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Toll-Like Receptor 2 (TLR2)-Dependent-Positive and TLR2-Independent-Negative Regulation of Proinflammatory Cytokines by Mycobacterial Lipomannans

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Lipoarabinomannans (LAM) and lipomannans (LM) are integral parts of the mycobacterial cell wall recognized by cells involved in the innate immune response and have been found to modulate the cytokine response. Typically, mannosylated LAM from pathogenic mycobacteria have been reported to be anti-inflammatory, whereas phosphoinositol-substituted LAM from nonpathogenic species are proinflammatory molecules. In this study, we show that LM from several mycobacterial species, including Mycobacterium chelonae, Mycobacterium kansasii, and Mycobacterium bovis bacillus Calmette-Guérin, display a dual function by stimulating or inhibiting proinflammatory cytokine synthesis through different pathways in murine primary macrophages. LM, but none of the corresponding LAM, induce macrophage activation characterized by cell surface expression of CD40 and CD86 and by TNF and NO secretion. This activation is dependent on the presence of Toll-like receptor (TLR) 2 and mediated through the adaptor protein myeloid differentiation factor 88 (MyD88), but independent of either TLR4 or TLR6 recognition. Surprisingly, LM exerted also a potent inhibitory effect on TNF, IL-12p40, and NO production by LPS-activated macrophages. This TLR2-, TLR6-, and MyD88-independent inhibitory effect is also mediated by LAM from M. bovis bacillus Calmette-Guérin but not by LAM derived from M. chelonae and M. kansasii. This study provides evidence that mycobacterial LM bear structural motifs susceptible to interact with different pattern recognition receptors with pro- or anti-inflammatory effects. Thus, the ultimate response of the host may therefore depend on the prevailing LM or LAM in the mycobacterial envelope and the local host cell receptor availability. The Journal of Immunology, 2004, 172: 4425–4434.

Immunity to Mycobacterium tuberculosis is a complex process that requires both macrophages and T cell-mediated immune responses (reviewed in Refs. 1–3). Resolution of the infection normally involves the sequestration of the surviving pathogen in macrophages within the complex and dynamic structure of a granuloma. The use of gene-targeted mice and studies using Ab neutralization have helped to identify a number of mediators that are essential for control of M. tuberculosis infection, including IFN-γ, IL-12, IL-23, TNF, lymphotoxin α, lymphotoxin β, and NO (1–3). Several of these mediators are also important for controlling the infection during latency, as neutralization of TNF or inducible NO synthase inhibition leads to a flare of the infection (4–6). We propose that a continuous, smoldering activation of the phagocytes is required to maintain an active immunological pressure and that mycobacterial lipoglycans, such as lipomannan (LM)4 and lipoarabinomannan (LAM), may contribute to the regulation of macrophage and dendritic cell activation through their modulin effects on the inflammatory response.

The initial recognition of mycobacterial components by the innate immune system may involve several different pattern recognition receptors (PRR) including Toll-like receptors (TLR), each of which contributes to host resistance and loss of any one of them may tip the balance against the host (7–10). Several reports have described a TLR2-dependent cell activation by mycobacterial cell wall lipoglycans (phosphoinositol-capped LAM (PILAM), phosphatidylinositol-bound mannosides (PIM)) or the 19-kDa mycobacterial lipoprotein (11–13) and direct antimycobacterial activity (14), suggesting that TLR2 is crucially involved in the innate response to mycobacteria. TLR4 can also mediate cellular activation to soluble cell-associated mycobacterial factors distinct from the mycobacterial cell wall LAM (15) and M. tuberculosis-induced TNF production by murine macrophages is blocked by a TLR4 antagonist (16). Mice deficient for TLR4 or TLR2 are defective in their long-term control of the M. tuberculosis infection (17, 18). In addition, other PRR such as the mannose receptor,

Abbreviations used in this paper: LM, lipomannan; LAM, lipoarabinomannan; Ara-LAM, uncapped LAM; DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3; ManLAM, mannose-capped LAM; MPI, mannosyl-phosphatidylinositol; MyD88, myeloid differentiation factor 88; PILAM, phosphoinositol-capped LAM; PRR, pattern recognition receptor; TLR, Toll-like receptor; BCG, bacillus Calmette-Guérin; LTA, lipoteichoic acid; PIM, phosphatidylinositol-bound mannosides.
human pulmonary surfactant protein A, or dendritic cell-specific intracellular adhesion molecule 3 (DC-SIGN) have been implicated in binding and/or as key molecules participating in anti-inflammatory transduction signals from mannosose-capped LAM (ManLAM) in dendritic cells (19–23).

LAM are lipoglycans ubiquitously found in the envelope of mycobacteria. They are composed of a β-D-manan core and a β-arabinan domain, a mannosyl-phosphatidylinositol (MPI) anchor at the reducing end of the mannan core and capping motifs (see Fig. 1; Refs. 24 and 25). The arabinan domain is capped by either mannosyl (ManLAM) or phosphoinositol caps, has been described with the identification and structural determination of the LAM from Mycobacterium chelonae (26). LAM are heterogeneous in size, depending on structural features such as the number of arabinosyl or mannosyl units composing the homopolysaccharides, or the structure of the MPI anchor and the capping motifs (25).

There is an emerging consensus that PILAM are proinflammatory molecules stimulating the production of TNF and IL-12, while ManLAM are anti-inflammatory molecules inhibiting the production of IL-12 and TNF and increasing IL-10 production by dendritic cells or mononuclear cell lines (19, 22, 34). PILAM activate macrophages in a TLR-2-dependent manner by activating the NF-κB signaling pathway (11), whereas the anti-inflammatory effects of ManLAM have been attributed to their binding to the mannose receptor (19) or to DC-SIGN (22, 23). This paradigm provides an interesting correlation between LAM structure and their immunomodulatory effect.

However, comparatively little is known about the regulatory role of the mycobacterial cell wall-derived LM on macrophage activation. Since LM are biosynthetic precursors of LAM and represent abundant cell wall molecules, characterization of their pro- and/or anti-inflammatory properties as well as of the pathways involved in conveying their signals may provide new insights on the immunomodulatory signals of pathogenic mycobacteria during primary infection but also at “homeostasis” during latent infection.

In this study, we investigated the regulatory properties of LM on the innate immune response in macrophages. We compared the pro- and anti-inflammatory activities of LM and LAM from various mycobacterial species: M. chelonae as a prototype of uncapped LAM, M. kansasii as an emerging mycobacteria of clinical relevance with specific lipoglycan structural features recently unraveled, as well as M. bovis BCG and M. tuberculosis as typical ManLAM-containing species. Using primary macrophages derived from TLR- and MyD88-deficient mice, we demonstrate a dual potential for LM from different mycobacterial origin, with profound proinflammatory and anti-inflammatory effects. The stimulatory effect of LM on TNF and IL-12 production is mediated by TLR2 and MyD88, while their inhibitory effect on LPS-induced TNF production is TLR and MyD88 independent and presumably mediated through other PRRs.

### Materials and Methods

#### Purification of LM and LAM

Lipoglycans from M. chelonae and M. kansasii were purified by successive detergent and phenol extractions leading to the obtention of a protein, lipid, and nucleic acid-free material, as described previously (30, 33). After suspension in Tris deoxycholate buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.2 M NaCl, and 0.25% deoxycholate), LAM and LM were separated by gel filtration on a Sephacryl 200 column and extensively dialyzed. The purity of preparations was assessed by gas chromatography/mass spectrometry and SDS-PAGE analysis. The endotoxin content of the LM and LAM preparations was <20 pg LPS/10 μg, as measured in a chromogenic Limulus lyase assay. LM and ManLAM from M. bovis BCG were prepared as previously described (35). LM from M. tuberculosis H37Rv was kindly provided by J. Belisle (Colorado State University, Fort Collins, CO; endotoxin content of 17.6 pg/10 μg) or prepared as previously described (35, 36).

#### Preparation of M. kansasii total soluble Ags

Soluble M. kansasii Ags from total cellular extracts were prepared by sonication of the mycobacteria in PBS, followed by centrifugation at 27,000 × g for 30 min at 4°C and 100,000 × g for 90 min at 4°C. The soluble fraction was then recovered, and the total protein concentration was determined using the BCA Protein Assay Reagent kit (Pierce Europe, Oud Beijerland, The Netherlands).

#### Mice

Six- to 12-wk-old mice deficient for TLR4 and/or TLR2, obtained by intercross from TLR4-deficient mice (from S. Akira (37)) and TLR2-deficient mice (from C. Kirschning (38)), TLR6-deficient mice (39), and MyD88-deficient mice (40) and their control littermates were bred under specific pathogen-free conditions in the Transgeno Institute animal breeding facility (Orleans, France).

#### Primary macrophage cultures

Murine bone marrow cells were isolated from femurs and cultivated (10^6 /ml) for 7 days in DMEM supplemented with 2 mM t-glutamine and 2 × 10^-5 M 2-ME, 20% horse serum, and 30% L929 cell-conditioned medium (as source of M-CSF, as described in Ref. 41). After resuspension in cold PBS, washing, and reculturing 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 10^4 cells/well in DMEM supplemented with

![FIGURE 1. Summary table and schematic representation of the structure of the LM (A) and LAM (B) isolated from the different mycobacterial strains used in this study: M. chelonae, M. kansasii, M. bovis BCG, and M. tuberculosis. 5-MTP, 5-Methylthiopentose; MPI, mannosyl phosphorylaminosyl; Manp, manno-pyranoside; R1,2,3 represent acyl chains.](image-url)
2 mM L-glutamine and 2 × 10^{-5} M 2-ME and stimulated with 100 ng/ml LPS (Escherichia coli, serotype O111:B4; Sigma-Aldrich, St. Louis, MO), 0.5 μg/ml synthetic bacterial lipopeptide Pam3CSK4 ([S-[2,3-bis-(palmitoyloxy)-2-RS]-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys-OH), trihydrochloride; EMC Microcollections, Tübingen, Germany), 10 μg/ml lipoteichoic acid (LTA from Streptococcus sp.; Lunamed, Basel, Switzerland), LM, or LAM (at the concentrations indicated). The macrophages were activated with IFN-γ (500 U/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. Absence of cytotoxicity of the stimuli was controlled using MTT incorporation.

Cytokine ELISA

Supernatants were harvested and assayed for cytokine content using commercially available ELISA reagents for TNF, and IL-12p40 (Duoset R&D Systems, Abingdon, U.K.).

Nitrite measurements

Nitrite concentrations in cell supernatants were determined using the Griess reaction (3% phosphoric acid, 1% p-aminobenzenesulfonamide, 1% N-(1-naphthyl)ethylenediamine) as previously described (42).

Results

LM, but not LAM, stimulate primary macrophages to release TNF and IL-12p40

ManLAM is a complex lipoglycan considered as a major virulence factor of the mycobacterial cell wall that plays a crucial role in the immune system through its interactions with various cells (43, 44). Although LM is thought to be a direct biosynthetic precursor of...
LAM (24), few immunological studies using LM have been conducted (55, 56). We therefore explored the possibility of LM isolated from various species (Fig. 1) being modulins/virulence factors in mycobacteria. In this study, we first tested the capacity of both the noncapped LAM and the LM from *M. chelonae* (designated CheLAM and CheLM, respectively) and mannose-capped LAM and LM from *M. kansasii* (designated KanLAM and KanLM, respectively) to stimulate primary macrophages to produce TNF. Bone marrow-derived macrophages were stimulated in vitro with the different lipoglycans and cell supernatants were assessed for TNF production. As shown in Fig. 2A, both KanLM and CheLM stimulate primary macrophages to produce TNF, although no TNF was detected after stimulation with the respective LAM.

To investigate whether the stronger stimulatory effect of LM over LAM was a general phenomenon among different mycobacterial species or may be restricted to some particular species, we tested the capacity of LM vs LAM from *M. bovis* BCG (designated BCGLAM and BCGLM, respectively) and *M. tuberculosis* H37Rv (designated H37LAM and H37LM, respectively), to stimulate primary murine macrophages. BCGLM and H37LM were able to stimulate macrophages to produce high levels of both TNF (Fig. 2B) and IL-12p40 (Fig. 2C). In contrast, small amounts of TNF and no IL-12p40 could be detected after stimulation of the cells with the respective H37LAM and BCGLAM molecules, in agreement with previous studies (32, 45). Thus, unlike their respective LAM counterparts, LM from *M. chelonae*, *M. kansasii*, *M. tuberculosis*, and *M. bovis* BCG, which all lack the arabinan domain and the terminal capping motifs, are potent stimulators of proinflammatory cytokine secretion from primary macrophages.

### Functional activation of macrophages by LM

We next addressed the effect of LM on macrophage activation and effector functions with regard to CD40 and CD86 expression and NO release. Flow cytometry analysis revealed that BCGLM stimulated very efficiently CD11b+ bone marrow-derived macrophages (>97% CD11b+) were either unstimulated or incubated with LM or LAM at a concentration of 10 μg/ml for 12 h. CD40 and CD86 expression by unstimulated (thin line) or stimulated macrophages (thick line) was analyzed by flow cytometry. Results are from n = 2 mice and are from one representative experiment of two.

![FIGURE 3. LM induce functional cell activation primary macrophages.](http://www.jimmunol.org/)

LM from *M. bovis* BCG stimulated the expression of CD40 (A) and CD86 (B) on primary macrophages. LM, but not LAM, from *M. kansasii* and *M. chelonae* stimulated macrophage expression of CD40 (C). Murine bone marrow-derived macrophages (>97% CD11b+) were either unstimulated or incubated with LM or LAM at a concentration of 10 μg/ml for 12 h. CD40 and CD86 expression by unstimulated (thin line) or stimulated macrophages (thick line) was analyzed by flow cytometry. Results are from n = 2 mice and are from one representative experiment of two.

![FIGURE 4. TNF release by macrophages in response to LM from *M. kansasii* and *M. chelonae* is dependent on TLR2, not TLR4 and TLR6, and signals through MyD88.](http://www.jimmunol.org/)

Bone marrow-derived macrophages from mice deficient for TLR2 and/or TLR4 (A) or deficient for TLR6 or MyD88 (B) were incubated with medium alone, LM or LAM from *M. kansasii* and *M. chelonae* at a concentration of 10 μg/ml or with LPS (100 ng/ml) or a soluble fraction of *M. kansasii* Ags (supKan at 10 μg/ml) for 24 h. TNF was measured in the supernatants by ELISA. Results are mean ± SD from n = 2 mice per genotype and are from one representative experiment of three.
(Fig. 3C). CD86 expression was slightly induced by CheLM and KanLM but not by their respective LAM (data not shown).

Since reactive nitrogen intermediates play a critical role in the control of mycobacterial infection (46), we next investigated whether LM or LAM may induce the secretion of NO by primary murine macrophages. As shown in Fig. 2D, BCGLM and H37LM stimulated a strong production of NO, whereas essentially no NO was detected after stimulation with either BCGLM or H37LAM. Altogether these results suggest that LM, but not the respective LAM, functionally activate primary macrophages as evidenced by the expression of the costimulatory molecules CD40 and CD86 and the synthesis of the effector molecule NO.

Cytokine secretion by LM-stimulated macrophages is TLR2 and MyD88 dependent, but TLR4 and TLR6 independent

To identify the receptors present on primary macrophages that recognize LM and transmit a positive signal for cytokine release, we analyzed the TLR dependence of this response. Macrophages from mice deficient for TLR2, TLR4, or both TLR2 and TLR4 were stimulated with either KanLM or CheLM. After stimulation with LM, no TNF could be detected in the supernatant of macrophages deficient for TLR2, whereas TNF was efficiently released in the supernatant of TLR4-deficient macrophages (Fig. 4A). These results suggest that LM induce TNF secretion through a TLR2-dependent pathway. As expected, no cytokine production was detected in the supernatants of macrophages isolated from the double TLR2- and TLR4-deficient mice. The TNF concentration achieved upon LM stimulation was relatively high, as compared with the reference stimulation by LPS (0.1 µg/ml) or total soluble Ags isolated from M. kansasii cells. This latter fraction is likely to contain a complex mixture of all soluble proteins from M. kansasii along with PIMs, KanLM, KanLAM, and presumably other lipoglycans. Fig. 4A clearly indicates that this fraction activates macrophages through a TLR2-dependent mechanism. We next assessed whether TLR6, known to form heterodimers with TLR2 (47–50), may be involved in the immune response to LM. Macrophages deficient for TLR6 responded to KanLM and CheLM as efficiently as wild-type control cells (Fig. 4B), suggesting that TNF production by LM is TLR6 independent. Recent studies suggested the adaptor MyD88 may be involved in the signal pathways of most TLRs (40), although MyD88-independent pathways have also been identified (51). Using macrophages deficient for MyD88, we showed that KanLM and CheLM were unable to transmit the signal leading to the production of TNF (Fig. 4B) or NO (data not shown) in the absence of MyD88, suggesting that MyD88 participates in the LM-induced signaling pathway.

Similarly, induction of TNF, IL-12p40, and NO by BCGLM was strongly dependent on TLR2 and MyD88, but independent of TLR4 and TLR6 (see Fig. 7 and data not shown). Thus, the release of proinflammatory cytokines and NO by primary murine macrophages stimulated with CheLM, KanLM, or BCGLM is dependent on both TLR2 and MyD88 signaling, but appears independent of TLR4 and TLR6.

LM from M. chelonae and M. kansasii inhibit LPS-induced IL-12p40 in macrophages

ManLAM from M. tuberculosis and M. bovis BCG were reported to inhibit IL-12p40 in human dendritic cells stimulated with LPS (19). We next addressed whether LM from M. chelonae and M. kansasii, as well as the corresponding LAM counterparts, may also display inhibitory effects with regard to IL-12p40 secretion after stimulation of primary macrophages with LPS. As shown in Fig. 5A, the secretion of IL-12p40 by LPS-stimulated macrophages was strongly inhibited by KanLM and CheLM, whereas KanLAM and CheLAM had essentially no effect. The absence of cytotoxic effect of all the LM and LAM preparations, either alone or in combination with LPS, on macrophages in culture was verified (data not shown). Interestingly, the IL-12p40 released by macrophages in response to stimulation with known TLR2 agonists such as the synthetic bacterial lipopeptide Pam3CSK4 (49, 52) or LTA (53) was not inhibited by any of the LM or LAM tested (data not shown). Thus, IL-12p40 secreted by macrophages in response to LPS was potently inhibited by KanLM and CheLM, while their respective LAM molecules were found to be inactive.

The inhibition of LPS-induced IL-12p40 by LM is TLR2 and TLR6 independent

Our results indicate that both KanLM and CheLM induce macrophages to produce TNF through the TLR2 signaling. We then asked whether the inhibitory effect of the LM on IL-12p40 release induced by the TLR agonist LPS is mediated through TLR ligation. Both KanLM and CheLM potently inhibited the release of IL-12p40 induced by LPS in TLR2-deficient macrophages (Fig. 5B). As expected, no secretion of IL-12p40 was detected in TLR4 deficient macrophages. This latter fraction is likely to contain a complex mixture of all soluble proteins from M. kansasii along with PIMs, KanLM, KanLAM, and presumably other lipoglycans. These results suggest that this fraction activates macrophages through a TLR2-dependent mechanism. We next assessed whether TLR6, known to form heterodimers with TLR2 (47–50), may be involved in the immune response to LM. Macrophages deficient for TLR6 responded to KanLM and CheLM as efficiently as wild-type control cells (Fig. 4B), suggesting that TNF production by LM is TLR6 independent. Recent studies suggested the adaptor MyD88 may be involved in the signal pathways of most TLRs (40), although MyD88-independent pathways have also been identified (51). Using macrophages deficient for MyD88, we showed that KanLM and CheLM were unable to transmit the signal leading to the production of TNF (Fig. 4B) or NO (data not shown) in the absence of MyD88, suggesting that MyD88 participates in the LM-induced signaling pathway.

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or in TLR2/TLR4 double-deficient cells after stimulation with LPS (data not shown). Moreover, KanLM and CheLM inhibited LPS-induced IL-12p40 secretion in TLR6-deficient as efficiently as in wild-type macrophages (Fig. 5C). Therefore, the inhibition of LPS-induced IL-12p40 release by KanLM and CheLM is independent of functional TLR2 and TLR6.

**LM from M. chelonae and M. kansasii inhibit LPS-induced TNF and NO secretion**

We next addressed whether the inhibitory effect of LM was restricted to IL-12p40 or whether it may also be seen with another proinflammatory cytokine such as TNF. The release of TNF by macrophages stimulated with LPS was significantly inhibited by KanLM and CheLM, but not by KanLAM and CheLAM (Fig. 6A). It is noteworthy that the inhibition of TNF was less marked than that observed with IL-12p40 secretion. This relatively lower effect observed on TNF vs IL-12p40 secretion may be attributed to the fact that KanLM and CheLM are also strong TNF-inducing factors as shown above (Fig. 2) and that high levels of TNF, usually up to 10-fold higher than that of IL-12p40, are produced in these cultures. Pam,CSK, another TLR2 ligand, did not inhibit the LPS-induced TNF secretion (data not shown). LPS-induced NO production was also partially inhibited by KanLM and CheLM but not with KanLAM and CheLAM (Fig. 6C). When macrophages were stimulated with live M. bovis BCG or HKH37Rv, no reduction of the TNF levels could be detected after coinoculation with LM or LAM (data not shown). Thus, KanLM and CheLM, but not their respective LAM, inhibited the secretion of LPS-induced TNF and NO.

The inhibition of LPS-induced TNF by LM is independent of TLR2, TLR6, and MyD88.

Using the TLR4 agonist LPS as a stimulus for TNF release by macrophages, we next investigated whether the inhibitory effect of the LM on TNF release was mediated through TLR2 or TLR6. Fig. 6A shows that even in TLR2-deficient macrophages, KanLM and CheLM inhibit TNF release. As expected, TNF release was abrogated in TLR4- and TLR2/TLR4 double-deficient cells (data not shown). The absence of functional TLR6, in TLR6-deficient macrophages, did not affect the inhibition of LPS-induced TNF release by CheLM, although the inhibitory activity of KanLM was slightly reduced (Fig. 6B). LPS is known to stimulate macrophages through TLR4 via at least two independent signaling pathways (40, 51). The adaptor protein MyD88 is involved in the signaling of most TLRs, but some of the LPS response is MyD88 independent (51, 54). Therefore, we asked whether the inhibitory effect of LM on LPS-induced TNF was mediated by MyD88. In agreement with published results (51), a small fraction of the LPS-induced TNF release was found to be MyD88 independent when comparing macrophages from MyD88-deficient and wild-type mice (Fig. 4B). The level of TNF produced by MyD88-deficient macrophages was very low and represented at most 6% of the levels produced by wild-type cells. Interestingly, the MyD88-independent TNF response to LPS could be fully inhibited by KanLM and CheLM (data not shown). Together, these results suggest that KanLM and CheLM exert a dual effect on the release of TNF by primary macrophages, a stimulatory effect through TLR2, and an inhibitory effect on TNF induced by LPS that is independent of functional TLR2, TLR4, TLR6, and the MyD88 signaling pathway.

**BCGLM bears both TLR2-dependent stimulatory and TLR-2-independent inhibitory motifs for proinflammatory cytokine secretion**

Since KanLAM and CheLAM present no inhibitory effects on cytokine release by primary macrophages, we next examined the effect of ManLAM from M. kansasii and M. chelonae at a concentration of 10 μg/ml for 24 h. TNF (A and B) and NO (C) were measured in the cell supernatants. There was no detectable TNF production by unstimulated cells. Results are mean ± SD from n = 2 mice per genotype and are from one representative experiment of two independent experiments.

**FIGURE 6.** Inhibition of LPS-induced TNF and NO by LM from M. kansasii and M. chelonae, independent of TLR2 and TLR6. Macrophages from mice deficient in TLR2 (A), TLR6 (B), or control mice (C) were incubated with LPS alone (100 ng/ml) or with LPS plus LM or LAM from M. kansasii and M. chelonae at a concentration of 10 μg/ml for 24 h. TNF (A and B) and NO (C) were measured in the cell supernatants. There was no detectable TNF production by unstimulated cells. Results are mean ± SD from n = 2 mice per genotype and are from one representative experiment of two independent experiments.
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latory effect since H37LM did reduce IL-12p40 secretion in mac-
inhibitory effect of H37LM may be attributed to its strong stimu-
stimulated macrophages whereas H37LM had no apparent inhib-
were absent for their respective LAM, suggesting that the arabinan
was previously reported to induce expression of proinflammatory cytokines and IL-8 (56). ManLAM
from virulent M. tuberculosis H37Rv or Erdman strains induced only little TNF, IL-1, or IL-6, as compared with PILAM from rapidly growing mycobacteria strains, although ManLAM was
found to induce TGFβ (45). In this study, we extended the obser-
we showed that LM stimulates the expression not only of cytokine synthesis in murine primary macrophages. LM is
stimulating cytokine secretion. All LM tested, regardless of their mycobacterial origin, were
found to be strong proinflammatory molecules by inducing abundant levels of TNF and functional macrophage cell activation fac-
tors, including the CD40 and CD86 costimulatory molecules, as well as NO secretion. The stimulatory effects of the different LM were absent for their respective LAM, suggesting that the arabinan moiety blocked the stimulatory activity of the LM. This result is in
agreement with the fact that LM, but not LAM, from M. kansasii
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LM from Mycobacterium sp. was previously reported to induce expression of proinflammatory cytokines and IL-8 (56). ManLAM
from virulent M. tuberculosis H37Rv or Erdman strains induced
the presence of phosphoinositol caps have been isolated from non-
pathogenic species, Mycobacterium sp. and M. smegmatis (31, 32).

Third, LAM recently isolated from M. chelonae, a fast-growing pathogenic mycobacterial species, appears to be devoid of both mannose and phosphoinositol caps, therefore designating a new family of uncapped AraLAM (33). The different LM and LAM from M. bovis BCG, M. tuberculosis, M. chelonae, or M. kansasii that are used in this study and their main structural features are summarized in Fig. 1. Differences concern the three structural do-
main, mainly the nature of the fatty acids bound to the MPI an-
chor, the nature of the linkage and the degree of substitution of the mannoylarabino side chains that are linked to the mannan core, and the absence of the mannose cap that characterizes CheLAM.

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The present data demonstrate for the first time that LM display dual modulatory functions, a TLR2-dependent stimulation and/or a TLR2-independent inhibition of cell activation and proinflamma-
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LAM: we showed that LM stimulates the expression not only of inflammatory cytokines and IL-8 (56). ManLAM
from virulent M. tuberculosis H37Rv or Erdman strains induced
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FIGURE 7. The TLR2 agonist stimulatory effect of LM is masking its TLR2-independent inhibitory effect on LPS-induced TNF, which becomes apparent in TLR2-deficient mice. In contrast, BCGLAM, which is not a TLR2 agonist, inhibits LPS-induced TNF in wild-type cells. A and B, Stimul-
atory effect of LM. Macrophages from control mice (A) and mice defi-
cient for TLR2 (B) were incubated with medium, LPS (100 ng/ml), or LM or LAM from M. bovis BCG, C and D, Inhibitory effect of LM and LAM. Macrophages from control mice (C) and mice deficient for TLR2 (D) were incubated with LPS alone (100 ng/ml) or LPS in combination with LM or LAM from M. bovis BCG at a concentration of 10 μg/ml. TNF was mea-
sured in the supernatants after 24 h. Results are mean ± SD from n = 2 mice per genotype and are from one representative experiment of two.

shown). The stimulatory effects of BCGLM on TNF and IL-12p40 secretion were already visible at concentrations down to 1 μg/ml and the inhibitory effects of BCGLAM on TNF and IL-12p40 at concentrations of 3 μg/ml (Fig. 8).

H37LAM partially inhibited the secretion of IL-12p40 by LPS-
stimulated macrophages whereas H37LM had no apparent inhib-
hibitory effect (data not shown). Similar to BCGLM, the absence of an inhibitory effect of H37LM may be attributed to its strong stimu-
latory effect since H37LM did reduce IL-12p40 secretion in mac-
rophages devoid of functional TLR2 (data not shown).

Therefore, BCGLM bear motifs yielding to inhibition of TNF and IL-12p40 secretion by macrophages after LPS stimulation, an inhibitory effect that is TLR2 independent and appears to be masked by its strong TLR2-dependent immunostimulatory effect.
Second, we showed that KanLM and CheLM exert a potent inhibitory effect on IL-12p40 and TNF production in macrophages stimulated with the TLR4 agonist LPS. This inhibitory effect was neither mediated by TLR2 nor by TLR6 and also appears to be MyD88 independent.

LAM from *M. bovis* BCG or *M. tuberculosis*, but not LAM from *M. kansasii* and *M. chelonae*, inhibited LPS-induced TNF and IL-12p40 secretion by macrophages. This is in line with previous reports demonstrating a strong inhibition of TNF and IL-12 release by ManLAM from *M. bovis* BCG and *M. tuberculosis* in human dendritic cells or THP-1 stimulated with LPS (19, 22, 34). Nevertheless, the macrophages were still activated in terms of CD40 expression (data not shown). Interestingly, the corresponding BCGLM had essentially no apparent inhibitory effect in this system. We further characterized the potential of BCGLM and BCGLAM to modulate cytokine release by primary macrophages and found that LM strongly stimulated macrophage activation and TNF release to levels similar to those seen after LPS induction, whereas BCGLAM, as expected, was poorly active. The stimulatory effect of BCGLM was TLR2 dependent. This led us to hypothesize that the inhibitory effect of BCGLM may be masked by its strong stimulatory effect. Indeed, in the absence of functional TLR2, BCGLM was unable to trigger any proinflammatory cytokine secretion and its inhibitory effect on LPS-induced TNF secretion became apparent. Similarly, the inhibitory effect of CheLM and KanLM on LPS-induced cytokines was more pronounced in TLR2-deficient macrophages which have an ablated proinflammatory response (see Fig. 5). Thus, the dual TLR2-dependent, stimulatory, and TLR2-independent inhibitory effect on LPS-induced cytokine secretion appears to be common to the various mycobacterial LM studied.

Therefore, BCGLM, unlike BCGLAM, bear both pro- and anti-inflammatory motifs. Macrophage activation and cytokine production observed after LPS stimulation in the presence of BCGLAM is the result of a cross-talk between a LPS proinflammatory stimulation mediated by TLR4 and the BCGLAM inhibitory and TLR2- and MyD88-independent signal. This cross-talk seemed to bear some TLR4 specificity since macrophage stimulation through the TLR2 agonists Pam₃CSK₄ or LTA was not inhibited by BCGLAM. The situation is more complex for LM as cell activation and cytokine production observed after LPS stimulation in the presence of LM is the result of TLR4 agonist stimulation by LPS, TLR2 agonist stimulation by LM, plus a TLR- and MyD88-independent inhibition by LM. The net result, in terms of pro- or anti-inflammatory cellular response, will therefore depend on the balanced cellular expression of the different TLR and PRR receptors involved. LM inhibitory effect does not seem to be a mere cross-tolerance/desensitization between TLR2 and TLR4 agonists since LPS and LM were added together to avoid desensitization, and LM inhibition occurred in TLR2-deficient macrophages.

Mannose receptor ligation inhibits IL-12 production by human dendritic cells and the mannose receptor itself has been inferred to participate in the inhibitory effect of ManLAM on dendritic cells (19). The inhibitory effects of the LM and LAM described here may possibly be mediated by the mannose receptor, as mannan from *Saccharomyces cerevisiae*, which is a ligand of the mannose receptor and is structurally very similar to the mannan core of LM, showed partial inhibition of LPS-induced TNF release by murine

![FIGURE 8. Titration of the stimulatory effect of LM and of the inhibitory effect of LAM from *M. bovis* BCG on TNF and IL-12p40 secretion in wild-type macrophages. A and B, Stimulatory effect of BCGLM. Macrophages from wild-type mice were incubated with increasing concentrations of BCGLM (in micrograms per milliliter). C and D, Inhibitory effect of BCGLAM. Macrophages from wild-type mice were incubated with LPS alone (100 ng/ml) or along with increasing concentrations of LAM from *M. bovis* BCG. TNF and IL-12 p40 levels were measured in the supernatants after 24 h. Results are mean ± SD from *n* = 2 mice per genotype.](http://www.jimmunol.org/content/4432/Figure8)

macrophage (data not shown). However, earlier studies using coated polystyrene beads indicated that ManLAM, but neither PILAM nor LM, bound to mannose receptor (57). Another receptor, DC-SIGN, has recently been shown to be involved in ManLAM inhibition of mycobacteria- or LPS-induced dendritic cell maturation (22). ManLAM, but not PILAM, inhibited M. tuberculosis binding to DC-SIGN (23). We show here that although ManLAM from M. bovis BCG or M. tuberculosis are strong inhibitors of LPS-induced macrophage activation and cytokine secretion as previously reported (19), this activity is not shared by LAM from M. chelonae or M. kansasii. The incapacity of CheLAM to inhibit LPS-induced cytokine secretion is in agreement with the fact that CheLAM is devoid of mannose caps (33). Indeed, it has been previously demonstrated that mannose caps are crucial elements for LAM inhibitory activity since PILAM or ManLAM devoid of mannose caps after α-mannosidase treatment are not inhibitory (19) due to their inability to bind mannose receptor and/or DC-SIGN (23). In relation to these data, it is noteworthy that M. chelonae bound poorly to DC-SIGN-expressing HeLa cells (23). Concerning KanLAM, the situation is unclear since it does possess mannose caps (see Fig. 1 and Ref. 30). A precise interpretation of the functional data in terms of structure is hampered by the fact that LAM and LM are polydisperse, complex molecules, whose fine structure determination is susceptible to evolve with the performance of the available analytical technologies.

Despite the absence of oligomannosyl caps, we show here that LM are also strong inhibitors of proinflammatory cytokines. This can be explained by their ability to bind C-type lectins through the side chain terminal mannose units as demonstrated for the binding of LM from M. bovis BCG and M. tuberculosis to the C-lectins SP-A (20, 21) or DC-SIGN (G. Puzo, J. Nigou, and O. Neyrolles, unpublished data). In the case of ManLAM, only terminal mannose units of the caps are involved in binding, those of the mannan core being hidden by the arabinan domain (20, 21).

The consensus that PILAM are proinflammatory molecules, while ManLAM are anti-inflammatory molecules, has been corroborated by the intramacrophagic fate of the corresponding mycobacteria. Indeed, the inability of M. smegmatis to survive inside activated macrophages is associated with the proinflammatory effect of PILAM. Likewise, the capacity of M. tuberculosis and M. bovis BCG to survive and multiply inside macrophages is in agreement with the anti-inflammatory effect of ManLAM. Thus, ManLAM emerged as a major virulence factor that contributes via an immunosuppressive effect to the persistence of M. tuberculosis and M. bovis BCG within phagocytic cells. Since LM is considered as a direct biosynthetic precursor of LAM, few studies were aimed to analyze its potential biological functions (55, 56). In this study, we provide evidence that LM from several species have the ability to display proinflammatory and stimulatory activity, whereas ManLAM have a predominant anti-inflammatory activity in the presence of the TLR4 agonist LPS. Thus, LAM may also be viewed as “attenuated” forms of LM. One can speculate that the mycobacteria may influence the cytokine environment by favoring LAM vs LM synthesis, expression in the cell wall, and release out of the mycobacterial phagosome to the medium and bystander cells as previously shown for LAM and PIM (58). The LM:LAM ratio seems variable in different mycobacteria, from 9:1 (mol:mol) in M. chelonae (33), 3:1 in M. kansasii (30), and 1:1 in M. bovis BCG (35). Many arabinosyltransferases are likely to participate in the synthesis of the arabinan domain of LAM. However, it remains possible that the expression of the genes encoding these enzymes is regulated and that under specific circumstances, they are overexpressed to ensure arabinan synthesis. Thus, overproduction of arabinosyltransferases may lead to important changes regardless to the LM:LAM balance, which may be a critical step for directing the outcome of innate immunity against mycobacteria. It was recently demonstrated that an embC-deficient M. smegmatis strain was affected in arabinosylation of LAM (59). Inactivation of the embC gene resulted in complete cessation of LAM biosynthesis although LM and PIMs were still synthesized. Thus, such a deficient mutant in M. tuberculosis would represent an attractive tool to address whether LM:LAM balance is a critical factor in mycobacterial pathogenesis. Determination of the LM:LAM composition of the M. tuberculosis cell wall during acute or latent infection, as well as during confection, will help to measure the biological significance of the LM and LAM modulins in the establishment and the persistence of the host immune response during tuberculosis infection.

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References


