Structural Determination and Toll-like Receptor 2-dependent Proinflammatory Activity of Dimycolyl-di\textit{arbino-}glycerol from \textit{Mycobacterium marinum}*

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Background: Cell wall–associated glycolipids contribute to the pathogenesis of mycobacterial infections.

Results: The structure of dimycolyl-di\textit{arbino-}glycerol (DMAG) from \textit{Mycobacterium marinum} was determined, and this glycolipid stimulated a potent TLR-2-dependent proinflammatory response in macrophages.

Conclusion: These findings strongly suggest that DMAG modulates the host immune system.

Significance: The biological relevance of DMAG activity in immunopathogenesis of mycobacterial infection needs to be addressed.

Although it was identified in the cell wall of several pathogenic mycobacteria, the biological properties of dimycolyl-di\textit{arbino-}glycerol have not been documented yet. In this study an apolar glycolipid, presumably corresponding to dimycolyl-di\textit{arbino-}glycerol, was purified from \textit{Mycobacterium marinum} and subsequently identified as a 5-O-mycyl-\textit{\beta}-\textit{Araf}(1\rightarrow2)-3-O-mycyl-\textit{\alpha}-\textit{Araf}(1\rightarrow1\prime)-\textit{glycerol} (designated \textit{Mma\_DMAG}) using a combination of nuclear magnetic resonance spectroscopy and mass spectrometry analyses. Lipid composition analysis revealed that mycolic acids were dominated by oxygenated mycolates over \textit{\alpha}-mycolates and devoid of trans-cyclopropane functions. Highly purified \textit{Mma\_DMAG} was used to demonstrate its immunomodulatory activity. \textit{Mma\_DMAG} was found to induce the secretion of proinflammatory cytokines (TNF-\textit{\alpha}, IL-8, IL-1\beta) in human macrophage THP-1 cells and to trigger the expression of ICAM-1 and CD40 cell surface antigens. This activation mechanism was dependent on TLR2, but not on TLR4, as demonstrated by (i) the use of neutralizing anti-TLR2 and -TLR4 antibodies and by (ii) the detection of secreted alkaline phosphatase in HEK293 cells co-transfected with the human TLR2 and secreted embryonic alkaline phosphatase reporter genes. In addition, transcriptomic analyses indicated that various genes encoding proinflammatory factors were upregulated after exposure of THP-1 cells to \textit{Mma\_DMAG}. Importantly, a wealth of other regulated genes related to immune and inflammatory responses, including chemokines/cytokines and their respective receptors, adhesion molecules, and metalloproteinas, were found to be modulated by \textit{Mma\_DMAG}. Overall, this study suggests that DMAG may be an active cell wall glycoconjugate driving host-pathogen interactions and participating in the immunopathogenesis of mycobacterial infections.

Tuberculosis, caused by \textit{Mycobacterium tuberculosis}, remains a major threat to worldwide health. The incapacity to eradicate this disease is due in part to the unique mycobacterial cell envelope that plays a crucial role in widespread antibiotic resistance and pathogenesis (1). Phylogenetic studies have shown that \textit{Mycobacterium marinum} \textit{(Mma)}2 is closely related to \textit{Mycobacterium tuberculosis} (2). This species causes systemic tuberculosis-like in fish and other ectotherms involving persistent growth within macrophages (3) and induces “fish tank disease” in humans (4), characterized by the induction of a granulomatous infection. The systemic granulomatous diseases caused by \textit{Mma} in fish share many histological traits with human tuberculosis including the granuloma formation and the ability of \textit{Mma} to persist in a latent state without causing disease (5). Moreover, \textit{M. tuberculosis} and \textit{Mma} share many virulence factors, and in general, \textit{M. tuberculosis} virulence genes can complement orthologous-mutated \textit{Mma} genes (6, 7). Importantly, \textit{Mma} infection of its natural hosts, especially zebrafish, has recently emerged as a useful model to study tuberculosis (5, 6, 8–13). This well-established embryology model is turning into a prominent model for immunological studies and particularly to decipher the interactions between \textit{Mma} in its natural host as well as to address the contribution of

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** This article contains supplemental Tables S1 and S2.

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2 The abbreviations used are: \textit{Mma, M. marinum}; DMAG, dimycolyl-di\textit{arbino-}glycerol; LM, lipomannan; TDM, trehalose-dimycolate; GMG, glucose-monomycoclate; GroMM, glycerol monomycolate; mAGP, mycolyl-arabino-galactan-peptidoglycan; TLR, toll-like-receptor; ICAM-1, intercellular adhesion molecule 1; SEAP, secreted embryonic alkaline phosphatase; TOCSY, two-dimensional total correlation spectroscopy; HSQC, heteronuclear single quantum correlation; LOS, lipo oligosaccharide; PE, phosphatidylethanolamine.
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The mycobacterial cell wall comprises long-chain fatty acids (C_{20}-C_{40}), the mycolic acids, that can be associated to extractable glycolipids or linked to the arabinogalactan-peptidoglycan insoluble backbone to form mycolyl-arabinogalactan-peptidoglycan (mAGP). According to the presence of various chemical functions on the meromycolic chain, mycolic acids are generally subdivided into α-, keto-, and methoxy-mycolates (15). mAGP serves as an anchoring matrix for a vast array of (glyco)lipids that play a critical role in the modulation of the host immune system (16–19). Among them, mycolylated glycolipids such as trehalose-dimycoclates (TDM), glucose-monomycolate (GMM), or glycerol monomycolate (GroMM) have been extensively scrutinized with respect to their structures and immunological properties. TDM for instance is regarded as one of the most bioactive and granulomatogenic cell wall glycolipid, exerting a potent adjuvant effect and playing a key role in mycobacterial virulence, mainly by stimulating both the innate and adaptive immunity (16, 20–22). After exposure to TDM, macrophages produce a broad panel of proinflammatory cytokines (TNF-α, IL1-β, IL-12) and chemokines (IL-8, MCP-1, and MIP-1α) that are essential for granuloma formation in mice and guinea pigs (21–24). Macrophage surface C-type lectin Mincle or Toll-like receptor 2 (TLR2), in combination with MARCO scavenger receptor/CD14 complex, have been demonstrated to interact with TDM (25–28). The two mycolic acids on TDM reflect the mycolic acid composition of the mycobacterial strain from which the TDM is isolated. As such, the chemical structure of TDM varies substantially between strains because the mycolic acid composition differs according to mycobacterial strains. These strain differences provide a natural source of chemically distinct TDM mixtures that can be tested for biological activity. Interestingly, it was demonstrated that mycolic acid composition of TDM influences the inflammatory activity of this glycolipid. Therefore, TDM activities largely depend on the chemical nature of mycolates attached to the trehalose backbone (29, 30).

Two decades ago, another mycolic acid-containing glycolipid, designated dimycoly-diarabino-glycerol (DMAG), was found in the Mycobacterium avium-intracellulare complex and in Mycobacterium kansasi (31, 32). Recently, this amphiphatic hydrophobic glycolipid was identified in the cell wall of other slow-growing mycobacterial species including M. tuberculosis, Mycobacterium bovis BCG, Mycobacterium scrofulaceum, and Mma (33). Structural analyses demonstrated that DMAG from M. bovis BCG is analogous to the terminal portion of mycolyl-arabinogalactan-peptidoglycan, consisting in 5-O-mycyl-β-Araf(1→2)-5-O-mycyl-α-Araf(1→1)-Gro (DMAG) (33). Moreover, exposure to anti-tubercular drugs (thiacetazone, ethambutol) known to interfere with the mAGP and mycolate biosynthesis altered DMAG production in both Mma and M. bovis BCG, leading to the hypothesis of a metabolic relationship between DMAG and mAGP (33). In addition, the presence of anti-DMAG antibodies in patients infected with M. avium strongly suggested that DMAG is an immunogenic compound produced (or released from the cell wall) during infection (34). However, despite the high structural analogy between DMAG and other mycolylated glycolipids, no investigation has been conducted yet regarding the eventual immunomodulatory properties of DMAG and whether this glycoconjugate interacts with the host immune receptors. The relevance of this glycolipid during mycobacterial pathogenesis remains to be fully addressed.

This study was, therefore, undertaken as a first step to decipher DMAG biological properties by focusing on its capacity to modulate the macrophage immune response and the molecular basis for macrophage recognition of DMAG. To extend our previous studies on the structural variability of Mma glycolipids and to gain new insight with respect to their biological functions, we have performed the detailed structural analysis of a family of mycolylated arabin-o-glycerol lipids from Mma, which allowed us to assign three related molecules. The major member of this family, Mma_DMAG, was used to investigate the ability of the arabinosylated glycolipids to induce both proinflammatory cytokine secretion and expression of cell surface antigens in the human macrophage-like differentiated THP-1 cell line via a Toll-like receptor-dependent mechanism. Furthermore, these results were not only confirmed but also extended at a whole transcriptomic level in THP-1 cells exposed to Mma_DMAG. These findings provide the first report on the immunomodulatory activity of this apolar glycoconjugate.

EXPERIMENTAL PROCEDURES

Mycobacteria and Growth Culture Conditions—Mma strain 7 (E7) was originally isolated from butterfly fish (35). It was grown at 30 °C in Sauton’s medium or on plates containing Middlebrook 7H10 agar supplemented with 10% oleic acid, albumin, dextrose, and catalase enrichment.

Mma_DMAG Extraction and Purification—Apolar lipids containing DMAG were extracted from 40 g of wet cells (around 10 liters of culture) of Mma according to established procedures (36). Mma_DMAG was then purified by two successive rounds of absorption chromatography, as reported earlier (33). Briefly, the crude apolar extract was dissolved in chloroform and fractionated by silica gel column flash chromatography. The column was eluted with chloroform and chloroform/methanol with increasing volumes of methanol (1–50%). Purification of the Mma_DMAG was monitored by one-dimensional high performance TLC (Merck) using chloroform/methanol (96:4, v/v) or chloroform/methanol (90:10, v/v). Glycolipids were visualized by spraying plates with orcinol/sulfuric acid reagent followed by charring at 120 °C. Mma_DMAG fractions containing 5–7% of methanol were pooled and applied on a Florisil® column chromatography (Acros Organics). Elution was performed with chloroform followed by chloroform/methanol with increasing volumes of methanol (2–20%). Purification monitored by high performance TLC after spraying with orcinol/sulfuric acid. Mma_DMAG was eluted in fractions containing 7% of methanol and Mma_DMAG-containing fractions were pooled for structural analyses. Finally, the yield for the DMAG was about 770 µg/liter of culture.
Purification of Lipomannan—Lipomannan (LM), a known proinflammatory-inducing factor, was purified from Mma using procedures reported previously (37).

Endotoxin Levels—Potential contamination by endotoxin in Mma_DMAG and LM samples was evaluated using the Limulus amoebocyte lysate assay kit (QCL1000; Cambrex). No endotoxin was detected in Mma_DMAG sample, whereas the LM preparation contained insignificant amounts of endotoxin (<50 pg/10 μg of LM).

Extraction of Mycolic Acids from DMAG—Extraction of mycolic acid methyl esters was carried out as described previously (38, 39). Briefly, purified DMAG were subjected to alkaline hydrolysis in 1 ml of 15% tetrabutylammonium hydroxide at 100 °C overnight. After cooling, free mycolic acids were methyl-esterified by the additions of 2 ml of dichloromethane, 150 μl of iodomethane, and 1 ml of water. The mixture was sonicated and then centrifuged at 3500 rpm for 10 min. The aqueous phase was discarded, and the dichloromethane phase containing mycolic acid methyl esters was washed three times with 2 ml of water. Finally, the organic phase was evaporated under a stream of nitrogen, and the sample was diluted in chloroform before mass spectrometry analysis.

Matrix-assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) Analysis—The molecular masses of glycolipids were measured by MALDI-TOF on a Voyager Elite reflectron mass spectrometer (PerSeptiveBiosystems, Framingham, MA) equipped with a 337-nm UV laser. Samples were prepared by mixing 5 μl of glycolipid solution in chloroform and 5 μl of 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml dissolved in chloroform/methanol (1/1, v/v)). The mixtures (2 μl) were then spotted on the target.

NMR Analysis—NMR experiments were recorded at 300 K on a Bruker Avance 400 spectrometer equipped with a 5-mm broad-band inverse probe, respectively. Before NMR spectroscopic analyses, Mma_DMAG was repeatedly exchanged in CDCl₃/CD3OD (2:1, v/v) (99.97% purity, Euriso-top, Saint-Aubin, France) with intermediate drying and finally dissolved in CDCl₃ as an internal reference. The COSY, TOCSY, and ¹³C HSQC experiments were performed using the Bruker standard sequences.

Cell Culture Conditions—Human pro- monocytic leukemia THP-1 cells (ECACC no. 88081201) were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and 2 × 10⁻⁵ M β-mercaptoethanol in a 5% CO₂/air-humidified atmosphere at 37 °C. Differentiation into macrophage-like cells was induced with 50 nM 1,25-dihydroxyvitamin D₃ for 72 h in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Viability of the cells was checked by trypan blue dye exclusion and by flow cytometry (FACSCalibur flow cytometer) by using propidium iodide. HEK-Blue-hTLR2 cells and HEK-Blue-Null1 cells were purchased from Invivogen (Toulouse, France) and maintained in growth medium supplemented with HEK-blue selection according to the manufacturer’s instructions. HEK-Blue-hTLR2 cells were obtained by co-transfection of the hTLR2 and secreted embryonic alkaline phosphatase (SEAP) reporter genes into human embryonic kidney 293 (HEK293) cells. The SEAP reporter gene was placed under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. HEK-Blue Null1 cells, the parental cell line of HEK-Blue-hTLR2, carry the SEAP reporter gene alone.

Quantification of Cytokine Secretion by ELISA—To investigate the effect of Mma_DMAG on TNF-α, IL-8, and IL-1β secretion, differentiated THP-1 cells were seeded in 96-well plastic culture plates at a density of 25 × 10⁴ cells/well in RPMI 1640 medium supplemented with 2% FCS and L-glutamine. Because Mma_DMAG was insoluble in aqueous medium, it was resuspended in hexane at the indicated concentrations and coated on plates. Wells were subsequently dried at 37 °C to ensure complete solvent evaporation before cell addition. Control wells were layered with solvent without glycolipids. LM purified from Mma, dissolved in pyrogen water, and sonicated was used as a positive control. After 6 or 24 h of incubation, culture supernatants were collected and analyzed for the detection of TNF-α, IL-8, and IL-1β by sandwich ELISA according to the manufacturers’ instructions (Ozyme S.A.). Cytokine concentrations were determined using standard curves obtained with recombinant human TNF-α, IL-1β, or IL-8. Statistical significance was determined using Student’s t test (only values of p < 0.05 were considered to be significant).

Flow Cytometry Analysis—The expression level of the cell surface markers was determined after stimulation of differentiated THP-1 cells (at a density of 3.5 × 10⁴ cells/well) with either Mma_DMAG or LM in RPMI 1640 supplemented with 2% FCS and L-glutamine as previously reported (40). Control wells were layered without glycolipids. After 24 h of incubation, expression of human ICAM-1 (CD54) and CD40 was determined by flow cytometry. Briefly, 250,000 cells were incubated for 20 min at 4 °C with 20 μg/ml human IgG (Sigma), washed 3 times, and incubated for 40 min with 10 μl of PE-conjugated anti-ICAM-1 (CD54) or FITC-conjugated anti-CD40 mouse monoclonal IgG1κ antibodies (BD Biosciences) in PBS containing 0.04% NaN₃ and 0.05% BSA. Both PE- and FITC-conjugated mouse isotype control IgG (BD Biosciences) were used as negative controls. In all experiments, cells were washed twice. Data were monitored on a flow cytometer (FACSCalibur, BD Biosciences) and analyzed with the CellQuest software (Mountain View, CA). Cells were gated for forward- and side-angle light scatter, the fluorescence channels were set on a logarithmic scale, and the mean fluorescence intensity was determined.

TLR Neutralization—To address the participation of TLRs in the proinflammatory-inducing activity of Mma_DMAG, differentiated macrophages were pretreated with 15 μg/ml neutralizing monoclonal against anti-TLR2 or anti-TLR4 for 30 min at 37 °C. Mouse IgG2a anti-TLR2 (clone TL2–1) was purchased from Biolegend, whereas mouse IgG2aκ was from ebioscience. Cells were then stimulated with 20 μg/ml Mma_DMAG for 6 or 24 h to allow TNF-α secretion or ICAM-1 expression, respectively. The corresponding isotype antibodies were used as negative controls. Supernatants were processed for TNF-α and IL-8 quantification by ELISA, whereas ICAM-1 expression was determined by flow cytometry, as described above. Statistical significance between antibody-pretreated cells and untreated cells was calculated by using Student’s t test. Values with p < 0.05 were considered significant.
HEK-TLR2 Experiment—HEK-Blue-hTLR2 that stably expresses the human TLR2 gene along with a NF-κB-inducible reporter system (secreted alkaline phosphatase) and the parental HEK-blue-Null1 cell line were seeded at 5 × 10⁴ cells/well in 96-well plates and stimulated with either 20 μg/ml Mma_DMAG or 20 ng/ml lipopolysaccharide (LPS) (PamCys(SKKKK)₅(Pam-CSK₄), EMC Microcollections GmbH, Germany), a well known TLR2 agonist. Stimulation with a TLR2 ligand activates NF-κB and AP-1, which induce production of SEAP. After 20 h of incubation at 37 °C, SEAP activity was determined using the QUANTI-Blue detection kit by measuring the absorbance at 630 nm. Statistical significance of between the unstimulated and stimulated HEK-Blue-hTLR2 cells was determined using Student’s t test, and only p < 0.05 was considered significant.

Microarrays Data Processing and Statistical Analyses—Differentially expressed THP-1 cells (3 × 10⁶) were incubated for 8 h either with medium alone or medium supplemented with 20 μg/ml Mma_DMAG. Total RNA was extracted from the cells using the Nucleospin RNA II kit (Macherey-Nagel, Düren), according to the manufacturer’s instructions. RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Agilent Technologies). All RNA samples had RNA quality assessed using Agilent RNA Nano 6000 LabChip kits and an Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples had RNA integrity numbers of 9.7 or higher. A human gene expression microarray (Agilent Technologies) was used for global gene expression analysis. 2 Acidic, mycolyl-α-galactan or mycolic acid biosynthesis (33). Structural elucidation of this product in Mycobacterium marinum DMAG—We have recently shown that several slow-growing mycobacterial species, including M. bovis BCG, M. tuberculosis, and Mma produce an apolar cell wall-associated glycolipid whose synthesis was altered by several anti-tubercular drugs inhibiting arabinogalactan or mycolic acid biosynthesis (33). Structural elucidation of this product in M. bovis BCG identified it as 5-O-mycoaryl-β-Araf(1→2)-5-O-mycoaryl-α-Araf(1→3)-Gro, designated di-mycoaryl di-arabino-glycerol (DMAG). To extend these studies regarding the structural diversity of DMAG and to undertake structure/function relationship studies, we purified and determined the fine structure of DMAG in Mma strain 7. This natural strain, originally isolated from infected butterfly fish, has previously been reported to exhibit an altered lipopolysaccharide (LOS) profile compared with the standard M strain (45). As reported earlier, a glycolipid presumably corresponding to DMAG was purified from the apolar extract by adsorption chromatography on a silica gel column using a gradient of methanol in chloroform. This component yielded a major orcinol-reactive component with an Rf of 0.87 compared with 0.38 for TDM on TLC plates using chloroform/methanol (96:4; v/v) as a running solvent (Fig. 1A). In addition, two minor undefined glycolipids (Rf of 0.49 and 0.21, respectively) with chromatographic mobilities closer to TDM (Rf of 0.38) were also tentatively attributed to arabino-containing glycolipids based on their distinctive intense blue color upon orcinol staining.

MALDI-TOF-MS analysis indicated that the DMAG from Mma (Mma_DMAG) exhibited a heterogeneous molecular mass profile ranging from 2577 to 2947 Da, with the most abundant molecular species at 2790 and 2818 Da (Fig. 1B). MS analysis of the methyl-esterified lipid moiety released from Mma_DMAG by alkaline hydrolysis revealed a heterogeneous pattern attributed to a mixture of alpha (α), keto (k), and methoxy-mycolates (m) with m/z values ranging from 1132 (αC₉₇γ) to 1346 (mC₉₇γ) and dominated by two signals at m/z 1274 and 1302 attributed to [M + Na]+ adducts of C₉₄ and C₉₆ ketomycolates (Fig. 1C). Consistent with the prevalence of keto- and methoxy-mycolates over α-mycolates, Mma_DMAG subspecies were mainly found to be substituted by oxygenated mycolates. Indeed, based on the tentative presence of a Ara-Gro moiety, mycolate composition of major Mma_DMAG signal clusters around m/z 2790 and m/z 2818 were assigned to DMAGs substituted by a mixture of keto- and methoxy-mycolates bearing totals of 166 and 168 carbons, respectively. The presence of oxygenated keto- and methoxy-mycolates was confirmed by ¹H NMR analysis of intact Mma_DMAG (Fig. 1D). Furthermore, ¹H NMR analysis allowed us to detect the presence of trans- and cis-ethylene groups as well as cis-cyclopropane groups. The presence of cis-cyclopropane was established by the observation of CH₂ signals at δ = 0.33 and 0.57, whereas trans-cyclopropane functional groups could not be observed, in agreement with previous observations on total mycolic acids from Mma (46). The structure of the glycan moiety of Mma_DMAG was next investigated by ¹H/¹H COSY, TOCSY, and ¹³C/¹H HSQC NMR experiments, allowing us to attribute its individual ¹H NMR parameters based on those previously assigned for M. bovis BCG DMAG (Fig. 2, Table 1) (33). As observed in Fig. 2, two anomer signals were identified at ¹H/¹H δ 4.99/106.0 and δ 5.02/101.5 ppm, thus confirming the presence of two different monosaccharides. Their spin systems determined by ¹H/¹H NMR experiments typified them as 5-acetylated α-Araf and β-Araf residues, respectively (Fig. 2A). The deshielding of α-Araf-C2 (Δδ = +6.0) compared with that of non-reducing terminal α-Araf established that this residue was substituted in position C2 (Fig. 2B). Finally, the identification of a C1-substituted glycerol residue (Gro) confirmed the structure of Mma_DMAG as 5-O-mycaryl-β-Araf(1→2)-5-O-mycaryl-
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**FIGURE 1. Structural analysis of Mma_DMAG.** A, a TLC profile of purified arabino-glycero lipids from Mma shows the major compound identified as di-mycolyl di-arabino-glycerol (Mma_DMAG) and two minor related glycoconjugates identified as mono-mycolyl mono-arabino-glycerol (X1) and mono-mycolyl di-arabino-glycerol (X2). Rf are indicated in the left margin. MALDI-TOF-MS spectrum of intact Mma_DMAG (B) and mycolic acid methyl esters (MAMES) (C) derived from Mma_DMAG show the presence of α-, keto-, and methoxy-mycolic acids. D, 1H NMR spectrum of Mma_DMAG; a to n indicate the relevant signals used for the identification of functional groups of mycolates.

α-Araf-(1→1)-Gro. Although not demonstrated in this study, the arabinose residues are believed to be in a α configuration based on the exclusive identification of α-Ara in all mycobacterial glycoconjugates so far and because of its postulated filiation with mAGP (33). Overall, this analysis indicates that DMAG from *Mma* is extremely similar to the one from *M. bovis* BCG, with the notable exception for its lipid moiety comprising a mixture of α-, keto- and methoxy-mycolates in *Mma* instead of α- and keto-mycolates only in *M. bovis* BCG. In addition, mycolates of Mma_DMAG lack a trans-cyclopropane ring. Along with Mma_DMAG, the partial structural analysis of the two minor arabinose-containing glycolipids (Fig. 1A) permitted their identification as 5-O-mycolyl-α-Araf-(1→1)-Gro and a mixture of β-Araf-(1→2)-5-O-mycolyl-α-Araf-(1→1)-Gro and 5-O-mycolyl-β-Araf-(1→2)-α-Araf-(1→1)-Gro, respectively (data not shown). However, the limited amount of each of these compounds precluded further biological analyses.

**Proinflammatory Activity of Mma_DMAG**—A plethora of reports demonstrated the immunomodulatory properties of cell wall-associated mycolylated glycolipids, which may represent key effectors in the induction of the host defense through the activation of macrophage and antigen presenting cells. Most studies have focused on TDM, GroMM, and GMM (22, 27, 47). Considering the structural similarity between these lipids with DMAG regarding their mycolic acid composition, we reasoned that the DMAG family of glycolipids may also participate to the modulation the host immune response. To check this hypothesis, we first evaluated the ability of Mma_DMAG to induce the secretion of proinflammatory cytokines in macrophages and to stimulate the expression of cell surface antigens.

TNF-α is a key mediator involved in the initiation and the maintenance of the granulomatous response (48, 49), whereas IL-1β represents another important proinflammatory cytokine that contributes to anti-mycobacterial host defense mechanisms (50), whose production is induced by *M. tuberculosis* through different pathways involving TLR2/TLR6 and NOD2 receptors (51). IL-8 mediates the recruitment of neutrophiles during mycobacterial infection (49). The capacity of Mma_DMAG to trigger TNF-α, IL-1β, and IL-8 secretion was investigated on differentiated THP-1 cells, which have been extensively used to test the biological effects of mycobacterial glycolipids (52, 53). Mma_DMAG was found to induce the release of TNF-α (Fig. 3A), IL-1β (Fig. 3B), and IL-8 (Fig. 3C) from differentiated cells. The optimal response regarding TNF-α secretion was achieved with 30 μg/ml Mma_DMAG. The level of cytokines induced by DMAG was about 18-fold higher for TNF-α and 30-fold higher for IL1-β compared with un-stimulated cells (medium alone). IL-8 production was also significantly increased (9-fold) in the presence of various concentrations of DMAG (Fig. 3C). LM purified from *Mma* was included as a positive control because it has been reported to exhibit a significant proinflammatory response (52–55). Mma_DMAG exhibited, however a lower proinflammatory activity compared with LM (Fig. 3).

The interaction of ICAM-1 (CD54) with α1/β2 integrin (CD11a/CD18) leads to the activation and the proliferation of T cells, whereas the co-stimulatory protein CD40 is required for the activation of antigen presenting cells (56). To address whether DMAG stimulates the expression of macrophage cell surface markers, THP-1 macrophages were incubated with either LM or Mma_DMAG and analyzed by flow cytometry using either PE-conjugated anti-ICAM-1 or FITC-conjugated anti-CD40 antibodies. As shown in Fig. 4, Mma_DMAG induces the expression of both ICAM-1 and CD40 at the sur-
face of THP-1 cells, albeit to a lesser extent than LM. As expected, no significant fluorescence signal could be detected using isotype control antibodies.

Overall, these results indicate that, like other mycolic acid-containing glycolipids, DMAG is a biologically active molecule exhibiting potent proinflammatory activity and promoting macrophage activation, both being relevant to macrophage-lymphocyte interaction/recruitment and mycobacteria-induced immunopathogenesis.

Macrophage Activation and Proinflammatory Response by DMAG Are Dependent on TLR-2—TLRs are known as key receptors for promoting the inflammatory immune response during microbial infection (55). TLR2 recognizes mycolic acid-containing lipids, such as TDM, in combination with other receptors (MARCO/CD14) present on macrophages (26). We next investigated the possible participation of TLR2 and/or TLR4 in the signaling pathway leading to THP-1 activation by Mma_DMAG. Production of inflammatory cytokines/chemokines by THP-1 cells as well as expression of cell surface receptors were followed by incubating cells with specific neutralizing TLR2 or TLR4 antibodies or their corresponding isotype control antibodies. As shown in Fig. 5 A, pretreatment with anti-TLR2 antibodies, but not with anti-TLR4 or isotype control antibodies, was accompanied by a strong decrease in TNF-α and IL-8 secretion (with 71 and 62% inhibition in the presence of anti-TLR2 antibodies, respectively). Consistent with these findings, neutralization of TLR2 was also associated with a decrease in ICAM-1 cell surface expression induced by Mma_DMAG (Fig. 5 B). This effect was not observed when cells were pretreated with anti-TLR4 antibodies.

To further confirm the involvement of TLR2 in Mma_DMAG-induced activity, HEK293-TLR2 cells co-transfected with both the human TLR2 gene and the SEAP-inducible reporter system were stimulated for 20 h with Mma_DMAG. Lipopeptide Pam3CSK4 and LM were included as positive TLR2 agonists. As judged by QUANTI-Blue detection, Mma_DMAG exhibited dose-dependent NF-κB activation in HEK293-TLR2 cells (Fig. 5 C). As expected, no induction was observed after stimulation of the parental HEK-Blue Null1 cells, thus demonstrating the specificity of the TLR2-dependent effect. It is noteworthy that the level of NF-κB activation

**TABLE 1**

| H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 |
|--------|--------|--------|--------|--------|
| β-Araf | α-Araf |
| 5.02/101.5 | 4.07/77.8 | 4.06/76.6 | 4.07/79.6 | 4.18, 4.52/65.2 |

**FIGURE 2.** NMR analysis of the Mma_DMAG glycan moiety. $^{1}$H/$^{1}$H TOCSY (A) and $^{13}$C/$^{1}$H HSQC (B) NMR spectra of Mma_DMAG permitted us to establish the structure of Mma_DMAG as 5-O-mycolyl-β-Araf(1→2)-5-O-mycolyl-α-Araf(1→1")-Gro.
observed was lower with Mma_DMAG than with LM or Pam3CSK4. Taken together, these results clearly reveal that Mma_DMAG exerts its effects through ligation to TLR2, leading to the production of a prominent proinflammatory response and to macrophage activation.

Transcriptomic Analyses of Mma_DMAG-stimulated THP-1 Macrophages—To gain insight toward the mechanisms by which DMAG interferes with the host immune system, THP-1 gene expression was monitored at a transcriptional scale using whole human genome cDNA microarray after stimulation with Mma_DMAG. The binary logarithm of Mma_DMAG-stimulated cells versus nontreated cells ratios were considered, with adjusted p values <0.01. The statistical transcriptomic analysis from two independent experiments showed the alteration of 547 genes after 8 h of incubation (supplemental Table S1). The selected genes were classified in functional groups and according to gene ontology using PANTHER (supplemental Table S2).

Compared with a NCBI Homo sapiens reference list, the differentially regulated genes (corresponding to significant increased or decreased transcription levels) revealed that some biological processes were significantly over-represented (p value < 0.05). This was particularly the case for processes related to immune system (167 genes of 547), response to stimuli, cell surface receptor-linked signal transduction pathways and intracellular signaling cascade, cell communication, metabolic systems (particularly nucleic acid and lipid metabolisms), and developmental stages. Interestingly, several overexpressed genes involved in immune responses were relevant to macrophage activation, cell adhesion, endocytosis, apoptosis, angiogenesis, and response to stress and to IFN-γ. Cell signaling pathways analysis led to the identification of genes involved in inflammatory mechanisms mediated by chemokines/cytokines and TLR, apoptosis regulation (pro- or anti-), integrin and heterotrimeric G protein signaling pathways, and oxidative stress response as well as growth factors activities. Molecular function and protein class Gene ontology analyses indicated the enrichment of chemokines/cytokines, signaling molecules, transcription factors, enzymes (transferase, hydrolase, kinase, phosphatase, oxygenase), and receptors activities. As reported in Table 2, IL-8, TNF-α, and IL-1β were among the most highly expressed gene candidates (up-regulated 17, 10, and 45 times, respectively, compared with the nontreated macrophages), thus confirming our ELISA results (Fig. 3). In addition, several other genes from the “cytokine/chemokine” family were strongly up-regulated, with a sharp preference for the CCL and CXCL chemotactic factors. Several cell surface markers were also strongly up-regulated,
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FIGURE 5. TLR2-dependent activity of Mma_DMAG. Differentiated THP-1 cells were pretreated with 15 μg/ml concentrations of either anti-TLR2, anti-TLR4, or control isotype monoclonal antibodies (Ab) for 30 min at 37 °C in 2% FCS-RPMI 1640 medium before the addition of 20 μg/ml Mma_DMAG. Supernatants were collected after 6 or 24 h and assayed by ELISA for both TNF-α and IL-8 productions. Values represent the means ± S.D. of triplicates.

In conclusion, the microarray analysis not only confirms the proinflammatory-inducing activity of Mma_DMAG but also suggests that Mma_DMAG affects expression of a large panoply of macrophage genes that are connected to the immunopathogenesis of mycobacterial infections, with important pathways related to signaling events, inflammation, lipid antigen presentation, or tissue destruction.

DISCUSSION

Based on structural analogy with mAGP and studies on the action of anti-tubercular drugs, a metabolic relationship between DMAG from M. bovis BCG and mAGP has recently been highlighted (33). However, neither the metabolic pathway leading to DMAG nor its potential biological properties has been reported yet. We present here the detailed structural elucidation of the 5-O-mycyl-β-Araf-(1→2)-5-O-mycyl-α-Araf-(1→1)-Gro (DMAG) in Mma and provide the first biological functions of this cell wall-associated glycolipid. A combination of NMR spectroscopy and mass spectrometry revealed the presence of three mycolate subclasses (i.e., methoxy-mycolates and methoxy-mycolic acids) in Mma_DMAG (33). Furthermore, in contrast to M. tuberculosis that contains a ratio of α-mycylates/oxygenated mycolates (i.e. methoxy- and keto-mycylates) of 1/1 (59), oxygenated mycolates are predominant in Mma_DMAG. It is noteworthy that exogenous glycerol is required to stimulate DMAG production (33) as reported earlier for another glycolipid, GroMM (60). These observations and unstimulated HEK-Blue-tLR2 cells was determined using Student’s t test (p values were <0.03).
### TABLE 2

| Gene                      | Description                                      | Log<sub>2</sub> ratio |
|---------------------------|--------------------------------------------------|-----------------------|
| **Cell surface antigen**  |                                                  |                       |
| ICAM1                     | Intercellular adhesion molecule 1                | 4.20                  |
| VCAM1                     | Vascular cell adhesion molecule 1                | 2.84                  |
| CD40                      | CD40 molecule, TNF receptor superfamily member 5 | 2.49                  |
| CD83                      | CD83 molecule                                    | 3.06                  |
| CD44                      | CD44 molecule (Indian blood group)               | 2.07                  |
| ITGB8                     | Integrin, 8                                      | 2.04                  |
| SLAMF7                    | Signaling lymphocytic activation molecule family member 7 | 3.86 |
| **Protease/metalloproteinase** |                                              |                       |
| MMP1                      | Matrix metallopeptidase 1 (interstitial collagenase) | 4.95                  |
| MMP9                      | Matrix metallopeptidase9 (gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase) | 3.25                  |
| PCSK5                     | Proprotein convertase subtilisin/kexin type 5    | 2.91                  |
| **Chemokines-cytokines**  |                                                  |                       |
| CCL8 (MCP-2)              | Chemokine ligand 8                               | 5.94                  |
| CCL2 (MCP-1)              | Chemokine ligand 2                               | 4.91                  |
| CXCL1                     | Chemokine ligand 1                               | 4.62                  |
| CCL4 (MIP-1)              | Chemokine ligand 4                               | 4.31                  |
| CCL20                     | Chemokine ligand 20                              | 4.07                  |
| CXCL11                    | Chemokine ligand 11                              | 4.00                  |
| CXCL13 (GRO)              | Chemokine ligand 3                               | 3.70                  |
| CXCL10                    | Chemokine ligand 10                              | 3.58                  |
| CCL7                      | Chemokine ligand 7                               | 3.41                  |
| CCL3L3                    | Chemokine ligand 3-like                         | 3.37                  |
| CXCL9                     | Chemokine ligand 9                               | 2.02                  |
| IL-8                      | Interleukin 8                                    | 4.11                  |
| IL-1β                     | Interleukin 1, β                                 | 5.54                  |
| TNFα                      | Tumor necrosis factor                            | 3.33                  |
| IL-1α                     | Interleukin 1, α                                 | 2.42                  |
| **Cytokine receptors**    |                                                  |                       |
| CCR7                      | Chemokine (C-C motif) receptor 7                 | 4.18                  |
| IL18RAP                   | Interleukin 18 receptor accessory protein         | 3.17                  |
| CMKLR1                    | Chemokine-like receptor 1                        | 2.37                  |
| **Apoptosis**             |                                                  |                       |
| BCL2A1                    | BCL2-related protein A1                          | 3.34                  |
| TNFAIP3                   | Tumor necrosis factor, α-induced protein 3       | 3.25                  |
| IER3                      | Immediate early response 3                       | 3.33                  |
| **Redox-enzymes**         |                                                  |                       |
| CYP19A1                   | Cytochrome P450, family 19, subfamily A, polypeptide 1 | 3.27                  |
| SOD2                      | Superoxide dismutase 2, mitochondrial             | 3.18                  |
| **Cell signaling pathways/nuclear factors** |                                      |                       |
| RGS1                      | Regulator of G-protein signaling 1               | 4.01                  |
| NRAFT3                    | Nuclear receptor subfamily A, group A, member 3   | 3.92                  |
| RND3                      | Rho family GTPase 3                              | 3.70                  |
| TNIP3                     | TNFAIP3 interacting protein 3                    | 3.23                  |
| TNFAIP3                   | Tumor necrosis factor, α-induced protein 3       | 3.25                  |
| RCAN1                     | Regulator of calcineurin 1                       | 3.16                  |
| SOCS3                     | Suppressor of cytokine signaling 3               | 3.09                  |
| DUSP1                     | Dual specificity phosphatase 1                   | 3.04                  |
| RGS16                     | Regulator of G-protein signaling 16              | 2.96                  |
| STAT4                     | Signal transducer and activator of transcription 4 | 2.73                  |
| ATF3                      | Activating transcription factor 3                | 2.75                  |
| TRAF1                     | TNF receptor-associated factor 1                 | 2.68                  |
| BCL3                      | B-cell CLL/lymphoma 3                           | 2.28                  |
| SPHK1                     | Sphingosine kinase 1                             | 2.16                  |
| NFKB2                     | Nuclear factor of κ light polypeptide gene enhancer | 2.10 |
| NFKB1                     | Nuclear factor of κ light polypeptide gene enhancer | 2.19 |
| NFKB12                    | Nuclear factor of κ light polypeptide gene enhancer | 2.46 |
| **IFN-inducible protein** |                                                  |                       |
| IFIT3                     | Interferon-induced protein                       | 3.24                  |
| IFIT2                     | Interferon-induced protein                       | 2.50                  |
| IFIT1                     | Interferon-induced protein                       | 2.4                   |
| IFIT5                     | Interferon-induced protein                       | 2.17                  |
| **Transporters/channels** |                                                  |                       |
| AQP9                      | Aquaporin 9                                      | 3.66                  |
| SLC7A11                   | Solute carrier family 7                          | 2.51                  |
| SLC7A11                   | ATPase, Ca<sup>2+</sup> transporting, plasma membrane 1 | 2.27 |
| **Lipid<sup>a</sup> and amino acid<sup>b</sup> metabolism** |                             |                       |
| FABP4<sup>a</sup>         | Fatty acid-binding protein 4                     | 3.38                  |
| AGPAT9<sup>a</sup>        | 1-Acylglycerol-3-phosphate O-acyltransferase 9   | 3.13                  |
| PTGS2<sup>a</sup>         | Prostaglandin-endoperoxide synthase 2            | 3.17                  |
| MGLL<sup>a</sup>          | Monoglyceride lipase                             | 2.07                  |
| PLA2G7<sup>g</sup>        | Phospholipase A2, group VII                      | 2.78                  |
have led to the speculation that DMAG may result from the catabolism of already-synthesized mAGP following the action of an arabinoanase and subsequent transfer onto an endo- or exogenous substrate, such as glycerol (33). During infection of foamy macrophages, dynamic changes in the glycolipid composition of the mycobacterial cell wall have been reported to occur (61, 62). That these mechanisms may be responsible in vivo for the production of DMAG during infection remains, however, to be demonstrated.

Considering the high structural analogy between DMAG and TDM as well as its localization to the mycobacterial cell wall (presumably surface-exposed), we reasoned that DMAG may share with TDM several traits that are relevant to mycobacterial pathogenesis, such as proinflammatory cytokine production and formation of granuloma and tissue-destructive lesions (63). Mma_DMAG was found to stimulate a potent macrophage activation and formation of granuloma and tissue-destructive lesions typical to acute inflammatory response (64, 65). Our findings propose to participate to acute inflammatory response, essentially as a consequence of a constant and massive recruitment of polymorphonuclear leukocytes (69). Furthermore, cis- or trans-cyclopropanated mycolic acids in TDM were found to directly regulate the innate immune activation of macrophages by modulating TNF-α production (29, 71). Interestingly, the molecular structure of mycolic acid seems to influence the pattern of inflammatory response (72). Thus, TDM lacking trans-cyclopropane rings purified from a M. tuberculosis strain were more potent in stimulating macrophages than TDM obtained from the wild-type strain (29, 71). In contrast, cis-M. tuberculosis/ H9251/cmaA2/CWS (66) as well as synthesized arabino-mycolates (67) have been shown to induce TNF-α production in murine macrophage cell lines. Moreover, these compounds, described as potent adjuvant in vivo, enhanced delayed type hypersensitivity reactions against inactivated tumor cells (66). A 5-mycoloyl diarabinoside isolated from the cell wall of M. tuberculosis was also reported to act as potential endotoxins through an inhibitory activity on mitochondrial oxidative phosphorylation (68). Arabinosylated mycolic acid isolated from M. bovis BCG were proposed to participate to acute inflammatory response, essentially as a consequence of a constant and massive recruitment of polymorphonuclear leukocytes (69).

### Table 2—continued

| Gene       | Description                                                                 | Log₂ ratio* |
|------------|-----------------------------------------------------------------------------|-------------|
| ACSL1*     | Acyl-CoA synthetase long-chain family member 1                              | 2.36        |
| LRP12*     | Low density lipoprotein receptor-related protein 12                         | 2.11        |
| IDO1*      | Indoleamine 2,3-dioxygenase 1 (Tryp catalabolism)                            | 2.78        |
| KYNU       | Kynureninase (l-kynurene hydrolase)                                        | 2.36        |
| CHST7      | Carbohydrate (N-acetylglucosamine-6-O) sulnotransferase 7                   | 2.42        |
| CHST2      | Carbohydrate (N-acetylglucosamine-6-O) sulnotransferase 2                   | 2.12        |
| NEU14      | Sialidase 4                                                                 | 2.26        |
| SDC4       | Syndecan 4                                                                  | 2.22        |
| PDE4B      | Phosphodiesterase 4B, cAMP-specific                                          | 2.59        |
| NTSE       | 5’-Nucleotidase, ecto (CD73)                                                | 2.33        |
| NAMPT      | Nicotinamide phosphoribosyltransferase                                       | 2.27        |
| ADORA2A    | Adenosine A2a receptor                                                       | 2.85        |
| ADDAD2     | Adenosine deaminase domain containing 2                                     | 2.83        |
| EBI3       | Epstein-Barr virus-induced 3                                                | 3.65        |
| ECR3       | Early growth response 2                                                      | 3.29        |
| SERPINE2   | Serpin peptidase inhibitor, serpin peptidase inhibitor, clade E             | 3.24        |
| HBEGF      | Heparin binding EGF-like growth factor                                       | 2.03        |

* Binary log ratio of altered gene expression according to microarray analysis of macrophages after exposure for 8 h with DMAG compared to unstimulated cells. Results with a log₂ ratio >2 are shown and are representative of two independent experiments.

* Genes related to lipid metabolism.

* Genes related to amino acid metabolism.

### Table 2—continued

| Gene       | Description                                                                 | Log₂ ratio* |
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| CHST7      | Carbohydrate (N-acetylglucosamine-6-O) sulnotransferase 7                   | 2.42        |
| CHST2      | Carbohydrate (N-acetylglucosamine-6-O) sulnotransferase 2                   | 2.12        |
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| SDC4       | Syndecan 4                                                                  | 2.22        |
| PDE4B      | Phosphodiesterase 4B, cAMP-specific                                          | 2.59        |
| NTSE       | 5’-Nucleotidase, ecto (CD73)                                                | 2.33        |
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* Binary log ratio of altered gene expression according to microarray analysis of macrophages after exposure for 8 h with DMAG compared to unstimulated cells. Results with a log₂ ratio >2 are shown and are representative of two independent experiments.

* Genes related to lipid metabolism.

* Genes related to amino acid metabolism.
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were inactive (72). Therefore, the structure of three subclasses of cis-cyclopropanated mycolates (keto, methoxy, and α) found in Mma_DMAG and the lack of trans-cyclopropanation are in favor of our biological results, demonstrating that DMAG may display an inflammatory pattern on the immune innate response. From a mechanistic point of view, our results indicate that DMAG-induced activation of macrophages is dependent on TLR2, presumably through an interaction with mycolic acids, as reported for arabinono-mycolates of M. bovis BCG (BCG-CWS) (66). However, because both TNF-α and IL-8 responses were partially inhibited in the presence of neutralizing TLR-2 antibodies, one can presume that host recognition of DMAG involves additional receptors. This was reported for TDM, which binds to multiple receptors, including TLR2/MARCO/CD14 complex or the Mincle C-type lectin (25–28). However, that DMAG binds to Mincle is very unlikely, as the trehalose unit of TDM has been demonstrated to be crucial to the binding of TDM to Mincle (25, 27–29).

Besides TDM and arabinose monomycolate, other glycolipids display structural similarity to DMAG, such GroMM. GroMM purified from M. tuberculosis was presented as an antigentic glycolipid, potent stimulator of CD1b-restricted CD4+ T cell clones (47). Presentation of free mycolates, GMM or GroMM by CD1 molecules triggers the T cell responses, leading to the development of acquired immunity against mycobacteria (61, 74, 75). GroMM induces eosphilinophil hypersensitivity responses in guinea pigs, which led the authors to predict that the host response to this lipid produced by dormant mycobacteria contributes to their survival in the host through the expression of Thelper (Th)-2 type cytokines, such as IL-5 and IL-10 (60). GroMM biosynthesis pathway has not been elucidated yet; thus, the metabolic relationship between DMAG and GroMM cannot be inferred. Interestingly, we established that activation of differentiated THP-1 cells by Mma_DMAG was accompanied by the down-regulation of CD1d transcript, an antigen restricted to natural killer T cells activation. Although this result has to be confirmed experimentally, it suggests that DMAG could also modulate the effectiveness of lipide antigen presentation to natural killer T cells. In agreement with our results, Roura-Mir et al. (76) previously demonstrated that the mycobacterial cell wall lipids of M. tuberculosis that activate human monocytes through TLR2 up-regulated CD1a, CD1b, and CD1c gene and protein expression, whereas CD1d transcripts decreased on the first day after exposure to the lipids.

Overall, this study illustrates the broad inflammatory activity of Mma_DMAG. As such, it describes a new partner in the growing list of mycobacterial cell wall components able to modulate the host immune response. Whether this activity ultimately leads to the recruitment of immune cells, eventually conditioning the outcome of the infection, remains to be established. Future work will be dedicated to address the in vivo biological relevance of DMAG activity and its potential participation in formation and/or maintenance of the granuloma. This is now possible thanks to the use of zebrafish embryos, which enable the investigation the Mma infection process at a spatiotemporal level (3, 77). As an example of application, phagocyte recruitments and granuloma formation could be readily visualized in real time by intravenously injecting Mma_DMAG-conjugated fluorescent beads in zebrafish embryos.

Recent reports have highlighted the possibility of producing novel classes of chemically defined lipid adjuvants (66, 70). For instance, the immunostimulatory activities of arabino-mycolates (65) and GroMM from M. bovis BCG (70) have been proposed to enhance the activity of new vaccine formulations. Based on the structural similarity between these compounds and DMAG, one can presume that DMAG elicits a potent adjuvant activity to be exploited, although this perspective of application requires a more extensive characterization of DMAG immunostimulatory properties.

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