Acylation Determines the Toll-like receptor (TLR)-dependent Positive Versus TLR2-, Mannose Receptor-, and SIGNR1-independent Negative Regulation of Pro-inflammatory Cytokines by Mycobacterial Lipomannan*

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Mycobacterium tuberculosis lipomannans (LMs) modulate the host innate immune response. The total fraction of Mycobacterium bovis BCG LM was shown both to induce macrophage activation and pro-inflammatory cytokines through Toll-like receptor 2 (TLR2) and to inhibit pro-inflammatory cytokine production by lipopolysaccharide (LPS)-activated macrophages through a TLR2-dependent pathway. The pro-inflammatory activity was attributed to tri- and tetra-acylated forms of BCG LM but not the mono- and di-acylated ones. Here, we further characterize the negative activities of M. bovis BCG LM on primary murine macrophage activation. We show that di-acylated LMs exhibit a potent inhibitory effect on cytokine and NO secretion by LPS-activated macrophages. The inhibitory activity of mycobacterial mannose-capped lipoarabinomannans on human phagocytes was previously attributed to their binding to the C-type lectins mannose receptor or specific intracellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN). However, we found that di-acylated LM inhibition of LPS-induced tumor necrosis factor secretion by murine macrophages was independent of TLR2, mannose receptor, or the murine ortholog SIGNR1. We further determined that tri-acyl-LM, an agonist of TLR2/TLR1, promoted interleukin-12 p40 and NO secretion through the adaptor proteins MyD88 and TIRAP, whereas the fraction containing tetra-acylated LM activated macrophages in a MyD88-dependent fashion, mostly through TLR4. TLR4-dependent pro-inflammatory activity was also seen with M. tuberculosis LM, composed mostly of tri-acylated LM, suggesting that acylation degree per se might not be sufficient to determine TLR2 versus TLR4 usage. Therefore, LM acylation pattern determines the anti-inflammatory versus pro-inflammatory effects of LM through different pattern recognition receptors or signaling pathways and may represent an additional mean of regulating the host innate immunity by mycobacteria.

Control of Mycobacterium tuberculosis infection involves both phagocytes and T cell-mediated innate and adaptive immune responses (for review, see Refs. 1–3), and interactions between the bacillus and host phagocytes, macrophages and dendritic cells, are central to both immunity to M. tuberculosis and tuberculosis pathogenesis. In the lungs alveolar macrophages are primary host cells for M. tuberculosis, which has evolved mechanisms to persist and multiply within these cells. Dendritic cells are critical to carry mycobacterial antigens from the infection site to the draining lymph nodes and establish an efficacious T cell-mediated immune response. In addition, macrophages and dendritic cells participate in modulation of the innate immune response by secreting cytokines after recognition of microbial motives through various pattern recognition receptors. Cytokines such as TNF2 are an integral part of the pathological process, with induction of cachexia and necrosis, but TNF is also an essential mediator for granuloma formation and containment of M. tuberculosis infection. Similarly IL-12, a cytokine that polarizes T lymphocytes toward a protective interferon (IFN)-γ-secreting type 1 profile, IFNγ, but also IL-1, IL-18, IL-23, lymphotoxin α (LTα), LTβ, and nitric oxide are required for host defense as demonstrated both in murine experimental tuberculosis models (1–3, 77) and in some clinical situations (4, 5). These mediators may be important for controlling the infection during latency, as neutralization of TNF or inducible nitric-oxide synthase inhibition leads to a flare of the infection (6–8), as also seen after TNF-neutralizing therapies (9, 10). Phagocytes also produce immunomodulatory cytokines such as IL-10 and transforming growth factor-β that dampen the immune response and inflammation. Tuberculosis protection versus pathogenesis, thus, likely relies on a fine equilibrium between pro- and anti-inflammatory cytokines. How M. tuberculosis interferes with these inflammatory and immunomodulatory networks is still not fully understood. A better comprehension of the molecular mechanisms by which the tubercle modulates such immune responses should help in understanding the disease.

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2 The abbreviations used are: LAM, lipoarabinomannan; DC-SIGN, dendritic cell-specific intracellular adhesion molecule-3 grabbing nonintegrin; LM, lipomannan; ManLAM, mannose-capped LM; MyD88, myeloid differentiation protein 88; TLR, Toll-like receptor; PIM, phosphatidylinositol mannoside; LPS, lipopolysaccharide; IL, interleukin; MALDI-MS, matrix-assisted laser desorption ionization-mass spectroscopy.
the design of new strategies to prevent or treat tuberculosis primary infection or reactivation.

Macrophages and dendritic cells recognize mycobacterial structural motifs through various pattern recognition receptors, including Toll-like receptors (TLRs) (11–14) and C-type lectins such as the mannose receptor (CD207) and the dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN/CD209). M. tuberculosis binds to and is internalized by human dendritic cells through DC-SIGN that recognizes mannose-capped lipoarabinomannan (ManLAM), lipomannans (LMs), and other ligands within the mycobacterial envelope (15–18). DC-SIGN is also expressed by alveolar macrophages in tuberculosis patients (19). Interactions between M. tuberculosis and DC-SIGN on human dendritic cells lead to secretion of the anti-inflammatory cytokine IL-10 and partial deactivation of the cells (20).

Mycobacterial lipoglycans such as ManLAM and LM may contribute to modulate the regulation of macrophage and dendritic cell activation and, thus, control the inflammatory response. Mannose receptor and more recently DC-SIGN have been proposed to mediate the inhibition by ManLAM of LPS-induced IL-12 production in dendritic cells (20, 21). We showed previously that mycobacterial LMs have a dual potential for pro-inflammatory and anti-inflammatory effects. The stimulatory effect of LM on TNF and IL-12 production was mediated by TLR2 and MyD88, whereas their inhibitory effect on LPS-induced TNF, IL-12, and NO production was TLR-independent (22). These different studies led to the interesting hypothesis that tuberculosis protection versus pathogenesis may rely, at least in part, on the balance between TLRs versus C-type lectin signaling induced by mycobacterial motifs in phagocytes (23, 24). To address this point, we analyzed the molecular basis of mycobacterial LM stimulatory and inhibitory properties using a combination of purified LM acyl forms with genetically engineered macrophages lacking specific TLRs, TLR adaptors, or C-type lectins.

TLR2 seems crucially involved in the innate response to mycobacteria since TLR2-dependent cell activation by mycobacterial cell wall lipoglycans such as phosphinositol-capped LM but also LM, PIM₃, and PIM₅ or the 19-kDa mycobacterial lipoprotein have been described (22, 25–28). TLR4 can also mediate cellular activation to soluble cell-associated mycobacterial factors distinct from LAM (29), and M. tuberculosis-induced TNF production by murine macrophages is blocked by a TLR4 antagonist (30). Mice deficient for TLR4 or TLR2 are defective in their long-term control of the M. tuberculosis infection (31, 32). In addition, other pattern recognition receptors such as the C-type lectins mannose receptor, human pulmonary surfactant protein A, or DC-SIGN have been implicated in binding and/or as key molecules participating in anti-inflammatory transduction signals from ManLAM in dendritic cells (16, 20, 21, 33, 34).

LAMs are lipoglycans ubiquitously found in the envelope of mycobacteria. They may have different immunomodulatory activities, depending upon their structure. Phosphinositol-capped LAM from fast-growing and avirulent species, such as Mycobacterium smegmatis (35, 36) are pro-inflammatory molecules stimulating the production of TNF and IL-12, whereas LAM capped by mannosyl residues (ManLAM) from the slow-growing mycobacteria M. tuberculosis and Mycobacterium bovis BCG (37–39) are anti-inflammatory molecules, inhibiting the production of IL-12 and TNF and increasing IL-10 production by dendritic cells or mononuclear cell lines (20, 21, 40).

Although phosphinositol-capped LAM activates macrophages in a TLR2-dependent manner by activating the NF-κB signaling pathway (25), the anti-inflammatory effects of ManLAM have been attributed to their binding to the mannose receptor (21) or to DC-SIGN (16, 20). Within ManLAM, the critical motifs for recognition by DC-SIGN have been shown to be the mannose caps as well as the fatty acids involved in a supramolecular organization of the molecule associated with increased avidity for their receptors (16, 41).

LMs, the biosynthetic precursors of LAM, are composed of a carbohydrate backbone made of a α-D-mannan core and a mannosyl-phosphatidylinositol anchor at the reducing end of the mannan core but lack the α-arabinan domain and capping motifs found in LAM (42, 43). LM are pro-inflammatory, but we described recently that LM from different mycobacterial origins, including M. bovis BCG, M. tuberculosis, Mycobacterium chelonae, and Mycobacterium kansasii, also present strong anti-inflammatory properties (22). In particular, LM from M. bovis BCG was shown to induce macrophage activation and pro-inflammatory cytokines through TLR2 and the adaptor protein MyD88 and to inhibit pro-inflammatory cytokines production by LPS-activated macrophages through a TLR2- and MyD88-independent pathway. Recently, purification and structural characterization of four LM acyl forms from M. bovis BCG was reported using MALDI-MS and two-dimensional 1H, 31P NMR analyses (44). Tri- and tetra-acylated fractions were strongly pro-inflammatory, but not the mono- and di-acylated LM, and the tri-acylated LM form was identified as the main LM TLR2 agonist using TLR2/TLR1 heterodimers for signaling.

Here, we separate the negative and positive activities of M. bovis BCG LM. The purified mono-, di-, tri-, and tetra-acylated forms of M. bovis BCG LM were compared for anti-inflammatory or pro-inflammatory activities. Using primary macrophages derived from various TLR-, C-type lectins- and signal adaptor-deficient mice, we demonstrate a potent inhibitory effect of M. bovis BCG di-acylated LM on LPS-induced macrophage activation that turned out to be independent of TLR2 but also of mannose receptor and SIGNR1. Although mono-acylated LM was essentially inactive, the profound pro-inflammatory activity of M. bovis BCG tetra-acylated LM fraction was largely TLR4-dependent. The M. bovis BCG tri-acylated LM fraction exhibited both a strong TLR2/TLR1-dependent TNF-promoting activity mediated by MyD88 and TIRAP and some inhibition of LPS-induced TNF secretion that was independent of TLR2. This study demonstrates that mycobacterial LM acylation pattern determines the anti-inflammatory versus pro-inflammatory modulin effect of M. bovis BCG LM fractions.

**EXPERIMENTAL PROCEDURES**

**Purification of LM Acyl Forms**—LMs from M. bovis BCG was prepared as previously described (45, 46), and M. bovis BCG...
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L.M. mono-, di-, tri-, and tetra-acyl forms were further fractionated using hydrophobic interaction chromatography (44). The purity of the different acyl forms was assessed by $^{31}$P NMR and MALDI-MS and was estimated to be >95% with no other molecular species detected. LM from M. tuberculosis H37Rv purified according to established procedures (45, 46) was determined here by $^{31}$P NMR and MALDI-MS analysis to contain 88% tri-acylated and 12% di-acylated forms, and no other molecular contaminant was detectable (46, 47). The endotoxin content of the LM preparations was quantified by limulus ame-

Mice—6–12-week old mice deficient for TLR4 and/or TLR2, obtained by intercross from TLR4-deficient mice (from S. Akira (48)) and TLR2-deficient mice (from C. Kirschning (49)), for MyD88 (50), TIRAP (51), mannose receptor (52), or SIGNR1 (53) and their wild-type control littermates or C57Bl/6 mice were bred under specific-pathogen-free conditions in the Transgenose Institute animal breeding facility (Orleans, France).

Primary Macrophage Cultures—Murine bone marrow cells were isolated from femurs and cultured (10⁶/ml) for 7 days in Dulbecco’s minimal essential medium supplemented with 2 mM L-glutamine and 2 × 10⁻⁴ M β-mercaptoethanol, 20% horse serum, and 30% L929 cell-conditioned medium (as source of macrophage colony-stimulating factor, as described in Muller et al. (54)). After resuspension in cold phosphate-buffered saline, washing, and re-culturing for 3 days in fresh medium, the cell preparation contained a homogenous population of macrophages (verified periodically by Giemsa staining and CD11b expression). The bone marrow-derived macrophages were plated in 96-well microculture plates at a density of 10⁵ cells/well in Dulbecco’s minimal essential medium supplemented with 2 mM L-glutamine and 2 × 10⁻⁴ M β-mercaptoethanol and stimulated with 100 ng/ml LPS (Escherichia coli, serotype O111:B4, Sigma), 0.5 μg/ml synthetic bacterial lipopeptide Pam3CSK4 ([S-[2,3-bis(palmitoyloxy)-[2-βRS]-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys OH] trihydrochloride, EMC Microcollections, Tuebingen, Germany), 0.125 μM CpG ODN1826 (tcctagcgcttctgacctgct), and LM or LAM (at the concentrations indicated). The macrophages were activated with interferon-γ (500 units/ml) to study IL-12 expression. After 6–24 h of stimulation, the supernatants were harvested and analyzed immediately or stored at −20 °C until further use. The absence of cytotoxicity of the stimuli was controlled using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide incorporation.

SIGNR1-expressing RAW Cells—Murine macrophage RAW 264.7 cells expressing SIGN-R1 were prepared as described using SIGNR1 cDNA subcloned into the retroviral vector pFB (neo) (Stratagene, La Jolla, CA), and RAW-FB cells were used as controls (55), both kind gifts from Prof. S. Gordon (University of Oxford, Oxford, UK).

RESULTS

Separation of the Inhibitory and the Stimulatory Activities of M. bovis BCG LM on Cytokine and NO Production after Purification of the Different Acyl Forms—ManLAM is a complex lipoglycan considered as a major virulence factor of the mycobacterial cell envelope (57, 58), and LAM biosynthetic precursors (42), LMs, from various mycobacterial species have been shown
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Phages to produce cytokines and nitric oxide (NO), a potent anti-mycobacterial effector.

In our attempt to separate the inhibitory from the stimulatory activity of BCG LM, we first asked whether the BCG LM acyl forms displayed inhibitory effects with regard to TNF or IL-12p40 secretion after stimulation of primary macrophages with LPS. As shown in Fig. 2A, the secretion of TNF by LPS-stimulated wild-type macrophages was strongly inhibited by Ac2LM whereas the Ac1LM, Ac3LM, and Ac4LM fractions had essentially no effect. Because reactive nitrogen intermediates play a critical role in the control of mycobacterial infection (62), we investigated the effect of the purified LM acyl forms on the secretion of NO by primary murine macrophages. Similarly, LPS-induced NO production was also partially inhibited by Ac2LM but not with Ac1LM, Ac3LM, or Ac4LM (Fig. 2B).

We then confirmed and further characterized the pro-inflammatory effect of the BCG LM acyl fractions. Ac3LM and Ac4LM strongly stimulate primary macrophages to produce IL-12p40, whereas little IL-12p40 was detected after stimulation with Ac1LM and almost none with Ac2LM. The IL-12p40 concentrations achieved upon stimulation by Ac3LM and particularly with Ac4LM were relatively high, as compared with the reference stimulation by LPS or bacterial lipopeptide (Fig. 3A). Ac3LM and Ac4LM also stimulated a strong production of NO, whereas little NO was detected after stimulation with Ac1LM and Ac2LM (Fig. 3B). Therefore, purification of M. bovis BCG LM subfractions according to the acylation state allowed the separation of an inhibitory component, Ac2LM, which is not stimulatory but clearly exhibits an inhibitory activity on TNF and NO secretion by LPS-stimulated macrophages from tri- and tetra-acylated forms that induce the functional activation of primary macrophages to produce pro-inflammatory cytokines and the effector molecule NO.

The Inhibition of LPS-induced TNF by Ac2LM Is TLR2-independent—The inhibition of LPS-induced IL-12p40 and TNF release by M. kansasii LM and M. chelonae LM was shown to be independent of functional TLR2 and TLR6 as these LM were inhibitory in TLR2- or TLR6-deficient macrophages (22). Here, we verified that inhibition of TNF release by M. bovis BCG Ac2LM was TLR2-independent. Indeed, TNF and NO release induced by LPS were also to exert both pro- and anti-inflammatory activities (22, 59). LM from M. bovis BCG and M. tuberculosis H37Rv were able to stimulate macrophages to produce high levels of TNF and IL-12p40, whereas little amounts of TNF and no IL-12p40 could be detected after stimulation of the cells with the respective LAM molecules (22, 36, 59–61). Interestingly, we showed previously that LM also exhibits a strong inhibitory activity of pro-inflammatory cytokine release (22), reminiscent of that described for ManLAM. This suggested that LM fractions might contain two types of epitopes or structural entities with either positive and negative regulatory effects. We, therefore, explored the possibility to separate the inhibitory activity from the stimulatory one in BCG LM subfractions. Recently, four acyl forms of BCG LM were identified, purified, and characterized using MALDI-MS and two-dimensional NMR analyses, and a strong TNF-inducer activity was reported for tri- and tetra-acylated LM (44). In the present study we characterized the capacity of the respective mono-, di-, tri-, and tetra-acylated forms of LM from M. bovis BCG (designated Ac1LM to Ac4LM, respectively; see Fig. 1) to repress primary macrophages from M. bovis BCG (designated Ac1LM to Ac4LM, respectively; see Fig. 1) to repress primary macrophages (22). Here, we verified that inhibition of TNF release by M. bovis BCG Ac2LM was TLR2-independent. Indeed, TNF and NO release induced by LPS were also...
potently inhibited by Ac2LM in TLR2-deficient macrophages (Fig. 2, C and D). Therefore, TLR2 is not required for mediating the Ac2LM-induced signal that impairs the LPS-TLR4-induced activation cascade.

Inhibition of LPS-induced TNF Production by M. bovis Ac3LM in the Absence of TLR2—We reported previously that the inhibitory activity of M. bovis BCG LM on LPS-induced TNF and IL-12 secretion was masked by its strong TLR2-dependent pro-inflammatory activity in wild-type macrophages. Only the use of TLR2-deficient macrophages revealed the inhibitory activity of BCG LM in the absence of TLR2-dependent pro-inflammatory stimulation (22).

Because Ac3LM is a TLR2 agonist, we next asked whether this stimulatory activity may mask a potential inhibitory activity of TNF release. When all M. bovis BCG LM acylated fractions were tested for their inhibitory activity on TLR2-deficient macrophages, Ac3LM indeed partially inhibited the production of TNF (Fig. 2C) and NO (Fig. 2D) in response to LPS stimulation.

Therefore, in addition to Ac2LM, which is inhibitory and is devoid of stimulatory activity, the Ac3LM fraction still contains both a TLR2-dependent stimulatory and a TLR2-independent inhibitory activity, as originally described for BCG LM total fraction. Because no currently available technology allows further separation of the two activities in Ac3LM, we further concentrated in characterizing the inhibitory activity of Ac2LM.

Role of C-type Lectins in the Inhibition of LPS-induced TNF by Ac2LM—C-type lectins such as DC-SIGN or mannose receptor have been implicated in the negative regulation mediated by ManLAM on dendritic cells (20, 21). Because DC-SIGN, an important receptor for mycobacterial recognition, binds not only ManLAM but also LM (17, 18), we next addressed whether the inhibitory effect of Ac2LM was mediated through C-type lectins. In mice, five DC-SIGN homologues, DC-SIGN and SIGN-R1–4, that are differentially expressed were originally described (63), and more genes are being unraveled (64). Here we concentrated on addressing the role of SIGN-R1, since SIGN-R1 was reported to associate with TLR4 and MD2 and modulate downstream signaling under specific conditions (65). Monocytic murine RAW cells transfected with the signr1 gene (55) were first used to see whether SIGNR1 overexpression influences the inhibition of LPS-induced response by Ac2LM. Control RAW cells stimulated with LPS expressed TNF, and this response was partially inhibited in the presence of Ac2LM (Fig. 4A), in line with what was observed on primary bone marrow macrophages (Fig. 2A). However, overexpression of SIGNR1 had little effect on the TNF release inhibition induced by Ac2LM (Fig. 4B; 31 ± 8% in control versus 53 ± 11% inhibition in SIGNR1 expressing RAW cells in two experiments). Similar results were obtained for NO production (Fig. 4, C and D), suggesting little contribution of SIGNR1 overexpression in Ac2LM inhibitory activity. We verified the absence of Ac2LM cytotoxicity on RAW cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). To confirm these data and further address the potential implication of SIGNR1, we tested Ac2LM inhibitory activity in the absence of SIGNR1 using macrophages derived from the bone marrow of mice deficient for SIGNR1 expression (53). The inhibition of LPS-induced TNF release by Ac2LM was similar in SIGNR1-deficient macrophages and wild-type control cells (Fig. 4E).

Mannose receptor was also reported to deliver a negative signal that might account for ManLAM inhibition of IL-12 production in human dendritic cells (21). We next asked whether the inhibitory effect of Ac2LM was mediated through murine mannose receptor. Macrophages derived from the bone marrow of mannose receptor-deficient mice (31) were stimulated with LPS in the presence of Ac2LM. As shown in Fig. 5, the absence of mannose receptor on the macrophages did not prevent the inhibition of LPS-induced TNF secretion by Ac2LM. The inhibition was similar to that shown in wild-type or in TLR2-deficient macrophages. TLR4 agonist LPS, TLR2 agonist MALP2, and a mannosylated bovine serum albumin conjugate that binds mannose receptor (66) were used as specificity controls. Therefore, the murine C-type lectins SIGNR1 and mannose receptor are dispensable for the inhibitory effect of Ac2LM on LPS-induced TNF expression.
Cytokine and NO Secretion by M. bovis BCG Ac3LM and Ac4LM Fraction-stimulated Macrophages Is TLR2- and TLR4-dependent, Respectively—We showed recently that the release of pro-inflammatory cytokines by primary murine macrophages stimulated with Ac3LM is mediated through both TLR2 and TLR1, which may function as heterodimers, but not through TLR6 (44). To further identify whether other receptors present on primary macrophages recognize the different LM-acylated forms and the signaling pathways involved in transmitting a positive signal for cytokine and NO release, we further analyzed the TLR- and TLR adaptor dependence of these responses. Macrophages from mice deficient for TLR2, TLR4, both TLR2 and TLR4, TIRAP, or MyD88 were stimulated with the different BCG LM acyl forms.

Very little or no TNF was detected after stimulation of any of the macrophages by Ac1LM, and this was absent in macrophages deficient for TLR4. Ac2LM was not stimulatory for any of the macrophages tested (Fig. 6). As reported recently, Ac3LM induced a strong TNF release in the supernatant of wild-type macrophages that was impaired in macrophages deficient for TLR2 (Fig. 6, A and B (44)) or TLR4 (Fig. 6C (44)). In contrast, Ac4LM induced a strong TNF release by TLR2-deficient macrophages, although macrophages deficient for TLR4 were largely unresponsive (Fig. 6, B and C). Similar results were obtained for NO secretion (Fig. 6, F–H). In the absence of both TLR2 and TLR4 there was no TNF secretion detected (data not shown), and NO release was fully impaired (Fig. 6f), confirming that TLR2 and TLR4 represent the main receptors mediating the stimulatory effects of the different BCG LM acyl forms.

To exclude a contribution of endotoxins to the TLR4-mediated activity of Ac4LM, endotoxin levels were quantified in the different LM-acylated forms by a limulus amebocyte lysate kinetic turbidimetric assay. The endotoxin levels detected were of 0.37 ng/10^6 g of Ac1LM, 0.09 ng/10^6 g of Ac2LM, 18.9 ng/10^6 g of Ac3LM, and 1.7 ng/10^6 g for Ac4LM and the effective endotoxin concentration in the assay evaluated for each LM acyl-form fraction (Fig. 6E). LPS was down-titrated and tested side-by-side with Ac4LM to evaluate the contribution of endotoxins in the induction of TNF and NO measured. Although the TLR4 agonist activity of Ac3LM could be due to the level of endotoxin present in the preparation (the level of endotoxin possibly accounting for ~50% of the TNF and NO produced) as reported (44), this was not the case for Ac4LM. Ac4LM stimulated a secretion of TNF and NO 70–75-fold higher than the effective dose of LPS present in the sample (Fig. 6D). These results, therefore, suggest that Ac4LM-containing fraction induces TNF and NO secretion through TLR4, whereas the Ac3LM fraction does so mostly through a TLR2-dependent pathway.

We further confirmed the TLR4 dependence of Ac4LM fraction stimulation using a human HEK293 cell line transfected with hTLR4, CD14, and MD2 (Fig. 7A). The only TLR4-de-
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FIGURE 5. Mannose receptor is dispensable for inhibition of LPS-induced TNF by M. bovis di-acylated LM. Macrophages from control mice (A), mice deficient in TLR2 (B), or in mannose receptor (C) were incubated with LPS alone (100 ng/ml) or with LPS plus M. bovis BCG di-acylated LM (Ac2LM) at a concentration of 3 μg/ml. TNF was measured in the cell supernatants after 24 h. Results are expressed as the mean ± S.D. from n = 2 mice per genotype and are from 1 representative of 4 independent experiments.

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**DISCUSSION**

LM from *M. bovis* BCG was recently shown to exert a dual effect on the release of TNF and IL-12 by primary macrophages, a stimulatory effect through TLR2 signaling, and an inhibitory effect on LPS-induced TNF that was independent of functional TLR2 (22, 44). LM is composed of α-D-mannan carbohydrate backbone and a mannosyl-phosphatidylinositol anchor bearing one to four fatty acyl moieties (44). In an attempt to decipher the molecular bases of LM anti- and pro-inflammatory effects, four acyl forms of *M. bovis* BCG LM differing by their degree of substitution by fatty acids (1–4) were tested for their modulin activity. Here, we separate and characterize for the first time the

FIGURE 6. Macrophages response to *M. bovis* BCG tri- and tetra-acylated LM fractions are dependent on TLR2 and TLR4, respectively. Bone marrow-derived macrophages from wild-type mice (A, D, and F) or mice deficient for TLR2 (B and G), TLR4 (C and H), or both (I) were incubated with medium alone, with *M. bovis* BCG LM, or the different LM-acylated forms of *M. bovis* BCG at a concentration of 3 μg/ml or with LPS (100 ng/ml) or Pam3CSK4 (bacterial lipopeptide, BLP; 0.5 μg/ml) as TLR4 and TLR2 reference agonists, respectively. TNF (A–D) and NO (F–I) were measured after 24 h in the supernatants. In J, the TLR4-dependent activity of *M. tuberculosis* H37Rv LM (3 and 10 μg/ml) was tested on TNF secretion by TLR2- and/or TLR4-deficient macrophages as above. Results are the mean ± S.D. from *n* = 2 mice per genotype and are representative of 3–4 independent experiments. In D, LPS was down titrated and tested on wild-type bone marrow-derived macrophages in parallel to Ac3LM and Ac4LM (at 3 μg/ml). TNF was measured as above. In E, the endotoxin content of the purified LM acyl fractions determined by limulus amebocyte lysate kinetic turbidimetric assay was expressed as ng of endotoxin per 10 μg of LM acyl fraction or as the endotoxin concentration present in the assay when LM acyl are used at 3 μg/ml.
anti-versus pro-inflammatory activity and the pattern recognition receptor usage of the different acyl forms of *M. bovis* BCG LM, namely the anti-inflammatory activity of di-acyl LM that turned out to be TLR2-, mannose receptor-, and SIGNR1-independent on the one side and the TLR2/1- and MyD88/TIRAP-dependent stimulation by tri-acyl LM- and TLR4-dependent stimulation of the tetra-acyl LM fraction on the other side.

ManLAM from *M. tuberculosis* and *M. bovis* BCG were reported to inhibit IL-12p40 in human dendritic cells stimulated with LPS (21). We showed that LM from different mycobacterial sources, including *M. chelonae*, *M. kansasii*, or *M. bovis* BCG inhibited IL-12p40 and TNF secretion by murine primary macrophages in response to LPS (22). The intrinsic capacity of *M. bovis* BCG LM to inhibit IL-12p40 and TNF-α secretion by murine primary macrophages in response to stimulation by LPS (22) was masked by its strong TLR2-dependent pro-inflammatory activity. Here we show that among the four acyl forms of *M. bovis* BCG LM isolated, only Ac2LM was able to inhibit the LPS-induced production of TNF-α by wild-type macrophages, whereas Ac3LM also significantly inhibited cytokine or NO production in the absence of TLR2. Therefore, purification according to the acylation state of LM allowed the separation of the inhibitory Ac2LM, which is devoid of stimulatory activity, from the LM stimulatory activity, present mostly in tri- and tetra-acylated LM forms.

*M. tuberculosis* activates macrophages via TLR2 and to a lesser extent via TLR4 (13). We and others have shown that
activation of macrophages and dendritic cells by mycobacterial fractions depends mostly on TLR2 and TLR4, since antigen-presenting cells derived from TLR2–4 deficient mice are impaired in the production of pro-inflammatory cytokines and nitric oxide and in the expression of costimulatory molecules CD40 and CD86 (for review, see Refs. 14 and 70). Most purified mycobacterial ligands identified so far are TLR2 agonists, including the 19-kDa lipoprotein, PIM2, PIM6, and LM (22, 27, 44, 71). The pro-inflammatory activity of M. bovis BCG total LM preparation was reported to be mainly TLR2-dependent (22), and the purification and separation of different acyl forms of M. bovis BCG LM allowed characterization of Ac3LM as a TLR2 agonist (44). However, we show here that the M. bovis BCG Ac4LM-containing fraction signals through TLR4. From the four major M. bovis BCG LM acyl forms differing by their degree of acylation, the most abundant are the di-acylated and the tri-acylated forms (44). Ac3LM being proportionally more abundant than Ac4LM may account for the prominent TLR2 agonist of the M. bovis BCG total LM. Because the Ac4LM fraction is a powerful TLR4-dependent pro-inflammatory stimulus, whereas tri-acyl LM is a TLR2 agonist, it was tempting to propose that TLR2 versus TLR4 recognition might be determined by the LM acylation degree. To test this hypothesis, we analyzed the acylation pattern of M. tuberculosis H37Rv LM, which we characterized as a TLR4 agonist, expecting to find a predominance of tetra-acylated forms. However, as determined by 31P NMR, H37Rv LM is composed mostly of the tri-acylated acyl form (88%) with a small proportion of di-acylated form (12%) but no traces of tetra-acylated form (not shown). Thus, TLR4-dependent pro-inflammatory activities were triggered by either the tetra-acylated fraction of M. bovis BCG LM or the prominent tri-acylated form in M. tuberculosis H37Rv LM. Conversely, tri-acylated LM from M. bovis BCG or M. tuberculosis H37Rv activated either TLR2 or TLR4, respectively. With the analytical methods available so far, Ac3LM from M. tuberculosis H37Rv cannot be distinguished from M. bovis BCG Ac3LM (Fig. 1). Therefore, acylation degree per se does not seem sufficient to determine TLR2 versus TLR4 usage by LM.

Although more information is being unraveled on mycobacterial determinants through various pattern recognition receptors, including TLRs and C-type lectins such as the mannose receptor and DC-SIGN, C-type lectins have been associated with anti-inflammatory effects. ManLAM binding to DC-SIGN induces secretion of the anti-inflammatory cytokine IL-10 and leads to partial deactivation of human dendritic cells stimulated by LPS (20). IL-10 transcription was recently reported to be both prolonged and increased after acetylation of NF-κB p65 in response to ManLAM/DC-SIGN activation of Raf-1 (73). ManLAM induced Ser and Tyr phosphorylation and activation of Raf-1 through Ras and the Src and Pak kinases (73). Mannose receptor was proposed to mediate the inhibition by ManLAM of IL-12 production in dendritic cells stimulated with LPS (21). Studies using the pulmonary surfactant protein A (SP-A) led to the proposal that ManLAM immunomodulatory activities are associated to a highly ordered supramolecular organization of the molecules allowing high affinity binding to C-type lectins. TLRs and C-type lectins may, thus, contribute to an equilibrium between pro- and anti-inflammatory signals, respectively (24, 74).

Interestingly, LM, as ManLAM, can interact with DC-SIGN (16, 17). In mice, eight DC-SIGN homologues that are differentially expressed have been described (63, 64). Because human DC-SIGN and DC-SIGNR are more related to each other than to any of the mouse SIGNR genes, it is difficult to identify the mouse DC-SIGN ortholog. Here we concentrated on addressing the role of SIGN-R1, reported to interact with dense arrays of sugar characteristic of microorganism surfaces, to have endocytic activity and a domain organization closest to human DC-SIGN (64), and to physically associate with the TLR4-MD2 complex (65). SIGN-R1-deficient mice have shown enhanced susceptibility to Streptococcus pneumoniae (53) but not to high dose, acute mycobacteria infection (75). We show here that neither SIGN-R1 overexpression nor deficiency notably affected the inhibition by LM of LPS-induced cytokine or NO secretion by murine macrophages. Therefore, SIGN-R1 is not essential for the recognition of LM or the downstream signaling that interferes with LPS/TLR4 induced pro-inflammatory response. The potential involvement of mannose receptor in this response...
was addressed in macrophages from mannose receptor-deficient mice. Again, the absence of mannose receptor had no effect on the inhibitory activity of Ac2LM, indicating that mannose receptor is dispensable for LM recognition or downstream signaling that interferes with LPS/TLR4 induced pro-inflammatory response. Therefore, Ac2LM anti-inflammatory activity characterized in murine macrophages seems to differ from the previously reported anti-inflammatory activity for ManLAM on human dendritic cells both in terms of the role of mannosylated caps, essential for ManLAM activity but absent in Ac2LM, and the C-type lectin involvement documented for human DC-SIGN and mannose receptor and dispensable for murine SIGN-R1 and mannose receptor. Because several homologues of DC-SIGN exist in the mouse genome, a systematic investigation of the role of these different receptors as well as of other pattern recognition receptors will be further required to understand these negative regulatory pathways at the molecular level. Di-acylated LM might represent another pattern–derived immunomodulatory molecule with potential as immunotherapeutics, as recently reviewed for pathogen originating proteins (76).

In conclusion, the present report shows for the first time the separation of anti-inflammatory activities from pro-inflammatory activities of LM fractions, documenting that modulation of the acylation degree of major lipoglycans such as LM by mycobacterial enzymes may represent an additional means of regulating the host innate immunity for mycobacteria.

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REFERENCES

Anti-inflammatory Activity of BCG Lipomannan Acyl Forms


