Novel Generation Mycobacterial Adjuvant Based on Liposome-Encapsulated Monomycoloyl Glycerol from *Mycobacterium bovis* Bacillus Calmette-Guérin

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*J Immunol* 2009; 183:2294-2302; Prepublished online 20 July 2009; doi: 10.4049/jimmunol.0804091
http://www.jimmunol.org/content/183/4/2294

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Mycobacterium species, including one of the world's most successful pathogens, *Mycobacterium tuberculosis*, constitute some of the most immunostimulatory organisms known to date. This is illustrated by infection with *tuberculosis* (TB), which gives rise to granulomatous inflammation at infection site and a powerful induction of T cell responses. The existence of mycobacterial components with substantial immunostimulatory activity is furthermore reflected in the prominent activity of Freund's complete adjuvant (FCA) based on mineral oil extract showing that the majority of the activity was attributable to the apolar lipids and more specifically to a single lipid, monomycoloyl glycerol (MMG), previously also shown to stimulate human dendritic cells. Delivered in cationic liposomes, MMG induced the most prominent Th1-biased immune response that provided significant protection against tuberculosis. Importantly, a simple synthetic analog of MMG, based on a 32 carbon mycolic acid, was found to give rise to comparable high Th1-biased responses with a major representation of polyfunctional CD4 T cells coexpressing IFN-γ, TNF-α, and IL-2. Furthermore, comparable activity was shown by an even simpler monoacyl glycerol analog, based on octadecanoic acid. The use of these synthetic analogs of MMG represents a promising new strategy for exploiting the immunostimulatory activity and adjuvant potential of components from the mycobacterial cell wall without the associated toxicity issues observed with complex mycobacterial preparations. The *Journal of Immunology*, 2009, 183: 2294–2302.
TB vaccine candidate Ag85B-ESAT-6 promoted strong humoral and cell-mediated immune responses and gave rise to high levels of protection against TB (6).

Herein, we dissected the specific lipid components responsible for the powerful adjuvant effect. The majority of the adjuvant activity of the total BCG lipids was attributable to the apolar lipids and in particular the lipid monomycoloyl glycerol (MMG). MMG is structurally relatively simple with a mycolic acid attached to the glycerol head group. When delivered in combination with cationic liposomes, MMG was found to give rise to a strong Th1 response, protective against TB. In addition, the biological activity could be reproduced with a synthetic analog of MMG based on 32 carbons atoms. The recent finding that natural MMG and a synthetic analog also activate human dendritic cells (DC) (7) further supports the use of MMG compounds as vaccine adjuvants.

Materials and Methods

Extraction and purification of lipids

*M. bovis* BCG Copenhagen 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 (6–8). Samples (10 μl) of rehydrated lipid extracts (1 mg/ml) were analyzed by SDS-PAGE and silver staining for residual protein content. The lipid preparations were then dried in vacuo, and the residue was chromatographed on a 20-g silica gel Varian Bond Elut 12256026 cartridge, using hexane to hexane:ethyl acetate (9:1) in 2% increments, to yield the desired product as a white solid.

Each fraction was analyzed by one-dimensional TLC on aluminum-backed plates (Merck 60 – 80°C) and carefully loaded onto a 20-g column of silica gel (Fluka 60741 Silica gel 60) prepared in petroleum ether. The column was eluted with a gradient of 0, 0.2, 0.5, 10, 15, 20, 25, 50, and 100% acetone in petroleum ether, followed by a single wash with methanol. Each fraction was analyzed by one-dimensional TLC on aluminum-backed plates (Merck 5554), using petroleum ether/acetone (98:2), toluene/acetone (95:5), or toluene/acetone (80:20). 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 (8).

Single lipids were purified by preparative one-dimensional TLC on plastic-backed plates (Merck 5735); detection with 0.01% ethanolic Rhodamine 6G was followed by examination under 366-nm UV light and diethyl ether extraction. PDIM and TAG were isolated from the 5 to 10% acetone fractions, using TLC run in petroleum ether/acetone (98:2); only the major component, based on phthiocerol A, was recovered. PGL and MMG were isolated together from the 15–20% fractions by TLC run in toluene/acetone (95:5). PGL and MMG were separated on one-dimensional TLC in chloroform:methanol:0.880 ammonia (97:3:0.5). The PDIM (18 mg), TAG (12 mg), PGL (11 mg), and MMG (10 mg) were characterized by 500 MHz 1H nuclear magnetic resonance (NMR) (Bruker drx300) and MALDI-TOF mass spectrometry (Bruker Biflex IV). The structures of these and the other lipids tested herein are shown in Fig. 1.

Individual MMG, based on α-mycolic acids and keto-mycolic acids, and a C32 synthetic MMG analog (3-hydroxy-2-tetradecyl-2,3-dihydroxypropyl ester) were prepared as described previously (7, 9). The same synthetic strategy was used to prepare a C16 mono-acetyl glycerol analog (C16 MAG; octadecanoic acid-2,3-dihydroxypropyl ester). Briefly, stearic acid (100 mg, 0.38 mmol, 1 eq), 4-pyridilinopropionic acid (100 mg, 3 eq), and 2,2-dimethyl-4-hydroxymethyl-1,3-dioxlan (sn-isopropylidene) (0.1 ml, 0.76 mmol, 2 eq) were dissolved in dichloromethane, were treated with N,N-dicyclohexylcarbodiimidazole (NHS), and the title compound was obtained by purification using normal-phase chromatography. The MMG compounds were characterized by high-resolution mass spectrometry calculated for C40H57O6Na (M+Na+H)+ 741.4; found 739.8.

Antigens

The fusion protein of Ag85B and ESAT-6 (in the following designated Ag85B-ESAT-6) was produced as recombinant proteins as described previously (10). CTH1 is a fusion of the two chlamydia Ags Cts21 and Cts44 (11, 12). The recombinant fusion protein of Cts21 and Cts44 was cloned and produced essentially as described in Ref. 10. TLR4-knockout mice (13) and BCG Copenhagen 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 (6–8). Samples (10 μl) of rehydrated lipid extracts (1 mg/ml) were analyzed by SDS-PAGE and silver staining for residual protein content. The lipid preparations were then dried in vacuo, and the residue was chromatographed on a 20-g silica gel Varian Bond Elut 12256026 cartridge, using hexane to hexane:ethyl acetate (9:1) in 2% increments, to yield the desired product as a white solid. Deprotection of the sn-glycerol unit was achieved using TFA:THF:H2O (8:17:3) (7, 9). Chromatography, as above, gave the title compound as a white solid in 65% yield (63 mg). Melting point 78–80°C; 1H NMR (CDCl3, 300 MHz) δ 0.90 (t, 3H, CH3), 1.30 (s, 32H, CH2), 1.55 (t, 2H, CH), 2.45 (t, 2H, CH), 2.55–4.15 (m, 5H, CH, CH2), 1C NMR (CDCl3, 300 MHz) δ 15.0 (CH3), 22.7, 29, 29.4, 31.9, 34.1 (CH3), 63.3 (CH2), 65.2 (CH2O), 70.3 (CH), 173.5 (C = O); m/z (EI) 379.1 (M+Na+) (100%); high resolution mass spectrometry calculated for C31H59O5Na (M+Na+) 379.52 found 379.87.

Adjuvants and vaccines

Total or individual lipid extracts were prepared for in vivo use by rehydrating dry *M. bovis* lipids with Milli Q water at 1 or 5 mg/ml followed by probe sonication (two pulses of 30 s). DDA (Avanti) was prepared by adding DDA powder to autoclaved water (2.5 mg/ml) and heating at 80°C under continuous stirring for 20 min, followed by cooling to room temperature before use. The lipid vaccines were prepared by mixing the Ag with saline, followed by addition of rehydrated lipid extract and DDA, followed by vortex mixing. The vaccine was left over night to allow adsorption of the Ag.

Animals

Female BALB/c or C57BL/6 mice, 8–12 wk old, were obtained from Bomholtgaard or Harlan Scandinavia. TLR4-knockout mice (13) and...
TLR2-knockout mice (14), both backcrossed onto a C57BL/6 background, were interbred to obtain TLR2 and TLR4 double-deficient mice. Female TLR2/4-knockout and C57BL/6 control mice were used for vaccination. Mice, receiving a mycobacterial challenge, were housed in a BSL-3 facility. All experiments were conducted in accordance with the regulations set forward by the Danish Ministry of Justice and Animal Protection Committees and in compliance with EC Directive 86/609.

**Immunization**

Mice were immunized s.c. at the base of the tails up to three times with a 2-wk interval between each immunization. The vaccines (0.2 ml/mice) consisted of 2 μg of the fusion protein Ag85B-ESAT-6, 5 μg of the CTH1 administered in 250 μg of DDA, and 1–100 μg of rehydrated lipid. As a positive control for protective efficacy of experimental subunit vaccines compared with the conventional TB vaccine, BCG, a single group of mice received one dose of BCG Danish 1331, 5 × 10^6 CFU, injected s.c. at the base of the tail.

**Cellular assays**

Blood samples, inguinal lymph nodes, or spleens were taken from mice 7–21 days after the last immunization and prepared as described previously (6). Cell cultures were performed in triplicate in round-bottom microtiter wells, containing 2 × 10^5 cells in a volume of 200 μl of RPMI 1640 supplemented with 5 × 10^{-5} 2-ME, 1 mM glutamine, 1% penicillin-streptomycin, 1% HEPES, and 10% FCS (all from Invitrogen Life Technologies). Ags were used in concentrations ranging from 5 to 0.05 μg/ml. Wells containing medium only or 5 μg/ml ConA were included in all experiments as negative and positive controls, respectively. Culture supernatants were harvested from parallel cultures after 72 h of incubation in the presence of Ag, and the amount of IFN-γ was determined by ELISA (15) or by IFN-γ Duoset ELISA obtained from R&D Systems.

**Flow cytometry**

Splenocytes were stimulated for 1 h with 5 μg/ml Ag in the presence of 1 μg/ml anti-CD28 (clone 37.51) and anti-CD49d (clone 9C10(MFR4.B) (both BD Pharmingen) and subsequently incubated for 5–6 h at 37°C following addition of 10 μg/ml brefeldin A (Sigma-Alrich) and 0.7 μg/ml Monensin/GolgiStop (BD Pharmingen). Following overnight storage at 4°C, cells were washed in FACS buffer (PBS containing 0.1% sodium azide and 1% FCS) and subsequently stained 30 min at 4°C for surface markers with mAbs using 1/200 dilutions of anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), and anti-CD44 (clone IM7) (all BD Pharmingen). Cells were then washed in FACS buffer, permeabilized using the Cytofix/ Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions, and stained intracellularly for 30 min at 4°C in dilutions of 1/200 using anti-IFN-γ (clone XMG1.2), anti-TNF-α (clone MP6-XT22), or anti-IL-2 (clone JES6-5H4) mAbs. After washing, cells were resuspended in FACS buffer and analyzed on a six-color BD FACSCanto flow cytometer (BD Biosciences).

**Experimental infections**

For evaluation of vaccine efficacy, mice were challenged 10 wk after the first immunization by the aerosol route in a Glas-Col inhalation exposure system (Glas-Col) calibrated to deposit ~25 CFU of virulent M. tuberculosis Erdman in the lungs. The bacterial load in the lungs was determined by guest on July 29, 2017 http://www.jimmunol.org/ Downloaded from

MMG LIPID AS AN EFFECTIVE ADJUVANT (results not shown). The polar fraction was highly enriched in polar lipids, including PIM, phosphatidylinositol, diphasphatidylglycerol (DPG), phosphatidylglycerol, and phosphatidylethanolamine. In the apolar fraction, the major lipids identified were PDIM, TAG, PGL, and MMG as previously shown (7). Trehalose mono- and dimycolates (“cord factor”) were not detectable in the lipid extracts. The total lipid extract included both apolar and polar lipids. All lipid extracts were confirmed to be free of protein contamination by silver-stained SDS-PAGE (data not shown).

The purified lipid fractions were compared for their ability to induce an immune response in two different strains of mice with different Th1/Th2 bias (BALB/c/Th2 and C57BL/6/Th1). The well-characterized vaccine Ag (Ag85B-ESAT-6) was used as a model Ag. We tested the lipid fractions both directly and co-formulated with cationic DDA liposomes. The vaccines were administered s.c. three times at 2-wk intervals, and the immune response was measured by restimulating PBMC with different concentrations of the vaccine Ag. Assessment of the IFN-γ response was measured by restimulating PBMC with different concentrations of the vaccine Ag. Assessment of the IFN-γ levels was further compared by immunizing BALB/c mice with Ag85B-ESAT-6 incorporated into liposomes, containing a wide dose range of polar and apolar lipids. Mice received three vaccinations, and 6 wk after the last vaccination, they were challenged by the aerosol challenge with virulent M. tuberculosis Erdman. Another 6 wk after the challenge, mice were sacrificed, and the numbers of bacteria in the lungs were enumerated. In agreement with the immunogenicity data (Fig. 2), the liposomes based on the apolar lipids were highly active and, combined with Ag85B-ESAT-6, they promoted a significant protective activity over a dose range of 0.1–100 μg with log_{10} reduction from 0.44 to 1.03 (Fig. 3). In contrast, the polar fraction was considerably less active and even at a dose of 100 μg in the DDA liposomes it only induced protection corresponding to a 0.55 ± 0.25 log_{10} reduction of bacteria. This level was comparable to that observed with a dose of 0.1 μg of apolar lipids (0.44 ± 0.1 log_{10} CFU). No significant levels of protection were observed in mice receiving the lipids fractions/DDA in the absence of Ag (results not shown). Furthermore, no significant protection was observed with the lipid fraction administered without DDA or with Ag alone with a log_{10} reduction ranging from 0.0 to 0.19 compared with nonimmunized mice (results not shown). Taken together, these results indicated that the majority of the prominent adjuvant activity found with BCG cell wall lipids can be attributed to the apolar lipid fraction.

**Identification and characterization of individual immunostimulatory apolar lipids**

To identify the individual molecules responsible for the high activity of the apolar lipids, the lipid extract was further separated based on polarity. A silica gel column was run with an increasing gradient of acetone in petroleum ether to sequentially elute the
lipids, including a final methanol wash for completion. The individual lipid fractions were subsequently administered with liposomes and used to promote a Th1 response to Ag85B-ESAT-6. The adjuvant activity (Fig. 4A) was centered around the 10–25% acetone fractions, whereas the more polar lipid fractions had limited stimulatory ability and promoted a modest IFN-γ/H9253 recall response. Lipids from the 5–10% acetone fractions (Fig. 4B) were isolated, using preparative TLC, to give pure samples of phthiocerol A dimycocerosates (PDIM) and TAG; PGL and MMG were purified from the 15–20% acetone fractions. The purity of these lipids is demonstrated in Fig. 4D and confirmed by 1H NMR and mass spectroscopy (data not shown).

The isolated lipids were incorporated into DDA-based liposomes, and the resulting adjuvants were compared for their ability to drive a Th1 response to Ag85B-ESAT-6 (Fig. 4C). The highest IFN-γ response was obtained with the DDA/MMG-adjuvanted preparation resulting in levels of 5 ng/ml upon restimulation of PBMCs. PGL-based liposomes also induced IFN-γ production, albeit to a lower level, whereas DDA liposomes incorporating PDIM or TAG promoted only a modest IFN-γ recall response from

![Figure 2](image-url)  
**FIGURE 2.** Immune responses induced by apolar and polar lipids derived from *M. bovis* BCG Copenhagen. A, BALB/c mice (n = 6) were immunized s.c. with 2 μg of Ag85B-ESAT-6 in combination with the indicated lipid fraction alone or administered in DDA liposomes three times with a 2-wk interval in between. Three weeks after the last immunization, splenocyte cultures were restimulated with Ag85B-ESAT-6 (5, 0.5, and 0.05 μg/ml) and the IFN-γ release ± SEM measured by ELISA. B, C57BL/6 mice (n = 3) were immunized once s.c. with 2 μg of Ag85B-ESAT-6 in the indicated lipid fraction, and the draining lymph nodes were isolated and restimulated with Ag85B-ESAT-6 (5, 0.5, and 0.05 μg/ml) 7 days postvaccination. The IFN-γ release ± SEM is measured by ELISA. *, p < 0.05; and ***, p < 0.001 compared with polar lipids.

![Figure 3](image-url)  
**FIGURE 3.** Apolar lipids derived from *M. bovis* BCG induce protection to TB. BALB/c mice (n = 6) were immunized three times with 2 μg of Ag85B-ESAT-6 adjuvanted with DDA liposomes in combination with different doses of the indicated lipid fraction. Six weeks after the last immunization, mice were subjected to an aerosol challenge with *M. tuberculosis* Erdman, and the number of bacteria in the lungs (expressed as log_{10} CFU) was measured another 6 wk later. Groups of unvaccinated naive mice were included in each round of aerosol infection and have been included in each graph for comparison. The protective efficacy (log_{10} protection) is expressed as the log_{10} reduction in bacterial load compared with unimmunized mice. *, p < 0.05 compared with naive control animals.
spleen cells restimulated with Ag85B-ESAT-6 comparable to the level seen with the DDA vehicle alone. Increasing the dose of lipid in the vaccine to 50 μg/H9262 g did not change the overall pattern with DDA/MMG, giving rise to the highest responses (results not shown).

Vaccination with MMG-based adjuvant induce a protective Th1 immune response

To further characterize the adjuvant activity of MMG, the immune response upon vaccination was compared with that of DDA/MPL and DDA/TDB, both of which have consistently been demonstrated as prominent inducers of Th1 responses protective against TB (16, 17). As shown in Fig. 5A, liposome formulations of MMG, TDB, and MPL promoted comparable responses to Ag85B-ESAT-6 in terms of IFN-γ release. The response was primarily a CD4 T cell response, whereas the CD8 T cell induction was negligible (results not shown). As a number of studies have demonstrated a role for TLR2 and TLR4 in recognition of mycobacterial lipid components (18), TLR2/4 double-knockout mice or wild-type controls were therefore vaccinated with the MMG-based adjuvant, and the cellular response was measured. The TLR2/4-deficient mice exhibited a prominent IFN-γ production, which was only slightly lower than the response observed in wild-type animals (p = 0.453) (Fig. 5B), suggesting that MMG is not signaling through these TLR. Finally, the ability of a MMG-adjuvanted vaccine to stimulate a protective immune response against a live M. tuberculosis challenge was assessed. Vaccination with Ag85B-ESAT-6 in DDA/MMG led to a significant reduction of the mycobacterial burden compared with unvaccinated mice (0.87 ± 0.15 log CFU; p < 0.01) and compared with a vaccine adjuvanted with only DDA (0.39 ± 0.17 log CFU; p < 0.05) and with a level of protection comparable to the reduction seen with a standard BCG vaccination (Fig. 5C). No protection was seen in mice vaccinated with DDA/MMG without Ag.

The potent immunostimulatory activity can be mimicked by simple synthetic MMG analogs

MMG is a relatively simple lipid with a mycolic acid being esterified to one of the primary hydroxyl groups of glycerol (Fig. 1). Natural M. bovis BCG MMG contains α- and ketomycolic acids, and to dissect the structures important for the observed adjuvant activity, we purified these two forms of MMG and evaluated their adjuvant activity by immunization studies. No differences in activity between the two forms were observed, indicating that the form of the mycolic acid seems to play a minor role (results not shown). Subsequently, we evaluated the more simplified synthetic MMG analog based on 32 carbons (7, 9). Immunization with Ag85B-ESAT-6 in DDA with 10 μg of naturally purified MMG or...
with the synthetic C\textsubscript{32} MMG induced strong and comparable IFN-\gamma responses upon restimulation of splenocytes (Fig. 6A) with different doses of Ag85B-ESAT-6. The theoretical molar weight of natural MMG is \textasciitilde 1300 g/mol, whereas the molar weight of C\textsubscript{32} MMG is 570.93 g/mol. Consequently, we further evaluated lower doses of C\textsubscript{32} MMG but found equivalent high responses down to 1 \mu g of C\textsubscript{32} MMG/dose (results not shown), reflecting lower molar concentration than 10 \mu g of natural MMG. In a separate experiment, we also analyzed a simple fatty acid glycerol ester (C\textsubscript{18} MAG) as shown in Fig. 1. Also with this simple synthetic construct, we obtained highly significant levels of IFN-\gamma (Fig. 6B), suggesting that the most critical part for the immunostimulation relates to the glycerol head group and/or the linkage of the glycerol to the lipid part. To further study the adjuvant activity of MMG in combination with a nonmycobacterial Ag, we used a fusion protein of two chlamydia Ags (Ct443 and Ct521, the fusion designated CTH1) for immunization studies. Significant levels of IFN-\gamma were recorded with CTH1, again demonstrating that MMG-based adjuvants can be used for enhancing the immune responses to proteins from other sources than mycobacteria (Fig. 6C). Finally, we dissected the phenotype of the activated T cells by analyzing the expression of IL-2, TNF-\alpha, and IFN-\gamma in various combinations by flow cytometry. The major population identified was characterized by the expression of multiple cytokines: IFN-\gamma\textsuperscript{+}, IL-2\textsuperscript{+}, and TNF-\alpha\textsuperscript{+} CD4 T cells, which are a population found to correlate with the immune response of the host toward mycobacteria with several advantages in terms of purity and reproducibility of the product and during the production process. In this regard, synthetic C\textsubscript{32} MMG and C\textsubscript{18} MAG are attractive molecules; their simple structures allowing facile chemical synthesis.

The most prominent and well-known example of using synthetic analogs of an immunostimulatory molecule is the large panel of different lipid A mimetics known as aminoalkyl glucosaminide phosphates, which have retained some of the immunostimulatory activity of LPS but display a more tolerable reactogenicity profile (20). Synthetic mycobacterial lipid adjuvant formulations has also previously been exploited successfully, e.g., TDB, which contains shorter, less saturated acid chains and is less reactogenic than the native compound TDM (21, 22). However, as it has retained much of the biological activity, it is highly effective in adjuvant formulations and has shown promising results in a range of animal models, including TB and chlamydia (23, 24). As also demonstrated for MMG herein, the induction of immune responses by these trehalose mycolates seems to occur independent of TLR signaling (TLR2, 3, 4, and 7) (4, 16). This finding is highly interesting in relation to data currently coming from basic studies of host pathogen interactions in TB. Although the family of TLR is involved in the immune response of the host toward mycobacteria with several mycobacterial components interacting with different TLR (25), the very vigorous T cell responses occurring during a TB

**Discussion**

It has long been recognized that heat-killed mycobacteria or various subfractions of mycobacteria have potent immunostimulatory activity, which can be harnessed to create Th1-inducing adjuvants; however, the associated reactogenicity has hampered the further use of these constituents in prophylactic vaccines. Herein, we describe the use of a single lipid, MMG, with potent immunostimulatory activity comparable to that seen with the well-known immunomodulators MPL and TDB (Fig. 5). The use of a defined lipid opens the possibility of designing a synthetic analog, which retains most of the immunostimulatory activity but with a more acceptable toxicity profile compared with whole cells or various subfractions of mycobacterial cell wall components. Adding to this, a synthetic component offers a number of advantages in terms of purity and reproducibility of the product and during the production process. In this regard, synthetic C\textsubscript{32} MMG and C\textsubscript{18} MAG are attractive molecules; their simple structures allowing facile chemical synthesis.

**FIGURE 5.** Th1-inducing ability of MMG in vivo in mice. A, C57BL/6 mice (n = 6) were immunized once with 2 \mu g of Ag85B-ESAT-6 in DDA alone, DDA/MPL, DDA/TDB, or DDA/MMG. The immune response was monitored in individual draining inguinal lymph nodes 1 wk after the last immunization after in vitro restimulation with Ag85B-ESAT-6 (5 \mu g/ml) by measuring the IFN-\gamma release by ELISA. B, TLR2/4 knockout or wild-type C57BL/6 mice were immunized twice as above and the IFN-\gamma response measured in splenocyte cultures (5 \mu g/ml Ag85B-ESAT-6 for restimulation) 18 days after the second vaccination. Mean \pm SEM of three mice per group is shown. C, C57BL/6 mice (n = 6) was immunized three times with 2 \mu g of Ag85B-ESAT-6 in indicated adjuvant and subjected to an aerosol challenge with M. tuberculosis Erdman 6 wk after the last immunization. The number of bacteria in the lungs was measured another 6 wk later (expressed as log\textsubscript{10} CFU). *, p < 0.05; **, p < 0.01; and ***, p < 0.001 compared with naive control animals.
infection are TLR independent. In these experiments, TLR-disabled knockout mice were incapable of controlling a TB infection; however, the T cell responses were unaffected and comparable to that seen in wild-type mice (26, 27). This suggests that, as adaptive immune responses develop even in the absence of TLR involvement, an alternative route of activation is most likely involved in the priming of the cellular immune responses. For vaccine and adjuvant research, this observation is of major significance and could also explain the recent finding that many classical adjuvants, including FCA, are capable of inducing a potent adaptive immune response in the absence of TLR signaling (28, 29). Although there is still an ongoing debate on the significance of TLR in generating immune responses, what becomes increasingly clear is that not only is it possible to generate potent adaptive immune responses in the absence of TLR but also that alternative pathways and potentially more potent than TLR signaling may exist with TDM and MMG being candidate ligands.

**FIGURE 6.** The activity of MMG can be mimicked by a C32 synthetic analog. A, C57BL/6 mice (*n = 3*) were immunized three times with 2 μg of Ag85B-ESAT-6 administered in DDA alone, DDA/MMG (10 μg), or DDA/C32 MMG (10 μg). The immune response was monitored in spleens 3 wk after the last immunization after in vitro restimulation with Ag85B-ESAT-6 (5, 0.5, and 0.05 μg/ml) by measuring the IFN-γ release by ELISA (mean ± SEM). B, C57BL/6 mice (*n = 3*) were immunized three times with 2 μg of Ag85B-ESAT-6 in DDA/MMG (10 μg) and DDA/C18 MAG (10 μg). PBMC were isolated 3 wk after the third vaccination, restimulated with Ag85B-ESAT-6 (5, 0.5, and 0.05 μg/ml) and the release of IFN-γ measured by ELISA. *p < 0.05; **p < 0.01; and ***p < 0.001 compared with naive control animals. C, C57BL/6 mice (*n = 3*) were immunized twice with 5 μg of CTH1 in DDA/MMG and DDA/C32 MMG. PBMC were isolated 1 wk after the second vaccination and restimulated with CTH1 (5, 0.5, and 0.05 μg/ml), and the release of IFN-γ was measured by ELISA. D, Production of IFN-γ, TNF-α, and IL-2 was assessed by flow cytometry following stimulation of splenocytes 10 days after vaccination with 5 μg of CTH1 in DDA/MMG or DDA/C32 MMG. The percentage of CD4+ T cells expressing any possible cytokine combination upon restimulation with 5 μg/ml CTH1. The percentages obtained in medium control wells have been shown for comparison. E, The total number of CD4 T cells/spleen expressing any possible cytokine combination upon restimulation with 5 μg/ml CTH1. F, The pie charts are coded according to the cytokine production profile and summarize the fractions of the CD4+ T cell response that are positive for a given cytokine production profile. No responses were seen in the CD8+ T cell subset (results not shown).
At present, studies are being undertaken to identify the signaling pathway stimulated by MMG, including also a direct comparison to TDM and its synthetic analog. Recently, the immunostimulatory activity of TDM and TDB was reported to rely on the FcRy-Syk-Card9 pathway, and MMG could potentially also mediate its protective efforts through Syk-Card9 signaling (30). However, although both components contain mycolic acids, the head groups, which presumably is the most important part for the observed activity, are different, indicating that the innate immune pathway and receptor(s) involved is not necessarily identical. In support of this, Silva (31) in 1985 injected charcoal particles coated with different mycobacterial glycolipids, including TDM and MMG preparations. Although the preparations were generally incompletely characterized, upon injection with the TDM and MMG preparations, quite distinct infiltrates in the lungs following injection was observed (31). Hence, TDM induced an increase in the number of mononuclear cells and the appearance of classical granulomatous lesions, whereas MMG gave rise to a more rapid and dense infiltrate of polymorphonuclear cells, which resolved after 4 wk. Further evidence supporting the differences between MMG and TDM comes from recent studies using human monocyte-derived DC (7). In these studies, stimulation with MMG resulted in enhanced expression of activation markers and the release of proinflammatory cytokines, whereas stimulation with TDM resulted in negligible activation comparable to that obtained with medium alone. Future studies will be undertaken to show whether MMG and TDM have complementary effects in vivo and whether there will be any advantage of combining these two components in the same adjuvant formulation.

From the studies herein, it is clear that the most interesting and bioactive lipids should be found among the apolar lipids. In contrast to our data, Sprott et al. (3) demonstrated that liposomes prepared from BCG polar lipids were able to promote CTL and Ab responses while also activating murine BMDC. However, the study by Sprott et al. (3) was only focused on the polar lipids and made no comparison between the polarity of the lipids and the stimulatory effects. In general, there have been very few studies on the whole polarity range of mycobacterial lipids and, in particular, on studies focused on the activity of the highly insoluble apolar lipids. Indeed, we also observed a critical dependence on a vehicle such as DDA liposomes, serving not only to enhance Ag acquisition by APC (32), but also to be able to solubilize the apolar lipids. The incorporation of apolar mycolic acids into the lipid bilayers by heating above the gel-to-liquid crystalline-phase transition temperature of DDA is an attractive formulation method when using the more insoluble (and thereby less accessible) apolar lipids or individual components thereof.

In conclusion, the identification in this study of the mycobacterial-derived proinflammatory lipid, MMG, with strong adjuvant activity makes the development of modern synthetic TB vaccines wherein both the immunostimulant and the pathogen-specific Ag are derived from mycobacteria itself an appealing possibility. Removing all the irrelevant signals from the plethora of molecules and complex lipids of the mycobacterial cell wall and focusing on simple synthetic derivatives of key importance for the innate and adaptive immune response to TB represents in many ways an ideal solution for a vaccine in urgent demand. The encouraging preliminary results with synthetic C32 MMG and C18 MAG demand extensive further studies on various ranges of such analogs.

Acknowledgments
We gratefully acknowledge the very skilful technical help of Linda Chris- tensen, Maria Noertoft, and Harald Dietrich.

Disclosures
Claire A. Swetman Andersen, David E. Minnikin, Gurdyal S. Besra, Peter Andersen and Else Marie Agger are co-inventors on a patent covering MMG for use in adjuvant formulations.

References


