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Lipomannans, But Not Lipoarabinomannans, Purified from *Mycobacterium chelonae* and *Mycobacterium kansasii* Induce TNF-α and IL-8 Secretion by a CD14-Toll-Like Receptor 2-Dependent Mechanism[1]

Cécile Vignal,* Yann Guérardel,* Laurent Kremer,† Maryse Masson,* Dominique Legrand,* Joël Mazurier,* and Elisabeth Elass[2]*

Lipoarabinomannans (LAMs) are glycolipids from the mycobacterial cell wall that exhibit various biological activities, including proinflammatory and anti-inflammatory responses. However, little is known about the properties of lipomannans (LMs), considered to be precursors of LAMs. In this study, we provide evidence that LMs purified from *Mycobacterium chelonae* and a clinical strain of *Mycobacterium kansasii* stimulated mRNA expression and secretion of TNF-α and IL-8 from human macrophage-like differentiated THP-1 cells. In contrast to LMs, LAMs were not able to induce a significant cytokine-inducing effect. The mechanism of activation by LMs was investigated using various Abs raised against surface receptors for multiple bacterial products. The presence of anti-CD14 or anti-Toll-like receptor 2 (TLR2) Abs profoundly affected production of TNF-α and IL-8, suggesting that both CD14 and TLR2 participate in the LM-mediated activation process. Furthermore, stimulation of cells was dependent on the presence of the LPS-binding protein, a plasma protein that transfers glycolipids to CD14. Chemical degradation of the arabinan domain of mannose-capped LAM from *M. kansasii*, which presented no cytokine-eliciting effect, restored the cytokine-inducing activity at a level similar to those of LMs. These results support the hypothesis that the presence of an arabinan in LAMs prevents the interaction of these glycolipids with TLR2/CD14 receptors. In addition, we found that phosphatidylinositol dimannosides isolated from *M. kansasii* did not induce cytokine secretion. This study suggests that LMs isolated from different mycobacterial species participate in the immunomodulation of the infected host and that the D-mannan core of this glycolipid is essential for this function. *The Journal of Immunology*, 2003, 171: 2014–2023.

Lipoarabinomannans (LAMs)[3] and lipomannans (LMs) are complex lipoglycans considered as major constituents of the mycobacterial cell wall (1, 2). These glycoconjugates are virulence factors that play a key role in the human immune system through their interactions with various cells (3, 4). Indeed, they exhibit a wide array of biological activities that enhance antimycobacterial immune defenses or facilitate mycobacterial survival through inhibition of the immune response. These effects include regulation of the proinflammatory and anti-inflammatory cytokine production (5–10), inhibition of microbicidal activity of macrophages (11), and suppression of T lymphocyte proliferation (12).

So far, studies have focused on LAMs isolated from pathogenic species, such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), or from fast-growing and nonvirulent mycobacteria such as *Mycobacterium smegmatis*. However, the biological properties of these glycolipids depend on their structural features (13, 14). LAM is composed of a phosphatidyl-*myo*-inositol anchor to which is attached a D-mannan core branched by single mannopyrannosyl residues followed by a D-arabinan domain (15–17). LM, which is considered as being a direct precursor of LAM, lacks the arabinan domain. According to the structure of caps located at the terminal extremity of arabinan domain, LAMs were classified into two types: mannosylated LAMs (ManLAMs), which are characterized by the presence of α(1–2) manno-oligosaccharides, as found in *M. tuberculosis*, *M. leprae*, *M. bovis* BCG, and *Mycobacterium avium*, and phosphoinositol-capped LAMs (PILAM) identified in *M. smegmatis* (13, 14, 16, 18, 19). These glycolipids are also heterogeneous in their acylation state and branching patterns of both arabinan and mannan domains.

Toll-like receptors (TLRs), essentially TLR2 and TLR4, have been shown to be essential for the recognition of distinct bacterial cell wall components (20). These bacterial products elicit the activation of an intracellular signaling cascade via TLR, which ultimately leads to the activation of transcription factors that initiate

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[3] Abbreviations used in this paper: LAM, lipoarabinomannan; LM, lipomannan; BCG, bacillus Calmette-Guérin; ManLAM, mannosylated LAM; PILAM, phosphoinositol-capped LAM; TLR, Toll-like receptor; DC-SIGN, dendritic cell-specific ICAM 3-grabbing nonintegrin; CheLAM, LAM purified from *Mycobacterium chelonae*; CheLM, LM purified from *M. chelonae*; KanLAM, LAM purified from *Mycobacterium kansasii*; KanLM, LM purified from *M. kansasii*; mCD14, GPI-anchored protein CD14; LBP, LPS-binding protein; PIM, phosphatidyl-*myo*-inositol mannoside; GC/MS, gas chromatography/mass spectrometry; Man*p*, mannopyranosyl; MR, mannose receptor.

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the transcription of proinflammatory cytokine genes. Therefore, TLRs play a key role in innate immune recognition and in subsequent activation of adaptive immunity (21–23). Underhill et al. (24) have demonstrated that upon exposure to *M. tuberculosis*, macrophages are stimulated to produce TNF-α in a TLR2-dependent manner. These authors also showed that TLR2-dependent signaling mediates responses to mycobacterial cell wall fractions enriched for mycolyl-arabinogalactan-peptidoglycan, *M. tuberculosis* total lipids, or LAM. In addition, CD14 associated to TLR2 has previously been described as a receptor for PILAM (25–27). CD14 recognizes a variety of microbial products and functions as a pattern recognition receptor on macrophages, monocytes, and neutrophils (28–30). Activation of various cells by PILAM leads to the production of TNF-α and IL-8 (6, 7, 18, 27). In contrast to PILAM, ManLAM failed to induce proinflammatory cytokines through a CD14/TLR2-dependent mechanism. Moreover, ManLAM from *M. tuberculosis* inhibited TNF-α and IL-12 production in LPS-stimulated human mononuclear phagocytes through its binding to the mannose receptor (MR) (10, 31, 32). Recently, the dendritic cell-specific ICAM 3-grabbing nonintegrin (DC-SIGN) molecule, highly expressed on dendritic cells, has been also identified as a receptor for ManLAM of *M. tuberculosis*, through selective recognition of mannose caps (33). In particular, the binding of ManLAM to DC-SIGN prevents LPS-induced dendritic cell maturation (34). Paradoxically, despite the relative abundance of LM in the mycobacterial cell wall, the biological activities of LMs have been poorly investigated.

In this context, a further insight into the relationship between structure and possible properties of LMs was needed. As experimental models, we used LMs isolated from two pathogenic mycobacteria, *Mycobacterium chelonae* and *Mycobacterium kansasii*, commonly found in soil and water. *M. kansasii* is involved in serious pulmonary diseases in patients with immunodeficiencies and systemic illness, particularly those infected with HIV (35–37). *M. chelonae* infections are more frequently associated with cutaneous lesions in patients who are immunosuppressed by malignancy, corticosteroid therapy, or drugs (38, 39). We have recently purified and determined the structures of both LM and LAM from *M. chelonae* (40) and from a clinical strain of *M. kansasii* isolated from an HIV-positive patient (63). LMs are 6-fold more abundant in the cell wall of *M. chelonae* than LAM, whereas the levels of LAM and LM in *M. kansasii* are similar. These structures displayed significant discrepancies in relation to those of LMs and LAMs from other mycobacterial species (Fig. 1). In particular, we found that the arabinan moiety of LAM from *M. chelonae* (CheLAM) was neither capped with oligomannosyl motifs nor with phosphoinositol, thus defining a novel class of LAM. In addition to the absence of capping, the mannan domain was characterized by the presence of unusual α-1,3-mannosyl side chains instead of α-1,2 commonly found in all other mycobacterial species analyzed to date. Lastly, in contrast to lipoglycans from other species that only contain two major types of fatty acids (C_{16} and C_{19}) (18, 41, 42), the phosphatidyl-*myo*-inositol anchor of both CheLAM and LM purified from *M. chelonae* (CheLM) showed a wide lipidic heterogeneity (40). From a biological standpoint, CheLAM did not induce secretion of TNF-α and IL-8 from differentiated THP-1 cells (40). The recent structural determination of LAM from *M. kansasii* (KanLAM) has shown that it belongs to the general ManLAM family of molecules, although it contains subtle differences compared with ManLAMs from *M. tuberculosis* or *M. bovis* BCG (63). For instance, we demonstrated that KanLAM and LM purified from *M. kansasii* (KanLM) possess an unusual mannan core characterized by the presence of monomannosidic and dimannosidic branches, which contrasts with the structures of the mannan domain of all mycobacterial LAM molecules studied so far, which are generally characterized by the presence of α-1,2-linked mono-Man*p* substitutions, with the exception of CheLAM carrying α-1,3-linked mono-Man*p* side chains. Moreover, several groups reported the widespread distribution of 5-methylthiopentose in the ManLAM of various *M. tuberculosis* strains, and it was postulated that this substituent could be implicated in some of the biological properties of ManLAMs (43, 44). We found 5-methylthiopentose to be linked to the mannan core of both KanLM and KanLAM (63).

In the present study, we have investigated the ability of LMs purified from *M. chelonae* and *M. kansasii* to stimulate mRNA expression as well as TNF-α and IL-8 secretion in the human monocytic cell line THP-1. Cells were first differentiated with 1,25-dihydroxy-vitamin D_3, to ensure expression at the cell surface of the GPI-anchored protein CD14 (mCD14), a pattern recognition receptor for multiple microbial products. Availability of LMs with defined structural variations prompted us to study their structure/activity relationships. Assays were performed with LMs from four

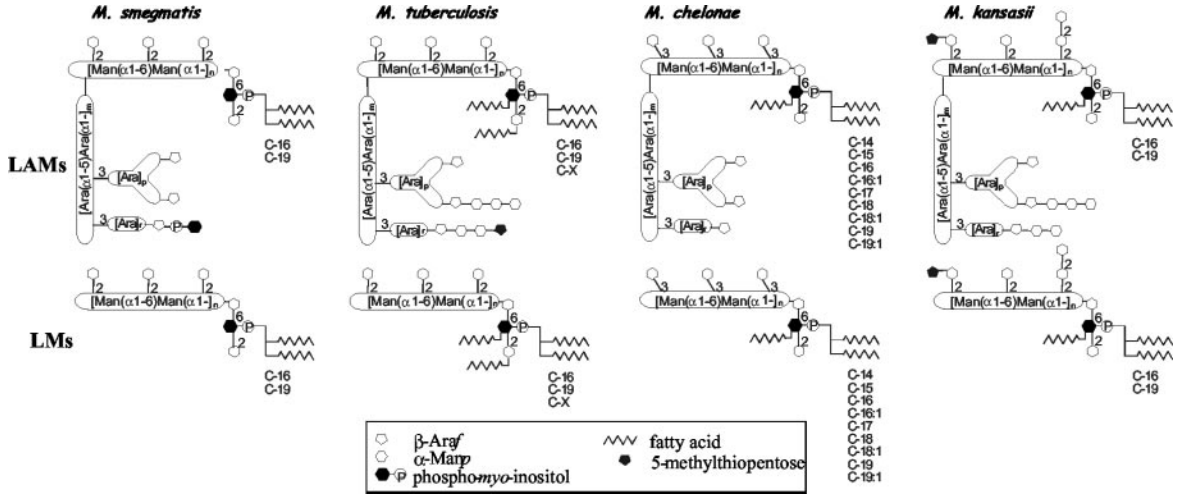

FIGURE 1. Structural models of LAMs and LMs from *M. chelonae*, *M. kansasii*, *M. tuberculosis/M. bovis* BCG, and *M. smegmatis*. The emphasis was put on the parts of the molecules showing interspecific heterogeneity, in particular the nature of capping, the substitution of the mannan core, and the acylation state of the GPI anchor.

species (*M. chelonae*, *M. kansasii*, *M. bovis* BCG, and *M. smegmatis*) in comparison with their respective LAM standards. The possible roles of mCD14, TLR2, and the LPS-binding protein (LBP), a serum acute-phase protein that facilitates the transfer of glycolipids to mCD14, in the stimulation of cells by LMs were also established. Moreover, to define the domain of LM that appears to be crucial for the cell activation process, a set of molecules presenting variable arabinan chain lengths was prepared by mild hydrolysis of KanLAM. The activities of the LAM-truncated variants as well as the phosphatidyl-*myo*-inositol dimannoside (PIM_2), a precursor molecule of LM, were compared with intact LM with regard to cytokine production.

Materials and Methods

Materials

RPMI 1640 medium and L-glutamine were purchased from Life Technologies (Eragny, France), and FCS was purchased from Dominique Dutscher (Brumath, France). Dulbecco's phosphate saline (PBS), BSA, isotype control mouse IgG2a and IgG2b, human IgG, FITC-conjugated goat anti-mouse IgG, and peroxidase-conjugated goat anti-rabbit IgG were obtained from Sigma-Aldrich (St. Louis, MO). Anti-CD14 monoclonal mouse IgG2b (clone MY4) and anti-TLR2 monoclonal mouse IgG2a (clone TL2-1) were from Coulter Immunology (Miami, FL) and Cascade Biosciences (Winchester, MA), respectively. Anti-human TLR4 monoclonal mouse IG2a (clone HTA125) was purchased from BD Biosciences (San Diego, CA). The 1,25-dihydroxy-vitamin D3 was obtained from Calbiochem (Darmstadt, Germany), and the apyrogen water was from Cooper (Melun, France). Human IL-8/Nap-1 module set (BSM204 MST) was from Bender Medsystems Diagnostic (Vienna, Austria). Human rLBP, neutralizing goat anti-human TNF-α, human TNF-α protein detection kit, and Quantikine mRNA kits were from R&D Systems (Minneapolis, MN). All chemicals used were of the highest analytical grade, and LPS contamination was evaluated with the *Limulus* amebocyte lysate assay kit (QCL1000; BioWhittaker, Walkersville, MD).

LMs isolated from *M. bovis* BCG and *M. smegmatis* as well as PILAM purified from *M. smegmatis* were kindly provided by Prof. G. Besra (School of Biosciences, University of Birmingham, Birmingham, U.K.).

Extraction and purification of LMs and LAMs from M. chelonae *and* M. kansasii

Lipoglycans from these two pathogenic mycobacterial species were purified, by successive detergent and phenol extractions, leading to the obtainment of a protein-, lipid-, and nucleic acid-free material, as described previously (40). Then, lipoglycans were resuspended in Tris deoxycholate buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.2 M NaCl, and 0.25% deoxycholate), and LAMs, LMs, PIMs were separated by gel filtration on a Sephacryl 200 column (Amersham Biosciences, Uppsala, Sweden). LAMs and LMs were extensively dialyzed. Purity of preparations was assessed by gas chromatography/mass spectrometry (GC/MS) routine experiments, nuclear magnetic resonance spectra, and SDS-PAGE following silver nitrate staining (40). No contamination with either lipoproteins or lipopeptides could be detected. Moreover, the endotoxin content of all of the reagents was measured in a chromogenic *Limulus* lysate assay. The LM and LAM preparations contained insignificant amounts of endotoxin (<20 pg LPS per 10 μg of KanLM or KanLAM; <150 pg LPS per 10 μg of CheLM or CheLAM).

Purification of PIM_2 from M. kansasii

PIMs were obtained from the Tris deoxycholate-insoluble lipid fraction. This material was repetitively extracted by chloroform/methanol/water (50/50/5) and applied to a silica gel (KG60; 0.063–0.200 mm; Merck, Strasbourg, France) column irrigated with chloroform, and then with chloroform/methanol/water eluant by increasing proportions of methanol/water. Glycolipids, including important quantities of lipooligosaccharides and PIMs were eluted from the 50/50/5 eluant. PIMs were finally separated on a DEAE cellulose (acetate form) column irrigated with chloroform/methanol/ammonium acetate eluant by increasing proportions of methanol/ammonium acetate. Fractions with purified PIMs were eluted with chloroform/methanol (1/2) containing 0.05 M ammonium acetate. Purity of the samples was assessed by GC/MS analysis, and ^{1}H homonuclear and ^{13}C-^{1}H heteronuclear multiple quantum coherence experiments were used to typify this fraction as PIM_2.

Preparation of truncated LAM fractions

Five milligrams of purified KanLAM were submitted to 2 M acetic acid hydrolysis at 80°C for 2 h and 30 min. Sample was lyophilized and resuspended in Tris deoxycholate buffer, and hydrolyzed compounds were fractionated on a Sephacryl 200 column according to their size. The extent of hydrolysis was assessed by SDS-PAGE. Collected fractions were pooled in five fractions (F1–F5), and their composition was determined by GC/MS analysis.

Cell culture

Human promonocytic leukemia THP-1 cells (88081201; European Cell Culture Collection, Porton Down, U.K.) were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 2×10^{-5} M 2-ME in 5% CO_2-air humidified atmosphere at 37°C. To express a high amount of mCD14 at the cell surface, THP-1 cells were differentiated by treatment for 72 h with 50 nM 1,25-dihydroxy-vitamin D3 (45). Viability of cells was >96% as determined by trypan blue dye exclusion.

mCD14 and TLR2 expressions at the surface of THP-1 cells

mCD14 and TLR2 expression on differentiated and undifferentiated THP-1 cells was analyzed by flow cytometry. Cells (300,000) were incubated for 20 min at 4°C with 20 μg/ml human IgG, washed three times, and incubated for 45 min with 12 μg/ml either anti-CD14 (MY4) or anti-TLR2 (clone TL2-1) mAbs in PBS containing 0.04% NaN_3 and 0.05% BSA. Mouse isotype Igs were used as negative controls. After three washings, the cells were stained with FITC-conjugated goat anti-mouse IgG (1/64) for 30 min at 4°C. Cells were analyzed with a BD Biosciences FACSCalibur flow cytometer and gated for forward- and side-angle light scatters. Approximately 10,000 particles of the gated population were analyzed. The fluorescence channels were set on a logarithmic scale, and the mean fluorescence intensity was determined.

Induction of the secretion of TNF-α and IL-8 by LAM and LM preparations

Promonocytic and differentiated THP-1 cells were seeded in 96-well plastic culture plates at a density of 2×10^5 cells/well, in RPMI supplemented with 10% FCS and glutamine. Increasing concentrations of purified LAMs and LMs were added for 6 and 24 h, to induce TNF-α and IL-8 secretion, respectively. Culture supernatants of triplicates were then collected and centrifuged before quantitation of cytokines by sandwich ELISA, using commercially available kits and according to the manufacturers' instructions.

Morever, to determine to what extent IL-8 secretion is mediated by TNF-α released from LM-treated cells, differentiated THP-1 cells were stimulated for 24 h at 37°C with 1 or 10 μg/ml LMs, in the absence or presence of neutralizing goat polyclonal Abs directed against TNF-α.

In other experiments, monoclonal anti-CD14 (MY4), anti-TLR2 (clone TL2-1), and anti-TLR4 (clone HTA125) Abs were used to investigate whether the LM-inducing cytokine activity was CD14/TLR2 dependent. Differentiated cells were pretreated for 30 min at 37°C, with either 10 μg/ml anti-CD14, anti-TLR2, or isotype Abs as controls. CheLM or KanLM (10 μg/ml) were then added to the cells for 6 and 24 h to allow production of TNF-α and IL-8, respectively.

The effect of recombinant human LBP on TNF-α and IL-8 production induced by LMs was also investigated. Purified recombinant human LBP (0.5 ng/ml) was preincubated for 30 min at room temperature with 1 or 10 μg/ml LMs and then added to differentiated cells (200,000 cells/well) at 37°C in RPMI without FCS. After 6 or 24 h of incubation, TNF-α and IL-8 secretion was measured in the supernatants of triplicates. Controls were performed in the absence of recombinant LBP.

Cytokines mRNA expression induced by LMs and LAMs

To investigate the effect of LAMs and LMs on TNF-α and IL-8 mRNA expression in differentiated THP-1 cells, 1×10^6 cells/ml were incubated in six-well plates in RPMI/10% FCS/glutamine in the presence of 10 μg/ml each glycoconjugate. After 2 and 5 h incubations, at 37°C, the cells were harvested by centrifugation, and the level of human TNF and IL-8 mRNA was measured by colorimetric mRNA quantification (R&D Systems). This method uses a conventional microplate reader with colorimetric detection to quantify gene-specific mRNA at low levels. Briefly, stimulated cells were resuspended in Quantikine mRNA cell lysis diluent (2×10^6 cells/ml). Cell lysates were diluted, and RNA contained in samples were hybridized with gene-specific biotin-labeled capture probes and digoxigenin-labeled detection probes in a microplate for 1 h at 65°C. The hybridization solution was then transferred to a streptavidin-coated microplate, and the

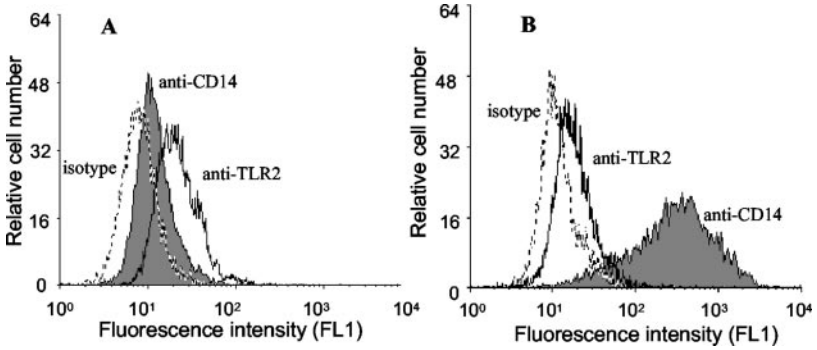

FIGURE 2. Cell surface expression of TLR2 and mCD14. Promonocytic THP-1 cells were exposed to 1,25-dihydroxy-vitamin D_3 for 72 h, as described in *Materials and Methods*. Analysis of mCD14 and TLR-2 expression at the surface of undifferentiated (*A*) and differentiated (*B*) cells was performed by flow cytometry using anti-CD14 (MY4) and anti-TLR2 (clone TL2-1) mouse Abs or an isotype control Ab, followed by incubation with FITC-labeled anti-mouse IgG. Expression of mCD14 is represented by shaded area, whereas expression of TLR2 is shown as a solid line. Cells incubated with the control isotype are shown as a dotted line. Results are shown as linear-log scale fluorescence histogram. Results shown are representative of two independent experiments.

RNA/probe hybrid was captured at room temperature for 1 h. After washing to remove unbound material, anti-digoxigenin alkaline phosphatase was added. In the last step, a substrate solution and then an amplifier solution were added. Intensity of the color developed in proportion to the amount of gene-specific mRNA in the original sample was measured spectrophotometrically. A serial dilution of specific TNF-α and IL-8 mRNA calibrators was performed to determine the concentration of mRNA in each sample, and results were expressed in 10^{-18} moles per milliliter (attomoles per milliliter).

Statistical analysis

Statistical significance was analyzed with Student's *t* test for unpaired data. Values of $p < 0.05$ were considered to be significant.

Results

LMs, but not LAMs, induce secretion of proinflammatory cytokines in differentiated THP-1 cells

The human myelomonocytic leukemia cell line THP-1 is known to overexpress mCD14 during differentiation of cells with 1,25-dihydroxy-vitamin D3 and to release cytokines after stimulation with pathogen-derived molecules (45). As illustrated in Fig. 2, flow cytometric analysis has confirmed the presence of higher levels of mCD14 at the surface of differentiated THP-1 cells, compared with undifferentiated THP-1 cells. In contrast, cell differentiation did not affect the expression level of TLR2.

The capacity of LAMs and LMs, isolated from *M. kansasii* and *M. chelonae*, to trigger TNF-α and IL-8 secretion was investigated on both undifferentiated and differentiated THP-1 cells. As shown in Fig. 3, various concentrations of LMs were found to induce the release of TNF-α and IL-8 from differentiated cells, whereas no effect could be detected with undifferentiated THP-1. In differentiated cells, a peak of induction was obtained in the presence of 10 μg/ml LMs. The level of cytokines induced by LMs was ~17-fold higher for TNF-α and 8-fold higher for IL-8, compared with unstimulated cells (medium alone). Moreover, CheLM and KanLM displayed similar cytokine-inducing responses. In contrast to LMs, the mannose-capped KanLAM or the noncapped CheLAM did not induce any cytokine release even in the presence of 20 μg/ml. When polymyxin B, a cationic peptide that blocks the biological function of LPS (46), was added to the cultures, it did not inhibit cytokine secretion (data not shown). This suggests that the cytokine-inducing responses observed with the various LM preparations could not be attributed to an eventual LPS contamination.

Because TNF-α stimulates the secretion of IL-8 in monocytes/macrophages (47), we investigated whether the IL-8 production detected in the supernatants of THP-1 cells stimulated with LMs may be a consequence of the induction of TNF-α. To address this question, cells were incubated with LMs as well as a neutralizing anti-TNF Ab. As reported in Table I, addition of this Ab only partially reduced (21 or 6%) the IL-8 production induced by 10

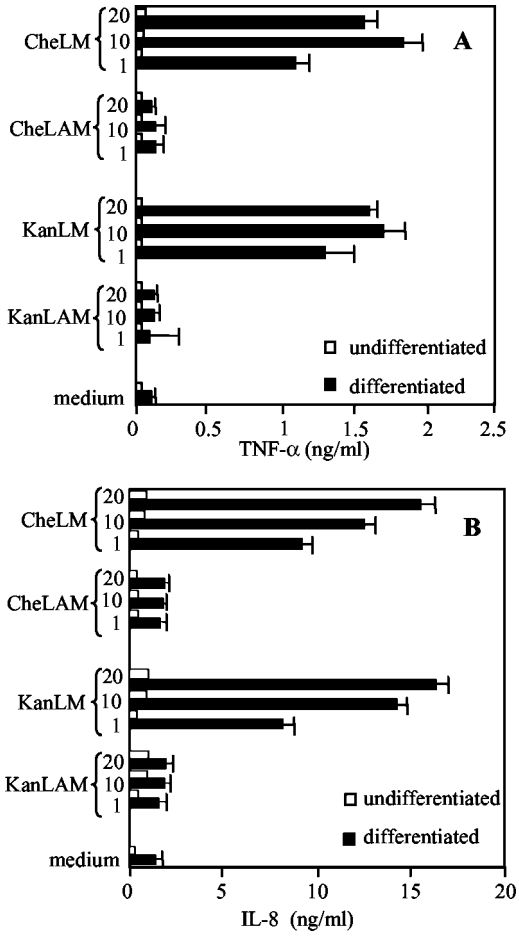

FIGURE 3. Proinflammatory cytokine response in THP-1 cells stimulated either with LMs or LAMs. Undifferentiated or differentiated human promonocytic THP-1 cells were incubated in 10% FCS-RPMI 1640 medium with increasing concentrations of CheLM, KanLM, CheLAM, or KanLAM (1, 10, and 20 μg/ml). Culture supernatants were collected after 6 or 24 h and assayed by ELISA for TNF-α (*A*) or IL-8 (*B*) secretion, respectively. The results presented are from one representative experiment of three independent experiments with similar results. Data are expressed as means ± SD of triplicate wells.

Table I. *Effect of TNF-α neutralization on IL-8 secretion in the supernatant of THP-1 cells stimulated with LMs*[a]

	No Abs	Anti-TNF-α	% of Inhibition
Unstimulated cells	1.63 ± 0.22		
CheLM			
1 μg/ml	3.70 ± 0.12	2.63 ± 0.18	29.03
10 μg/ml	7.03 ± 0.11	5.53 ± 0.15	21.33
KanLM			
1 μg/ml	3.01 ± 0.24	2.31 ± 0.2	23.23
10 μg/ml	6.06 ± 0.11	5.66 ± 0.31	6.7

[a] Differentiated THP-1 cells were stimulated for 24 h with CheLM or KanLM in the absence or presence of anti-TNF-α neutralizing Abs. Supernatants of triplicates were collected, and IL-8 secretion (nanograms per milliliter) was measured by ELISA. The results were expressed as the mean ± SD and are representative of three independent experiments.

μg/ml CheLM or KanLM, respectively. This result suggests that the vast majority of IL-8 released upon stimulation with LMs does not result from the secretion of TNF-α. We next compared the cytokine-inducing activities of various LMs purified from four mycobacterial species. PILAM from *M. smegmatis* was also added to this experiment as a positive control, because it induces a strong TNF-α production in THP-1 cells (18). As shown in Fig. 4, LMs from *M. bovis* BCG, *M. smegmatis*, *M. chelonae*, and *M. kansasii* were all potent TNF-α- and IL-8-inducing molecules, and no significant differences were observed with respect to their induction capacity. An optimal response was obtained with 10 μg/ml LM, which could not be further increased with higher concentrations. At 1 μg/ml, LMs were more potent in inducing cytokines than was PILAM. However, taking into account the different molecular masses of LMs and PILAM—an average of 17 kDa for PILAM and 6 kDa for LMs—these molecules exhibit comparable activities toward cytokine induction at a concentration of 10 μg/ml.

To address whether secretion of TNF-α and IL-8 by differentiated THP-1 cells stimulated with LMs results from an induction of specific mRNA synthesis, a colorimetric mRNA quantitation method was applied, as described in *Materials and Methods*. Total mRNA were isolated after 2 or 5 h of stimulation with LMs to determine their specific content in either TNF-α or IL-8 mRNA, respectively. Both CheLM and KanLM strongly up-regulated both TNF-α or IL-8 mRNA synthesis, whereas their respective LAMs failed to induce significant mRNA production (data not shown). This data indicates that the enhanced levels of cytokines detected in the supernatant of LM-stimulated cells are the consequence of specific mRNA up-regulation activities.

LMs induce cytokine secretion through a CD14/TLR2- and LBP-dependent pathway

Means et al. (26) reported that TLR2, but not TLR4, confers PILAM responsiveness to Chinese hamster ovary cells engineered to express mCD14. Given the importance of CD14 and TLR2 in inducing intracellular signaling to promote an inflammatory immune response during microbial infection, we investigated whether these two receptors are involved or not in the stimulation of THP-1 by LMs and account for the biological activities observed with LMs. Studies were conducted by measuring the inhibitory effect of cytokine production using specific anti-CD14 (MY4), anti-TLR2 (clone TL2-1), and anti-TLR4 (clone HTA125) mAbs. As demonstrated in Fig. 5, whereas differentiated THP-1 cells were not activated by anti-CD14 and anti-TLR2 Abs in the absence of LM, anti-CD14 Abs prevented the LM-induced expression of TNF-α and IL-8 by cells. TNF-α secretion was inhibited down to 90.2 and 78.7% for CheLM and KanLM, respectively. IL-8 expression was

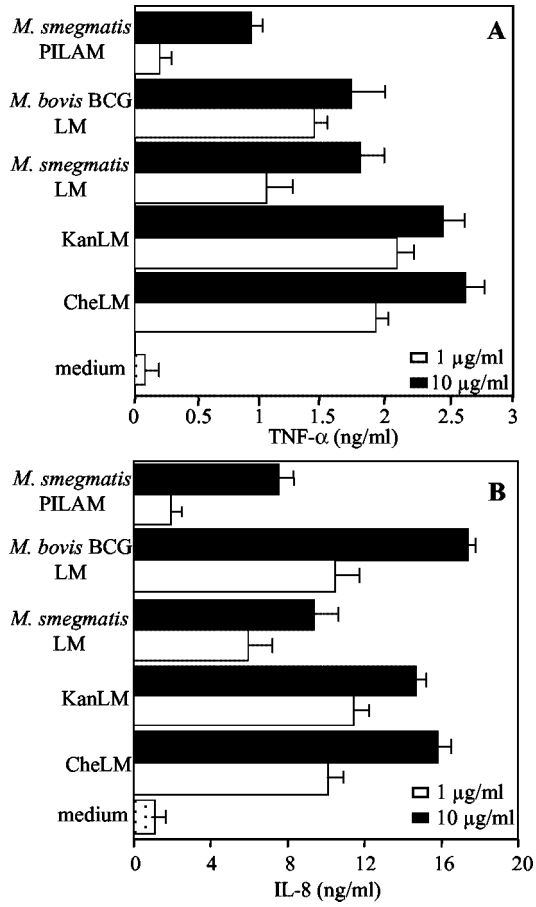

FIGURE 4. Release of proinflammatory cytokines from differentiated THP-1 cells in response to different LMs and PILAM. LMs isolated from *M. smegmatis*, *M. bovis* BCG, *M. chelonae*, or *M. kansasii*, as well as PILAM purified from *M. smegmatis*. Differentiated THP-1 cells were incubated with 1 and 10 μg/ml each glycolipid. Culture supernatants were collected after 6 or 24 h and assayed by ELISA for TNF-α (*A*) and IL-8 (*B*) secretion, respectively. The results presented are from one representative experiment of two independent experiments with similar results. Data are expressed as means ± SD of triplicate wells.

inhibited at 50.8 and 52.5% for CheLM and KanLM, respectively. Regarding the possible participation of TLR2 signaling, a strong inhibition of 86% was obtained when cells were pretreated with anti-TLR2 Abs. Whereas anti-TLR4 Abs blocked the LPS-mediated cytokine induction (data not shown), they did not significantly alter the activity of LMs. Incubation with the isotype mAbs did not affect cytokine production. These results clearly suggest the possible participation of both CD14 and TLR2, but not TLR4, in the cell activation process leading to TNF-α and IL-8 production by LMs.

We next examined whether LBP, an acute-phase protein present in serum that mediates the transfer of glycolipids to CD14, which ultimately leads to cell activation (25, 27, 48), may also modulate the cytokine-inducing activity of LMs. Experiments were performed in serum-free medium with 1 and 10 μg/ml LMs supplemented or not with 0.5 ng/ml human recombinant LBP. As shown in Fig. 6, preincubation of CheLM or KanLM with LBP resulted in a significant secretion of TNF-α and IL-8 from differentiated cells, whereas no stimulation was detected in the absence of rLBP, underlining an important function of LBP in inducing cytokine production by LMs. Altogether, these results suggest that LM-induced signaling events in differentiated THP-1 cells are mediated through a CD14/TLR2/LBP-dependent mechanism.

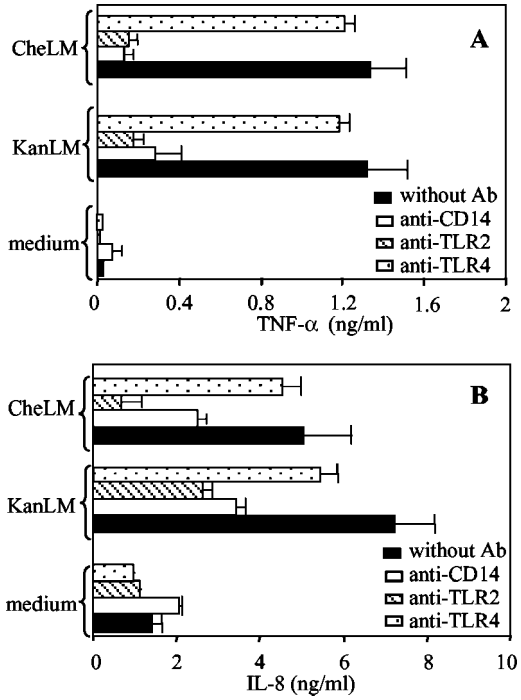

FIGURE 5. Inhibition of the TNF-α and IL-8 production using anti-CD14, anti-TLR-2, or anti-TLR-4 Abs. Differentiated THP-1 cells were pretreated with 10 μg/ml either an anti-CD14, an anti-TLR2, an anti-TLR4, or control isotype mAb (not shown) or without Abs, for 30 min at 37°C in 10% FCS-RPMI 1640 medium, before the addition of CheLM or KanLM (10 μg/ml). Supernatants were collected after 6 or 24 h and assayed by ELISA for TNF-α (*A*) and IL-8 (*B*) secretion, respectively. The results presented are from one representative experiment of three independent experiments with similar results. Data are expressed as means ± SD of triplicate wells.

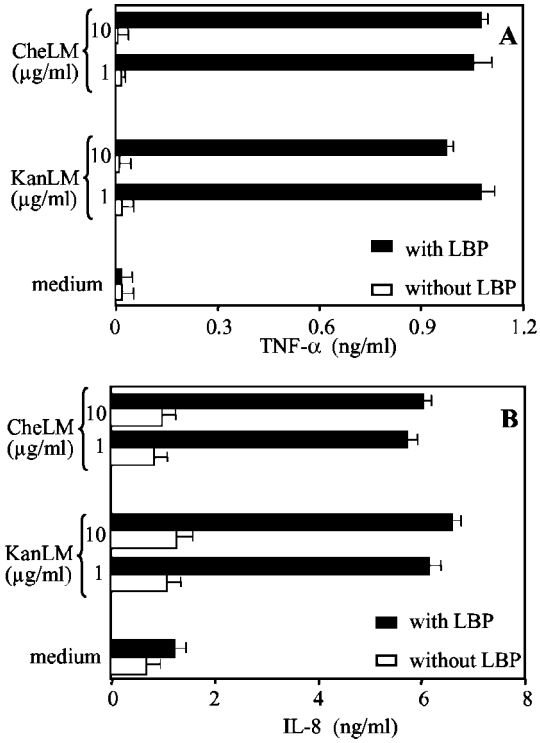

FIGURE 6. Effect of LBP in modulating the LM activity. LMs (1 and 10 μg/ml) were preincubated for 30 min at room temperature either alone or with 0.5 ng/ml purified human recombinant LBP, before addition to differentiated THP-1 cells in RPMI 1640 without FCS. Culture supernatants were collected after 6 or 24 h and assayed by ELISA for TNF-α (*A*) and IL-8 (*B*) secretion, respectively. The results presented are from one representative experiment of three independent experiments with similar results. Data are expressed as means ± SD of triplicate wells.

Removal of the arabinan moiety of KanLAM restores the ability to induce cytokines

Because LMs, but not LAMs, are able to stimulate TNF-α and IL-8 production, we have hypothesized that the presence of arabinan domain and/or the mannan cap in LAMs may prevent this mechanism of action. To address this question, we took advantage of the higher susceptibility of arabinose linkages to specifically release the arabinan chains from LAM by mild hydrolysis treatment. Therefore, we generated five fractions containing LAMs with partially truncated arabinan domains by gel exclusion chromatography on a S200 column and analyzed their mobilities by electrophoresis on a polyacrylamide gel (Fig. 7*A*). Purified KanLAM and KanLM were also loaded on the gel as standard reference molecules. Sizes of the five purified fractions ranged from nearly intact LAM (F1) to highly truncated molecules (F5) that ran even faster than purified KanLM (Fig. 7*A*). As expected, assessment of their mannose and arabinose contents by routine gas chromatography established that the decrease of molecular mass of fractions F1 to F5 correlated with a drastic shortening in their arabinan domain (Fig. 7*B*). Consequently, it was assumed that simultaneous reduction of their mannose content originated from the release of oligomannosyl domains capping arabinosyl side chains that followed degradation of arabinan domain. It is noteworthy that the most truncated molecules (F4 and F5) had smaller molecular masses than native LM. This suggests that the mannan domain was slightly reduced during hydrolysis, presumably through the loss of Man(α1–2) side chains due to the lower stability of α1,2 glycosidic linkage compared with α1,6 linkage under acidic conditions.

Analysis of the cytokine-inducing activity of these LAM-truncated molecules revealed that the longer variants corresponding to fractions F1 and F2 (arabinose/mannose ratios of 1.16 and 0.81, respectively) were unable to induce the TNF-α and IL-8 secretion, as reported for native KanLAM (Fig. 8). However, fractions F3 to F5, characterized by shorter arabinan domains (arabinose/mannose ratios ranging from 0.65 to 0.6), elicited a strong cytokine production response. In addition, the capacity of these three fractions to stimulate IL-8 secretion was found to be similar to that of native KanLM. Whereas mannan domain of F5 was partially degraded compared to native LM (Fig. 7*A*), fraction F5 showed an IL-8-inducing activity similar to the one detected with KanLM, F3, or F4 (Fig. 8*B*). These results clearly establish that the presence of arabinan blocks the induction of a proinflammatory response in differentiated THP-1 cells mediated by TLR2/CD14 receptors. Thus, we propose that the presence of arabinan domain of KanLAM, and eventually the terminal mannosyl caps, modulates its biological properties by masking the mannan domain, presumably through sterical hindrance.

PIM_2 fails to stimulate TNF-α and IL-8 secretion

Because the mannan core of LM seems to be required to induce an efficient proinflammatory response, we wanted to define more precisely the domain of LM that is important to explain this biological activity. PIMs are considered to be the precursors of LM (17). The biosynthesis of LM involves the addition of mannopyranosyl (Man*p*) residues to phosphatidyl-*myo*-inositol to produce the short PIMs (two to five Man*p* residues) which are further elongated to form an α1,6-linked Man*p* backbone substituted by Man*p* units.

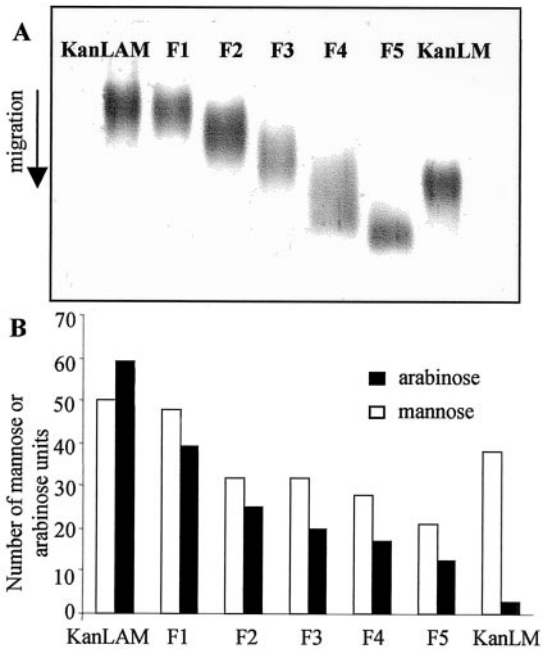

FIGURE 7. Generation of KanLAM truncated variants through mild hydrolysis treatment. KanLAM was hydrolyzed for 2 h and 30 min at 80°C with 2 M acetic acid, and fractionated using a Sephacryl 200 column, as described in *Materials and Methods*. The five fractions (F1–F5) collected were analyzed by SDS-13% PAGE, followed by silver staining after periodic oxidation treatment (*A*). Average mannose and arabinose contents per molecule were defined by routine gas chromatography (*B*). KanLAM and KanLM were also added as standard control molecules.

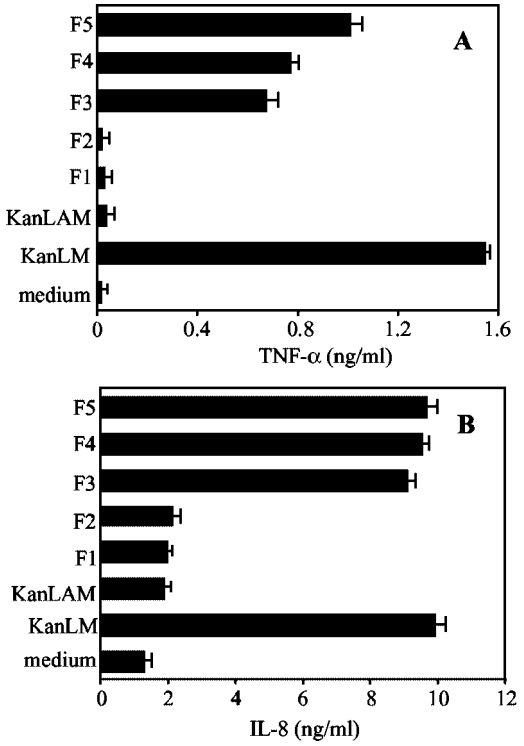

FIGURE 8. Cytokine-inducing activity of truncated KanLAM variants. Differentiated THP-1 cells were stimulated with 10 μg/ml each fraction (F1–F5) or with native KanLM or KanLAM, in RPMI 1640 supplemented with 10% FCS. Culture supernatants were collected after 6 or 24 h and assayed by ELISA for TNF-α (*A*) and IL-8 (*B*) secretion, respectively. The results presented are from one representative experiment of three independent experiments with similar results. Data are expressed as means ± SD of triplicate wells.

PIM_2 was purified from *M. kansasii* and used to stimulate differentiated THP-1 cells to evaluate the capacity of this minimal glycolipidic structure to induce cytokine production (Fig. 9). In contrast to LM, no production of TNF-α or IL-8 secretion could be detected in the supernatant from differentiated THP-1 stimulated with increasing concentrations of PIM_2, even at the highest concentration of 10 μg/ml. Levels of cytokines secreted in the presence of PIM_2 were comparable with those found in supernatants of unstimulated cells (medium alone). Therefore, the presence of only two Man*p* residues bound to the phosphatidylinositol anchor are not sufficient to trigger a proinflammatory response. This data supports the hypothesis that structures with a higher number of Man*p* residues are therefore necessary for eliciting this proinflammatory response.

Discussion

So far, most studies have focused on the functional properties of ManLAMs and PILAM (3, 4), which represent major glycolipids of the mycobacterial cell wall. LM is also a very abundant cell wall component and represents one of the major lipoglycan fractions. However, presumably because it is considered to be a direct precursor of LAM, the biological relevance of LM in the host defense has not been thoroughly investigated. In the present work, we have studied the activity of LMs purified from two pathogenic mycobacteria, *M. kansasii* and *M. chelonae*. We have demonstrated that LMs induced TNF-α and IL-8 secretion in the human macrophage-like THP-1 cell line in a dose-dependent manner. This response was dependent on the cell differentiation state, because undifferentiated promonocytic THP-1 cells were not activated by LMs. During the differentiation of THP-1 cells, mCD14 was overexpressed at the cell surface, as previously reported by Vey et al. (45). The involvement of mCD14 in the LM-mediated cell activation process was confirmed using specific anti-CD14 Abs (MY4). This mAb recognizes an epitope ranging from amino acid residues 39–44, which are involved in the binding to LPS (49). Because My4 also blocked the cytokine-inducing activity of LMs on THP-1 cells, one may suspect that residues 39–44 of CD14 also interact with LMs.

The TLR transmembrane signal-transducing proteins are involved in the activation of cells by LPS and other microbial products (50). In particular, PILAM activates macrophages through both TLR2 and CD14 receptors (26). In this study, differentiated cells were pretreated with monoclonal anti-TLR2 Abs (clone TL2-1) previously shown to interfere in the activation of cells with *M. avium* and purified lipoproteins/lipopeptides from spirochetes (51). These Abs also inhibited the proinflammatory response elicited by LMs, suggesting that the signaling responses mediated by LMs are CD14/TLR2 dependent. Recently, it has been proposed that TLR2 may dimerize with other TLRs to induce a signal (23, 52, 53). Thus, the involvement of other TLRs in host responses to LMs cannot be excluded. In contrast to the findings of Abel et al. (53), we have found that LMs exhibit a TLR4-independent effect. These authors demonstrated that PIM_{4-6} from *M. tuberculosis* H37Rv induces IL-6 production through both TLR2 and TLR4 in mice and in the human astrocytic U373 cell line. These differences may be explained by the nature of the glycolipids and the different cellular sources they were using. The availability of knockout mice defective in different TLRs will help us to define the eventual participation of these receptors in the inflammatory response induced by LMs.

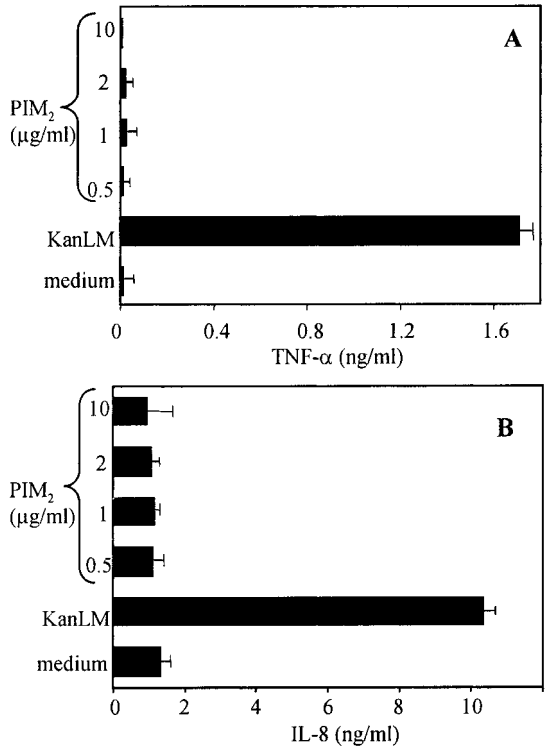

FIGURE 9. Capacity of PIM_2 to trigger TNF-α and IL-8 secretion. Differentiated THP-1 cells grown in 10% FCS-RPMI 1640 were stimulated with increasing concentrations of PIM_2 purified from *M. kansasii*. Culture supernatants were collected after 6 or 24 h and assayed by ELISA for TNF-α (*A*) and IL-8 (*B*) secretion, respectively. The results presented are from one representative experiment of three independent experiments with similar results. Data are expressed as means ± SD of triplicate wells.

LBP is a serum protein with a lipotransferase activity, which is required for endotoxin-induced cell activation (54, 55). More particularly, the importance of LBP in promoting the activity of PILAM on HL60 and THP-1 cell lines as well as astrocytes has been reported (25, 27, 48). We demonstrate here that LBP is also essential for LMs to activate differentiated THP-1 cells, leading to the hypothesis that LBP may mediate the transfer of LM to mCD14. Increasing serum concentrations of LBP have previously been reported in patients with active tuberculosis (56). Hence, in vivo, LBP may modulate the bioavailability of LM and therefore regulate the inflammatory response during mycobacterial infection.

Surprisingly, our data demonstrate that, despite significant structural variations both in polysaccharidic core and in lipid anchor, all LMs tested showed very similar inducing properties. This suggests that their immunological modulating properties are solely linked to the presence of either a mannan core or a phosphatidyl-*myo*-inositol anchor, independently of their structural variability. The fact that PIM_2 failed to induce any response favors the involvement of the sole mannan core in the activity mediated by LMs and excludes the possibility of the phosphatidylinositol anchor being responsible for this activity. However, numerous studies have reported that biological activities of lipoglycans were highly dependent on the integrity of their lipid moieties (6, 41). Deacylation of CheLM or KanLM completely abrogated their TNF-α secretion-inducing properties toward differentiated THP-1 cells at low concentrations (data not shown), whereas LMs were potent cytokine inducers even at 1 μg/ml. Unexpectedly, the activity of deacylated LMs was partially restored at higher concentrations (10 μg/ml). Two recent studies have established that the lipid anchor of ManLAMs exerts its influence on IL-12 induction and surfactant protein A fixation through the formation of micelles, and that it is not directly implicated in a receptor binding event (10, 57). Presentation of lipoglycans integrated in micelles would then be a prerequisite for the acquisition of multivalency and the efficient presentation of ManLAM to MR and to surfactant protein A. Indeed, multivalency appears to be a common feature in interactions involving carbohydrates, in both lectin-carbohydrate- and carbohydrate-carbohydrate-based interactions (58). In the light of these results, if multivalency is also important to explain the LM-inducing properties, partial activity restoration for high concentrations of deacylated LM may simply result from the acquisition of a pseudo-multivalency effect due to high local concentration of LM.

The lack of activity of the PIM_2 fraction is difficult to interpret with regard to previously published results. Indeed, some authors reported a stimulation of TNF-α secretion by PIMs in macrophages (6, 8, 53, 59), whereas others have demonstrated a 100-fold lower induction activity of PIM_6 compared with PILAM (5). It is noteworthy that most studies were conducted using mixtures of PIMs, which render direct comparisons of the results difficult to perform. If, as our results tend to demonstrate, the mannan domain is involved in the stimulation activity of LM, the heterogeneity of the mannan chain length of PIMs is expected to lead to various effects. In this context, the fact that PIM_2 did not show any activity established that the presence of only two mannose residues—one substituting the *myo*-inositol group in C-2 position and the other in C-6 position—was not sufficient to trigger TNF-α and IL-8 release from differentiated THP-1 cells. This suggests that the degree of mannosylation modulates the cytokine-inducing effect of lipoglycans, supporting the hypothesis that the biological properties of LM are associated with the mannan core. This was further supported by the demonstration that arabinan domain of LAM prevented the cytokine production induced by lipoglycans. Indeed, starting from a KanLAM that presented no cytokine-inducing effect, an optimal activity level similar to the one exhibited by LM was restored by progressively degrading the arabinan domain of KanLAM. Complete degradation of the arabinan domain was not necessary to restore the activity, an effect being observed from 70% of arabinan degradation and release of 20 mannose residues (fraction F3). Incidentally, 20 mannose residues correspond to the calculated average number of mannose residues of the oligomannoside-capping domain (63). We observed a dramatic increase in activity between F2 and F3, despite a minimal difference in arabinan chain length between these two components. This effect was observed for both IL-8 and TNF-α and may be explained by higher levels of active molecules in F3 than in F2. Indeed, each fraction contains a heterogeneous population of molecules. A minor proportion of molecules with a critical truncated arabinan domain may be sufficient to trigger the biological effect, suggesting the occurrence of a functional turning point between F2 and F3. From F3 to F5, the TNF-α release increased with the extent of arabinan degradation. On this basis, we hypothesize that the arabinan domain exerts its inhibiting effect through sterical hindrance that precisely prevents interaction of the underlying mannan domain. This is consistent with our previous observations that the arabinan domain of CheLAM prevented recognition of this molecule by lectins isolated from *Canavalia ensiformis* (Con A) and from *Galanthus nivalis* (*Galanthus nivalis* agglutinin), presumably due to steric hindrance of the mannan core by the arabinan polymer, although these two lectins were able to recognize CheLM (40).

As presented in this study, LMs and PILAM exhibited similar cytokine induction levels through pathways involving both CD14 and TLR2, in contrast to ManLAMs and noncapped LAMs. Presently, no available data allow us to understand the molecular bases

of the sorting out of the lipoglycans with fairly similar structures but with different functions. With respect to their structures, it is unlikely that LMs and PILAM exhibit a single common structural feature that would explain their specific activity. Previous studies strongly suggested that the inositol-phosphate capping may represent the major cytokine-inducing component of PILAM (18, 40). One tentative hypothesis would be that the *myo*-inositol-phosphate motif incorporated in the lipid anchor of lipoglycans, if accessible, may mimic the role of inositol-phosphate capping of PILAM, presumably accessible in LMs but not in LAMs due to steric hindrance by the arabinan domain. However, due to decoration by mannose residues in C-2 and C-6, and possibly by an additional acyl group in C-3 position, it is highly improbable that the *myo*-inositol-phosphate of the lipid anchor mimics the terminal, unsubstituted *myo*-inositol-phosphate. Furthermore, the lack of activity of PIM_2 definitely rules out this hypothesis. Therefore, it is difficult to define the exact recognition patterns of TLRs. This family of receptors seems to interact with a broad range of structurally unrelated microbial products including peptidoglycans, LPSs, lipoglycans, and lipoproteins (22). On this basis, it is likely that LMs and PILAM are recognized by a different panel of TLRs, including TLR2, presenting distinct structural requirements, in association with CD14 and LBP.

The inability of KanLAM to induce proinflammatory cytokines production is in agreement with the biological activity reported for ManLAM purified from *M. tuberculosis* or *M. bovis* BCG. ManLAM from virulent *M. tuberculosis* Erdman strain elicited very low TNF-α secretion (5, 60, 61). ManLAM is considered to be an anti-inflammatory molecule that down-regulates IL-12 production by LPS-stimulated macrophages and dendritic cells through MR and DC-SIGN ligation (4, 33, 34). These studies suggest that these MR and DC-SIGN interfere with TLR4-mediated signals through an inhibiting effect. However, as checked by immunofluorescence flow cytometry (data not shown), differentiated THP-1 cells express low levels of MR but not DC-SIGN. The absence of DC-SIGN on these cells was already reported (62). Additional experiments are required to investigate the potential inhibitory activity of KanLAM on LPS-stimulated dendritic cells.

In conclusion, our study strongly suggests that LMs are not only precursors of LAM biosynthesis but do indeed participate in the innate inflammatory response through a CD14/TLR2-dependent mechanism. The presence of large amounts of LM in the cell walls of various pathogenic mycobacteria may influence the host immune defenses. Our studies are also paving the way for further investigation into the functions of LM in virulence and mycobacterial pathogenesis and for the development of new therapeutic approaches.

Acknowledgments

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References

1. Daffe, M., and P. Draper. 1998. The envelope layers of mycobacteria with reference to their pathogenicity. *Adv. Microb. Physiol. 39:131.*
2. Chatterjee, D. 1997. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. *Curr. Opin. Chem. Biol. 1:579.*
3. Chatterjee, D., and K. H. Khoo. 1998. Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology 8:113.*
4. Nigou, J., M. Gilleron, M. Rojas, L. F. Garcia, M. Thurnher, and G. Puzo. 2002. Mycobacterial lipoarabinomannans: modulators of dendritic cell function and the apoptotic response. *Microbes Infect. 4:945.*
5. Chatterjee, D., A. D. Roberts, K. Lowell, P. J. Brennan, and I. M. Orme. 1992. Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor. *Infect. Immun. 60:1249.*
6. Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin. 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan: relationship to chemical structure. *J. Immunol. 149:541.*
7. Zhang, Y., M. Doerfler, T. C. Lee, B. Guillemin, and W. N. Rom. 1993. Mechanisms of stimulation of interleukin-1β and tumor necrosis factor-α by *Mycobacterium tuberculosis* components. *J. Clin. Invest. 91:2076.*
8. Zhang, Y., M. Broser, H. Cohen, M. Bodkin, K. Law, J. Reibman, and W. N. Rom. 1995. Enhanced interleukin-8 release and gene expression in macrophages after exposure to *Mycobacterium tuberculosis* and its components. *J. Clin. Invest. 95:586.*
9. Yoshida, A., and Y. Koide. 1997. Arabinofuranosyl-terminated and mannosylated lipoarabinomannans from *Mycobacterium tuberculosis* induce different levels of interleukin-12 expression in murine macrophages. *Infect. Immun. 65:1953.*
10. Nigou, J., C. Zelle-Rieser, M. Gilleron, M. Thurnher, and G. Puzo. 2001. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cell: evidence for a negative signal delivered through the mannose receptor. *J. Immunol. 166:7477.*
11. Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl. 1988. Mycobacterial lipoarabinomannan inhibits γ-interferon-mediated activation of macrophages. *Infect. Immun. 56:1232.*
12. Moreno, C., A. Mehlert, and J. Lamb. 1988. The inhibitory effects of mycobacterial lipoarabinomannan and polysaccharides upon polyclonal and monoclonal human T cell proliferation. *Clin. Exp. Immunol. 74:206.*
13. Chatterjee, D., K. Lowell, B. Rivoire, M. R. McNeil, and P. J. Brennan. 1992. Lipoarabinomannan of *Mycobacterium tuberculosis*: capping with mannosyl residues in some strains. *J. Biol. Chem. 267:6234.*
14. Khoo, K. H., A. Dell, H. R. Morris, P. J. Brennan, and D. Chatterjee. 1995. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium. J. Biol. Chem. 270:12380.*
15. Hunter, S. W., and P. J. Brennan. 1990. Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of *Mycobacterium tuberculosis. J. Biol. Chem. 265:9272.*
16. Venisse, A., J. M. Berjeaud, P. Chaurand, M. Gilleron, and G. Puzo. 1993. Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG: determination of a molecular mass by laser desorption mass spectrometry. *J. Biol. Chem. 268:12401.*
17. Khoo, K. H., A. Dell, H. R. Morris, P. J. Brennan, and D. Chatterjee. 1995. Structural definition of acylated phosphatidylinositol mannosides from *Mycobacterium tuberculosis*: definition of a common anchor for lipomannan and lipoarabinomannan. *Glycobiology 5:117.*
18. Gilleron, M., N. Himoudi, O. Adam, P. Constant, A. Venisse, M. Riviere, and G. Puzo. 1997. *Mycobacterium smegmatis* phosphoinositols-glyceroarabinomannans: structure and localization of alkali-labile and alkali-stable phosphoinositides. *J. Biol. Chem. 272:117.*
19. Khoo, K. H., J. B. Tang, and D. Chatterjee. 2001. Variation in mannose-capped terminal arabinan motifs of lipoarabinomannans from clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *J. Biol. Chem. 276: 3863.*
20. Takeuchi, O., and S. Akira. 2001. Toll-like receptors: their physiological role and signal transduction system. *Int. Immunopharmacol. 1:625.*
21. Heldwein, K. A., and M. J. Fenton. 2002. The role of Toll-like receptors in immunity against mycobacterial infection. *Microbes Infect. 4:937.*
22. Lien, E., and R. R. Ingalls. 2002. Toll-like receptors. *Crit. Care Med. 30:S1.*
23. Underhill, D. M., and A. Ozinsky. 2002. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol. 14:103.*
24. Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA 96:14459.*
25. Yu, W., E. Soprana, G. Cosentino, M. Volta, H. S. Lichenstein, G. Viale, and D. Vercelli. 1998. Soluble $CD14_{1-152}$ confers responsiveness to both lipoarabinomannan and lipopolysaccharide in a novel HL-60 cell bioassay. *J. Immunol. 161:4244.*
26. Means, T. K., E. Lien, A. Yoshimura, S. Wang, D. T. Golenbock, and M. J. Fenton. 1999. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J. Immunol. 163:6748.*
27. Savedra, R., R. L. Delude, R. R. Ingalls, M. J. Fenton, and D. T Golenbock. 1996. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signaling system. *J. Immunol. 157:2549.*
28. Pugin, J., D. Heumann, A. Tomasz, V. V. Kravchenko, Y. Akamatsu, M. Nishijima, M. P. Glauser, P. S. Tobias, and J. Ulevitch. 1994. CD14 is a pattern recognition receptor. *Immunity 1:509.*
29. Fenton, M. J., and D. T. Golenbock. 1998. LPS-binding proteins and receptors. *J. Leukocyte Biol. 64:25.*
30. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS-binding protein. *Science 249:1431.*
31. Venisse, A., J. J. Fournie, and G. Puzo. 1995. Mannosylated lipoarabinomannan interacts with phagocytes. *Eur. J. Biochem. 231:440.*
32. Knutson, K. L., Z. Hmama, P. Herrera-Velit, R. Rochford, and N. E. Reiner. 1998. Lipoarabinomannan of *Mycobacterium tuberculosis* promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes: role of the Src homology 2 containing tyrosine phosphatase. *J. Biol. Chem. 273:645.*
33. Maeda, N., J. Nigou, J. L. Herrmann, M. Jackson, A. Amara, P. H. Lagrange, G. Puzo, B. Gicquel, and O. Neyrolles. 2003. The cell surface receptor DC-SIGN

discriminates between *Mycobacterium* species through selective recognition of the mannose caps on lipoarabinomannan. *J. Biol. Chem. 278:5513.*
34. Geijtenbeek, T. B. H., S. J. van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. J. E. Vandenbroucke-Grauls, B. Appelmelk, and Y. van Kooyk. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med. 197:7.*
35. Rafael, E., and E. Carlos. 1997. *Mycobacterium kansasii* disease in patients infected with human immunodeficiency virus. *Clin. Infect. Dis. 24:1233.*
36. Pintado, V., E. Gomez-Mampaso, P. Martin-Davila, J. Cobo, E. Navas, C. Quereda, J. Fortun, and A. Guerrrero. 1999. *Mycobacterium kansasii* infection in patients infected with human immunodeficiency virus. *Eur. J. Clin. Microbiol. Infect. Dis. 18:582.*
37. Jacobson, K. L., R. Teira, H. I. Libshitz, I. Raad, K. V. I. Rolston, J. Tarrand, and E. Whimbey. 2000. *Mycobacterium kansasii* infections in patients with cancer. *Clin. Infect. Dis. 30:965.*
38. Wallace, R. J., Jr., B. A. Brown, and G. O. Onyi. 1992. Skin, soft tissue, and bone infections due to *Mycobacterium chelonae chelonae*: importance of prior corticosteroid therapy, frequency of disseminated infections, and resistance to oral antimicrobials other than clarithromycin. *J. Infect. Dis. 166:405.*
39. Ingram, C. W., D. C. Tanner, D. T. Durack, G. W. Kernodle, Jr., and G. R. Corey. 1993. Disseminated infection with rapidly growing mycobacteria. *Clin. Infect. Dis. 16:463.*
40. Guérardel, Y., E. Maes, E. Elass, Y. Leroy, P. Timmerman, G. S. Besra, C. Locht, G. Strecker, and L. Kremer. 2002. Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*: presence of unusual components with α1,3-mannopyranose side chain. *J. Biol. Chem. 277:30635.*
41. Nigou, J., M. Gilleron, B. Cahuzac, J. D. Bounéry, M. Herold, M. Thurnher, and G. Puzo. 1997. The phosphatidyl-*myo*-inositol anchor of the lipoarabinomannans from *Mycobacterium bovis* bacillus Calmette-Guérin: heterogeneity, structure and role in the regulation of cytokine production. *J. Biol. Chem. 272:23094.*
42. Gilleron, M., L. Bala, T. Brando, A. Vercellone, and G. Puzo. 2000. *Mycobacterium tuberculosis* H37Rv parietal and cellular lipoarabinomannans: characterization of the acyl- and glyco-forms. *J. Biol. Chem. 275:677.*
43. Treumann A., F. Xidong, L. McDonnell, P. J. Derrick, A. E. Ashcroft, D. Chatterjee, and S. W. Homans. 2002. 5-Methylthiopentose: a new substituent on lipoarabinomannan in *Mycobacterium tuberculosis. J. Mol. Biol. 316:89.*
44. Ludwiczak P., M. Gilleron, Y. Bordat, C. Martin, B. Gicquel, and G. Puzo. 2002. *Mycobacterium tuberculosis* phoP mutant: lipoarabinomannan molecular structure. *Microbiology 148:3029.*
45. Vey, E., J. H. Zhang, and J. M. Dayer. 1992. INF-γ and $1,25(OH)_2D_3$ induce on THP-1 cells distinct patterns of cell surface antigen expression, cytokine production, and responsiveness to contact with activated T cells. *J. Immunol. 149:2040.*
46. Stokes, D. C., J. L. Shenep, M. Fishman, W. K. Hildner, G. K. Bysani, and K. Rufus. 1989. Polymyxin B prevents lipopolysaccharide-induced release of tumor necrosis factor-α from alveolar macrophages. *J. Infect. Dis. 160:52.*
47. Friedland, J. S., D. G. Remick, R. Shattock, and G. E. Griffin. 1992. Secretion of interleukin-8 following phagocytosis of *Mycobacterium tuberculosis* by human monocyte cell lines. *Eur. J. Immunol. 22:1373.*
48. Orr, S. L., and P. Tobias. 2000. LPS and LAM activation of the U373 astrocytoma cell line: differential requirement for CD14. *J. Endotoxin Res. 6:215.*
49. Stelter, F., M. Bernheiden, R. Menzel, R. S. Jack, S. Witt, X. Fan, M. Pfister, and C. Schütt. 1997. Mutation of amino acids 39–44 of human CD14 abrogates binding of lipopolysaccharide and *Escherichia coli. Eur. J. Biochem. 243:100.*
50. Landmann, R., B. Muller, and W. Zimmerli. 2000. CD14, new aspects of ligand and signal diversity. *Microbes Infect. 2:295.*
51. Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem. 274:33419.*
52. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA 97:13766.*
53. Abel, B., N. Thieblemont, V. J. F. Quesniaux, N. Brown, J. Mpagi, K. Miyake, F. Bihl, and B. Ryffel. 2002. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J. Immunol. 169:3155.*
54. Tobias, P. S., J. C. Mathison, and R. J. Ulevitch. 1988. A family of lipopolysaccharide binding proteins involved in responses to Gram-negative sepsis. *J. Biol. Chem. 263:13479.*
55. Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science 249:1429.*
56. Juffermans, N. P., A. Verbon, S. J. H. van Deventer, W. A. Buurman, H. van Deutekom, P. Speelman, and T. Van Der Poll. 1998. Serum concentration of lipopolysaccharide activity-modulating proteins during tuberculosis. *J. Infect. Dis. 178:1839.*
57. Sidobre, S., G. Puzo, and M. Rivière. 2002. Lipid-restricted recognition of mycobacterial lipoglycans by human pulmonary surfactant protein A: a surface-plasmon-resonance study. *Biochem. J. 365:89.*
58. Bovin, N. V. 1997. Carbohydrate-carbohydrate interaction. In *Glycoscience: Status and Perspective.* H. J. Gabius and S. Gabius, eds. Chapman & Hall, Weinheim, Germany, p. 277.
59. Jones, B. W., T. K. Means, K. A. Heldwein, M. A. Keen, P. J. Hill, J. T. Belisle, and M. J. Fenton. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukocyte Biol. 69:1036.*
60. Roach, T. I., C. H. Barton, D. Chatterjee, and J. M. Blackwell. 1993. Macrophage activation: lipoarabinomannan from avirulent and virulent strains of *Mycobacterium tuberculosis* differentially induces the early genes c-*fos*, KC, JE, and tumor necrosis factor-α. *J. Immunol. 150:1886.*
61. Adams, L. B., Y. Fukutomi, and J. L. Krahenbuhl. 1993. Regulation of murine macrophage effector functions by lipoarabinomannan from mycobacterial strains with different degrees of virulence. *Infect. Immun. 61:4173.*
62. Geijtenbeek, T. B. H., R. Torensma, S. J. Van Vliet, G. C. F. Van Duijnhoven, G. J. Adema, Y. van Kooyk, and C. G. Figdor. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell 100:575.*
63. Guérardel, Y., E. Maes, V. Briken, F. Chirat, Y. Leroy, C. Locht, G. Strecker, and L. Kremer. Lipomannan and lipoarabinomannan from a clinical isolate of *Mycobacterium kansasii*: novel structural features and apoptosis-inducing properties. *J. Biol. Chem. In press.*