels of CD86 marker assessed. B. Culture supernatants obtained following treatment with 10 μg/ml MMG or C32 MMG were analyzed by ELISA for the presence of the cytokines IL-6 and TNF-α. Levels obtained after stimulation with LPS (0.1 μg/ml) is shown for comparison. C. The iDC (1 × 10⁴) were stimulated with 10 μg/ml MMG, C32 MMG, or LPS for 24 h. Autologous T cells from a PPD-positive donor were added in the presence or absence of PPD (5 μg/ml) and IFN-γ release measured after 6 days. Significant difference from unstimulated controls as assessed by ANOVA and Dunnett’s multiple comparison test. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
The adjuvant effect of MMG was finally analyzed in vivo using the tuberculosis vaccine candidate, Ag85B-ESAT6 (a fusion of two immunodominant proteins from *M. tuberculosis* (12)) as the vaccine Ag. C57BL/6 mice were immunized with 2 μg of Ag85B-ESAT-6 in the indicated adjuvant and the immune response monitored by restimulation with purified PBMCs with the vaccine Ag. Upon immunization with Ag85B-ESAT-6 in MMG alone, the immune response was found to be negligible as assessed by the IFN-γ release (Fig. 8A). Using cationic liposomes formed of DDA previously shown effective for delivery of mycobacterial lipids (9), we obtained significantly enhanced immune responses with MMG as well as C32 MMG. This immune response was comparable to that obtained with the well-known combination of TDB and DDA (20). Analysis of the cytokine imprint obtained with MMG and DDA showed a predominating Th1-type of immune response with high levels of IFN-γ, detectable levels of TNF-α and IL-2 and only negligible levels of Th2 cytokines (IL-4 and IL-5) (data not shown). Comparing the adjuvant activity of the four mycobacterial lipids when delivered in cationic liposomes, again MMG was the most active lipid giving rise to enhanced responses compared with DDA alone, whereas addition of PDIM and PGL to DDA gave rise to a more modest enhancement (Fig. 8B).

**Discussion**

The immunostimulatory activity of mycobacterial-derived lipids has been recognized for many decades with an ever-expanding literature of lipids capable of stimulating immune responses in animal (murine) models; however, very few lipid compounds with activity in humans have been identified thus far. Although microbial lipoproteins have been demonstrated to be stimulators of proinflammatory cytokine production by human macrophages and both the naturally occurring and a synthetic version of the 19-kDa lipoprotein from *M. tuberculosis* induce the maturation of human DC (21, 22), with the lipid moiety being found to be essential for this process, data pertaining to individual lipids that activate human DC is lacking. In this study, we tested different mycobacterial lipid preparations as well as individual lipids for their ability to mature human DCs identifying the apolar lipids as the most active fraction and within this fraction MMG being responsible for the majority of this activity. In contrast, the polar fraction exhibited only negligible stimulatory activity on human DCs.

In a study by Sprott et al. (4), liposomes formed of total polar lipids extracted from *M. bovis* BCG were previously found to be capable of activating DC with the majority of this activity found to be attributable to the phosphatidylinositol dimannoside lipid. In addition, liposomes composed of phosphatidylinositol dimannoside and cholesterol have been shown to activate primed mouse peritoneal macrophages (23). Although apparently contrasting to the data presented in this study, it is worth noting that the polar phospholipids were presented as liposomes either obtained by a heating process above the phase transition of the lipids (4) or by mixing with cholesterol (23). Indeed, the liposomal delivery was considered critical for obtaining the subsequent activity of the formulations. In addition, none of the above studies have included a comparative analysis to the more insoluble (and thereby less accessible) apolar lipids or individual components thereof. Finally, both studies were conducted using either murine DC or IFN-γ primed macrophages and therefore add to the large body of evidence for lipids/lipid fractions with immunomodulatory activities in murine models.

As noted, MMG, as well as the other apolar lipid preparations tested in our experiments were of very low solubility, and different methods for stimulating iDCs were evaluated identifying sonication of the lipids in the medium as the most efficient method to solubilize the lipids (data not shown). Using this method, clearly MMG was identified as the most active of the four mycobacterial lipids, although quite large doses (25–100 μg/ml) were needed, which could be due to material that was not dispersed despite the sonication procedure. In the in vivo studies, MMG was found highly efficient when delivered in cationic liposomes, which will serve to keep the glycolipid in dispersion and present the MMG to the APCs. Cationic liposomes have previously proven very efficient for enhancing the immune responses of complex mycobacterial extracts (9) as well as the synthetic analog of TDM (24), whereas other Th1-inducing immunomodulators, e.g., muramyldipeptide, β-glucan, and saponin, completely failed to act in synergy with the liposomes (25). The underlying mechanism is unknown but it could be related to a less efficient presentation to the APCs whereas the mycobacterial lipid extracts and the TDM analog is...
adequately incorporated into the lipid bilayers of the liposomes, and therefore presented as encountered in nature.

Although mycobacterial MMGs were occasionally found in early studies (26), including in M. bovis BCG (27), their close chromatographic similarity with monoglycosyl PGL (Fig. 1, ii and iii) may have obscured their presence and at present only a modest literature on MMG exists. Limited surveys, however, have indicated that substantial amounts of MMG are only consistently found in M. bovis and its BCG variants (8). As pointed out in another study, the apparent association of PGL with mycolate substitutes (28) may be explained by this similarity in chromatographic behavior and could also explain the lack of data pertaining to this active lipid. Interestingly, although TDM and MMG both contain mycolate components, MMG is far more active in the stimulation of human DC (Fig. 2B). The two lipids are certainly very different in structure; TDM has two mycolate residues attached to a large hydrophobic trehalose disaccharide but MMG has a single mycolate linked to a much smaller glycerol unit (Fig. 4). α- and keto-mycolic acids and their methyl esters are also less active than MMG (data not shown), so the basis of the DC stimulation apparently lies in the linkage of the glycerol to the 2-branched, 3-hydroxy mycolate unit. The precise structures required for obtaining optimal immunostimulatory activity is currently being investigated.

The receptors on DC, through which mycolates signal, have yet to be identified. Various mycobacterial proteins and lipids have previously been shown to be recognized by TLR2 in association with TLR1/6 and TLR4 (29). TLR2-dependent activation has been described for a number of cell wall constituents including lipopaminomannan and the 38-kDa glycolipidprotein is involved in TLR4-dependent signaling (see review at Ref. 30 by Jo et al). A preliminary testing of MMG in TLR2/4 double knockout mice failed to demonstrate a significant role for TLR2 and TLR4 in the recognition of MMG (C. S. Andersen et al., manuscript in preparation). In this regard, recent in vivo evidence using various gene-deficient mice indicates a role for MyD88, the adaptor protein through which TLR signal transduction is mediated, in responses to trehalose mycolates or purified mycopic acids but not for TLR 2 or TLR4 (6, 31). Further studies to identify the relevant receptors involved in MMG recognition will provide valuable insight into signaling promoted by the mycolic acid associated lipids of the Mycobacterium genus.

In this study, we provide evidence that MMG is a very active lipid compared with three other mycobacterial lipids; capable of stimulating human DCs. The identification of the immunostimulatory activity of MMG and its component mycolates, documented here, will enable studies of their distribution in various mycobacterial strains with different immunological properties. In addition, the preliminary data with a simple synthetic analog based on 32 carbon advocates their possible use in the development of a new vaccine. Material strains with different immunological properties. In addition, the preliminary data with a simple synthetic analog based on 32 carbon advocates their possible use in the development of a new vaccine.


