Chemistry & Biology *13*, 39–47, January 2006 DOI 10.1016/j.chembiol.2005.10.013

The Acylation State of Mycobacterial Lipomannans Modulates Innate Immunity Response through Toll-like Receptor 2

Martine Gilleron,[1,*] Jérôme Nigou,[1] Delphine Nicolle,[2] Valérie Quesniaux,[2] and Germain Puzo[1]
[1] Institut de Pharmacologie et de Biologie Structurale
Centre National de la Recherche Scientifique
205 route de Narbonne
31077 Toulouse Cedex 4
France
[2] Centre National de la Recherche Scientifique
Transgenose Institute of Molecular Immunology and Embryology
3B rue de la Ferrolerie
45071 Orléans Cedex 2
France

Summary

Detection of *Mycobacterium tuberculosis* antigens by professional phagocytes via toll-like receptors (TLR) contributes to controlling chronic *M. tuberculosis* infection. Lipomannans (LM), which are major lipoglycans of the mycobacterial envelope, were recently described as agonists of TLR2 with potent activity on proinflammatory cytokine regulation. LM correspond to a heterogeneous population of acyl- and glycoforms. We report here the purification and the complete structural characterization of four LM acyl-forms from *Mycobacterium bovis* BCG using MALDI MS and 2D ^{1}H-^{31}P NMR analyses. All this biochemical work provided the tools to investigate the implication of LM acylation degree on its proinflammatory activity. The latter was ascribed to the triacylated LM form, essentially an agonist of TLR2, using TLR2/TLR1 heterodimers for signaling. Altogether, these findings shed more light on the molecular basis of LM recognition by TLR.

Introduction

Mycobacterium tuberculosis is one of the most effective human pathogens, with one-third of the world population being infected. *M. tuberculosis* targets professional phagocytes, including alveolar macrophages and dendritic cells, in which it survives and multiplies. The intracellular location of *M. tuberculosis* shields it from antibodies, so host protection is essentially mediated by adaptive immunity response via mainly two subpopulations of T cells, including Th1 and cytotoxic T cells [1]. An efficient T cell immune response against *M. tuberculosis* is critically dependent on the rapid detection of the invader by the innate immune system. Toll-like receptors (TLR) contribute to innate immunity by the recognition of mycobacteria-associated molecular patterns and by mediating the secretion by professional phagocytes of proinflammatory cytokines, such as TNF-α, IL-12, and chemokines.

*Correspondence: martine.gilleron@ipbs.fr

Macrophages express a large diversity of TLR. Viable or dead *M. tuberculosis* activate murine macrophages via TLR2 and, to a lesser extent, via TLR4 [2]; however, all the mycobacterial TLR ligands identified so far (i.e., the 19 kDa lipoprotein [3], PIM_2 [4], PIM_6 [5], and LM [6, 7]) are TLR2 agonists. Recently, it has been proposed that *M. tuberculosis* ligation, via mannosylated lipoarabinomannans (ManLAM), to the C-type lectin receptors (CLR) present at the surface of macrophages and dendritic cells might allow *M. tuberculosis* to escape immune surveillance [8–10]. Indeed, ManLAM ligation to the CLR, mannose receptor, or DC-SIGN alters the proinflammatory response induced by the TLR4 agonist LPS [8–10]. This inhibitory activity has been tentatively attributed to an increase in IL-10 secretion [10]. ManLAM is a 17 kDa macromolecule of the *M. tuberculosis* envelope exhibiting a tripartite structure composed of a mannosyl-phosphatidyl-*myo*-inositol anchor (MPI), a polysaccharidic core, and mannose caps. We have shown that ManLAM inhibitory activity requires both the presence of at least two fatty acyl substituents on the MPI anchor and the mannose caps [9]. In contrast to ManLAM, PIM_2, PIM_6, and LM, the structure of which is based on the same MPI anchor as that of ManLAM, are TLR2-dependent proinflammatory molecules [5, 7]. They are built from an MPI anchor bearing two or six Man*p* units or a much larger D-mannan homopolysaccharide extension, respectively.

NMR studies conducted on PIM_2 [11, 12], PIM_6 [5], LM [11], and LAM [13, 14] led us to discover the existence of different "acyl-forms." They correspond to molecules differing by the number and the position of the fatty acid residues on the MPI anchor. The NMR strategies developed for PIM studies allowed the characterization of the potential acylation sites, corresponding to both positions of the Gro, position 3 of the *myo*-Ins and position 6 of the Man*p* unit linked at C-2 of the *myo*-Ins. In this way, mono- to tetra-acylated forms of PIM_2 and PIM_6 have been described [5, 11, 12]. As previously mentioned, the acylation positions were determined thanks to 2D NMR studies, while the structure of fatty acids on each position was assessed by mass spectrometry (ESI or MALDI). The LM structural feature appeared more difficult to investigate due to both its higher molecular weight and heterogeneity, thus preventing a complete NMR analysis.

The present study was devised to determine the influence of LM acylation pattern on its TLR-mediated proinflammatory activity. The different LM acyl-forms were fractionated and structurally characterized using 2D NMR spectroscopy and MALDI-TOF MS. We provide evidence that the various acyl-forms have different abilities to trigger TLRs to induce the production of proinflammatory cytokines from murine and human macrophages. Indeed, in contrast to PIM that are proinflammatory irrespective of their acylation degree, tri- and tetra-acylated forms of LM (Ac_3LM and Ac_4LM, respectively) were active, but not mono- and diacylated LM (Ac_1LM and Ac_2LM, respectively). Thus, this study suggests a subtle molecular mechanism involving the

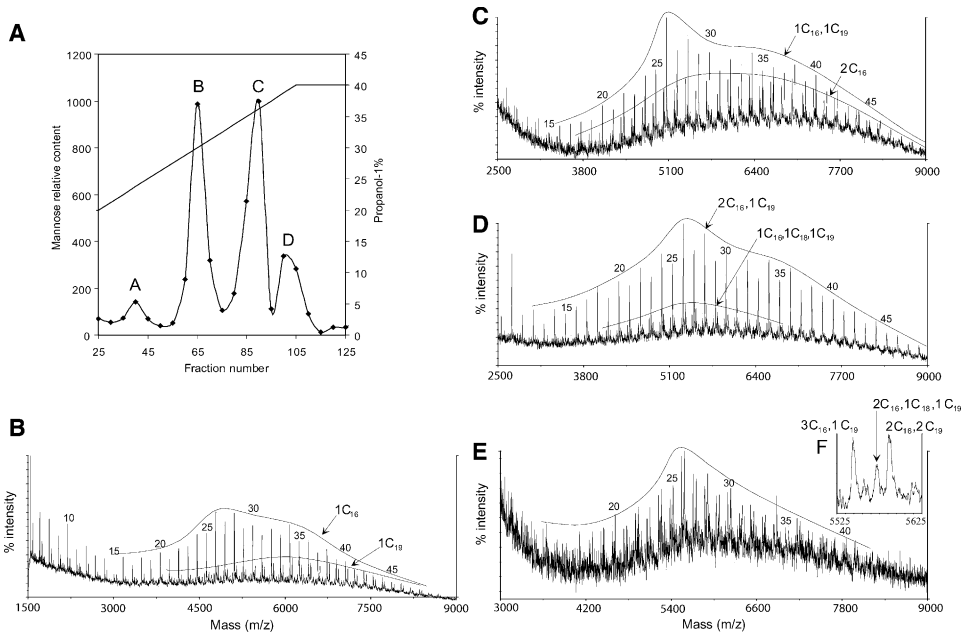


Figure 1. Purification of the *M. bovis* BCG LM Acyl-Forms Mixture and Negative MALDI Mass Spectra of the Purified Fractions A–D

(A) Hydrophobic interaction chromatography on octyl-sepharose of the *M. bovis* BCG LM acyl-forms mixture. The column was eluted with a propanol-1 linear gradient (20%–40%, v/v) in 0.1 M ammonium acetate in water. Fractions were analyzed by HPAEC for their mannose content.

(B) Negative MALDI mass spectra of the fraction A, which corresponded to mono-acylated forms, acylated by $1C_{16}$ or $1C_{19}$.

(C) Negative MALDI mass spectra of the fraction B, which corresponded to diacylated forms, acylated by $2C_{16}$ or $1C_{16}/1C_{19}$.

(D) Negative MALDI mass spectra of the fraction C, which corresponded to triacylated forms, acylated by $2C_{16}/1C_{19}$ or $1C_{16}/1C_{18}/1C_{19}$.

(E and F) Negative MALDI mass spectra of the fraction D, which corresponded to tetra-acylated forms, acylated by $3C_{16}/1C_{19}$, $2C_{16}/2C_{19}$ or, in small part, by $2C_{16}/1C_{18}/1C_{19}$. Fatty acyl compositions were based on the most abundant fatty acyl chains found by GC MS analysis (i.e., palmitate [C_{16}], tuberculostearate [C_{19}] and, to a lesser extent, stearate [C_{18}]).

MPI anchor acylation degree of LM by which *M. tuberculosis* might influence macrophage activation.

Results

Characterization of LM Acyl-Forms by NMR

LM were isolated from *Mycobacterium bovis* BCG and purified as previously described [11]. 1D ^{31}P NMR highlighted the presence of different LM acyl-forms. Indeed, the spectrum of LM solubilized in Me_2SO-d_6 showed five sharp phosphorus resonances at δ 1.73 (P1), δ 1.78 (P2), δ 1.90 (P3), δ 3.40 (P4) and δ 3.57 (P5), with relative integration values of 1.0 for P1 and P2, 0.9 for P3, 0.1 for P4, and 0.3 for P5, typifying at least five acyl-forms (as previously described in [11]). Using 2D ^{1}H-^{31}P HMQC-HOHAHA experiments, three of the four acylation sites of the MPI anchor were characterized, on both positions of the Gro, and on C3 of the *myo*-Ins [11]. P1, P2, and P3 acyl-forms were shown to contain diacyl Gro, while those of P4 and P5 contained mono-acyl Gro. In the case of P1 and P4 acyl-forms, an additional fatty acyl residue was found located at the C3 of the *myo*-Ins. LM thus appeared as a mixture of at least mono-, di-, and triacylated acyl-forms, with a putative additional fatty acyl appendage on the C6 of the Man*p* residue of the MPI anchor [11]. Integration of the phosphorus signals indicated that the most abundant acyl-forms were those that were at least triacylated (P1) and diacylated (P3) (32% and 38%, respectively). LM fatty acids composition was previously determined by GC as a combination of palmitic (C_{16}, 62%), tuberculostearic (10-methyloctadecanoic) (C_{19}, 30%), and stearic (C_{18}, 8%) acids [11]. Therefore, NMR studies identified four acyl-forms that were further investigated using MALDI-TOF MS. We intended to determine whether these LM acyl-forms contained an additional fatty acyl residue, and how the acyl substituents were distributed on each acyl-form.

To overcome the problems of species discrimination or ionization efficiency occurring during LM mixture analysis (unpublished data), we decided to purify the different LM acyl-forms prior to MALDI-TOF MS analysis.

Fractionation and Structural Characterization of LM Acyl-Forms

LM acyl-forms mixture was fractionated using hydrophobic interaction chromatography. The elution profile (Figure 1A) showed four peaks, two of weak intensity (A and D), eluting at approximately 24% and 40% propanol-1 in 0.1 M ammonium acetate in water, respectively, and two of higher intensity (B and C), eluting at approximately 30% and 35% propanol-1, respectively. The fractions corresponding to the different peaks were pooled (fractions A–D), dialyzed, and dried. The relative mass abundance of the different fractions was 7% for A, 29% for B, 56% for C, and 8% for D. Each fraction was analyzed by negative MALDI-TOF MS (Figures 1B–1E) using DHB solubilized in 1/1 (v/v) ethanol/water, and by 1D ^{31}P (Figure 2) and 2D ^{1}H-^{31}P NMR in Me_2SO-d_6 to localize the fatty acyl substituents on the MPI anchor of the different purified acyl-forms.

The MALDI-TOF mass spectrum of fraction A (Figure 1B) was dominated by one set of peaks assigned to monopalmitoylated LM with a mannan domain composed of up to 48 Man*p* units. The presence of another set of peaks with a much lower intensity was also observed and characterized as monotuberculostearoylated LM. The 1D ^{31}P spectrum of this fraction (Figure 2B) exhibited a single resonance at 3.50 ppm, characterizing an MPI anchor with a lyso-Gro, as previously described [11]. In summary, fraction A contained mono-acylated LM, mainly esterified by palmitoyl on the Gro unit.

Figure 1C shows the mass spectrum of fraction B, highlighting two distinct sets of diacylated LM. The most abundant LM acyl-form was assigned to LM containing one palmitoyl and one tuberculostearoyl appendage, while the other one corresponded to dipalmitoylated LM. As in the case of the Ac_1LM, the mannan domain was composed of 15–48 Man*p* units. The 1D ^{31}P spectrum of fraction B (Figure 2C) showed two resonances, revealing that Ac_2LM is composed of two isoforms differing by the positions of the fatty acids. The first resonance at 3.84 ppm (P5) revealed a lyso-Gro, while the other at 1.97 ppm (P2) typified a diacyl Gro. The 2D ^{1}H-^{31}P experiments (data not shown) indicated that the *myo*-Ins unit was not acylated. We thus deduced that in the case of the acyl-form with a lyso-Gro, the second acylation site was on the Man*p* unit of the MPI, assigning the acyl-form characterized by P5.

Figure 1D shows the mass spectrum of fraction C. It is dominated by one set of peaks assigned to triacylated LM containing two palmitoyl and one tuberculostearoyl substituents. The mannan domain is again composed of 15–48 Man*p* units. The 1D ^{31}P spectrum of the triacylated LM (Figure 2D) exhibited a single resonance at δ 1.81 ppm, corresponding to an MPI anchor with a diacyl Gro. By ^{1}H-^{31}P 2D NMR experiments (data not shown), we found that this phosphorus resonance corresponded to P3 (Figure 2A), the *myo*-Ins being non-acylated. We concluded that the third position of acylation was the C6 of the Man*p* unit of the MPI. A less abundant population was revealed by a weaker ^{31}P resonance at δ 3.45, attributed to an MPI anchor with a lyso-Gro. As this fraction corresponds to triacylated LM, both remaining acylation sites are thus substituted. This ^{31}P resonance is then likely to correspond to P4.

Finally, fraction D contained tetra-acylated LM (Figure 1E) composed of three or two palmitoyl combined to one or two tuberculostearoyl appendages, respectively (Figure 1F). Species containing two palmitoyl combined to one stearoyl and one tuberculostearoyl substituents were found in minor abundance (Figure 1F). The mannan domain was composed of 18–41 Man*p* units (Figure 1E). The 1D ^{31}P spectrum of the tetra-acylated LM (Figure 2E) showed a single resonance at δ 2.01 ppm, indicating the presence of a diacyl Gro. The resonance was assigned to P1 (Figure 2A). The presence of four fatty acyl groups implied that all the MPI acylation positions were acylated: both positions of Gro, C6 of the Man*p* unit, and C3 of *myo*-Ins unit. It is of note that ^{31}P chemical shifts of the purified acyl-form of LM (Figure 2E) were different from those observed within the mixture (Figure 2A) as a result of different environments. Therefore, the ^{31}P chemical shift value alone cannot be used to determine acylation degree of a molecular species, but is nevertheless informative on the degree of substitution of Gro (lyso- or diacylated).

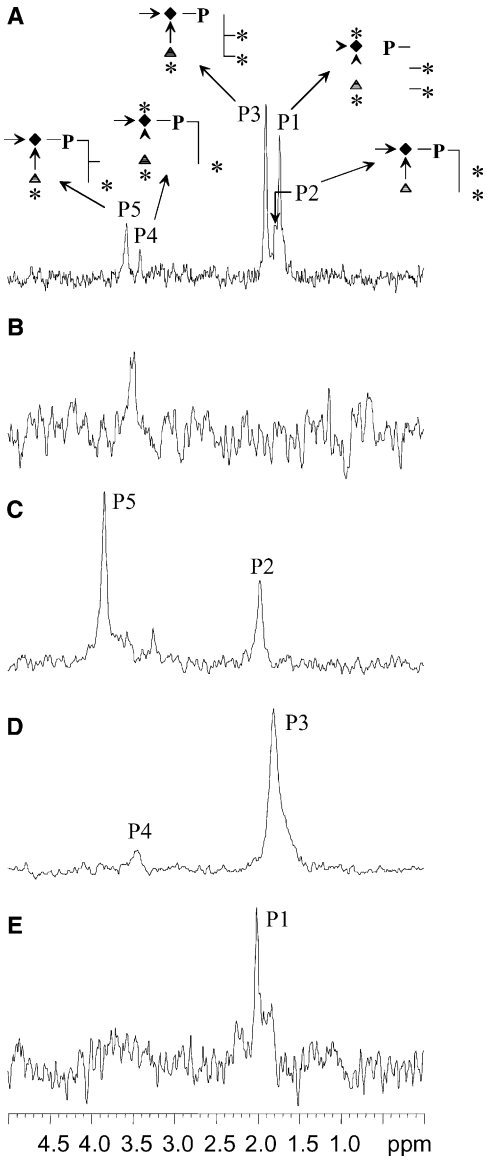
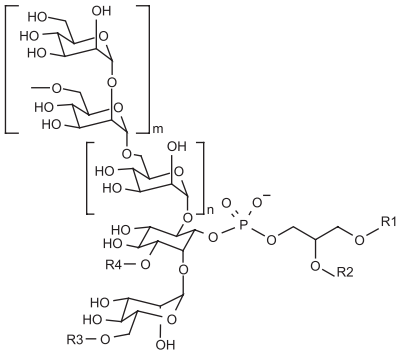


Figure 2. 1D ^{31}P Spectra of the LM Mixture and the Purified LM Acyl-Forms

Expanded regions of the 1D ^{31}P spectra of the LM mixture (A) and the purified LM acyl-forms (B–E) dissolved in Me_2SO-d_6 at 343 K (δ ^{31}P: 0.00–5.00): mono- (B), di- (C), tri- (D), and tetra- (E) acylated forms of LM (corresponding to the fractions A–D eluted from the hydrophobic interaction chromatography, respectively).

In summary, four major LM acyl-forms were characterized, differing by their degree of acylation, the most abundant being the diacylated and the triacylated forms. Each acyl-form, defined by the total number of fatty acids, is still heterogeneous according to its acylation pattern. Indeed, they are composed of subpopulations defined by two additional criteria: the type and the position of fatty acids substituents. As such, Ac_1LM consist of two subpopulations, acylated by either C_{16} or C_{19} fatty acyl residues on the Gro (Figure 3). Ac_2LM are composed of two molecular subspecies, comprising $1C_{16}$ and $1C_{19}$ or $2C_{16}$, and two isoforms



	Major species	R1	R2	R3	R4
Ac_1LM	$1C_{16}$	C_{16}	—	—	—
Ac_2LM	$1C_{16}$, $1C_{19}$	C_{19}	—	C_{16}	—
Ac_3LM	$2C_{16}$, $1C_{19}$	C_{19}	C_{16}	C_{16}	—
Ac_4LM	$3C_{16}$, $1C_{19}$	C_{19}	C_{16}	C_{16}	C_{16}

Figure 3. Proposed Structural Model of *M. bovis* BCG LM

This model is based on both MALDI and NMR data. MALDI-TOF MS experiments revealed glyco-forms containing 15–45 mannosyl units, with a systematic preponderance of the glyco-form with 26 Man*p* units, leading to 15 < 2m + n < 45. Integration of the anomeric signals on the ^1H NMR spectrum provided an average value for the substitution degree of the 6-*O*-linked mannan backbone, leading to an n value of 2 or 3 [11]. The proposed model for the mannan core does not account for the relative distribution of the mannobiose or mannosyl units along the backbone. The nature of the terminal residue remains to be elucidated. Fatty acids composition (C_{16} and C_{19}) is in agreement with GC data; the structure and the position of the fatty acyl appendages on the Gro were previously determined after acetolysis followed by GC/MS analysis [11].

differing by the site of acylation. The most abundant isoform contains lyso-Gro and acyl-Man*p* units (Figure 3), while the other is characterized by a diacylated Gro. Ac_3LM are dominated by a molecular species esterified by $2C_{16}$ and $1C_{19}$, distributed on the Gro and on the Man*p*, similarly to Ac_4LM that bear a fourth fatty acyl substituent, C_{16} or C_{19}, on the *myo*-Ins. All the acyl-forms showed a major glyco-form containing 26 Man*p* units. The mass values of the deprotonated molecular ions characterized LM containing around 15–45 Man*p* units.

Human and Murine Macrophages Stimulation by Purified *M. bovis* BCG LM Acyl-Forms

We have recently shown that *M. bovis* BCG LM total fraction stimulates murine primary macrophage activation and effector functions, such as the release of TNF-α and IL-12 [7]. Here, we tested the different purified *M. bovis* BCG LM acyl-forms for their capacity to induce the secretion of proinflammatory cytokines TNF-α and IL-12 by murine macrophages (Figure 4). As previously reported, LM mixture (3 μg/ml) induced elevated production of TNF-α (Figures 4A and 4B) and IL-12p40 (data not shown) by murine macrophages, reaching a plateau at a level obtained with classical macrophage activators, such as LPS (100 ng/ml) or the bacterial lipopeptide Pam_3CSK_4 (BLP) (0.5 μg/ml).

Purified Ac_4LM and Ac_3LM induced elevated production of TNF-α (Figures 4A and 4B) and IL-12p40 (data not shown). In contrast, Ac_1LM induced a much lower response, and Ac_2LM were essentially inactive, even at high concentration (Figure 4B). Therefore, of the four LM acyl-forms, Ac_3LM and Ac_4LM are the predominant inducers of the proinflammatory cytokines TNF and IL-12p40.

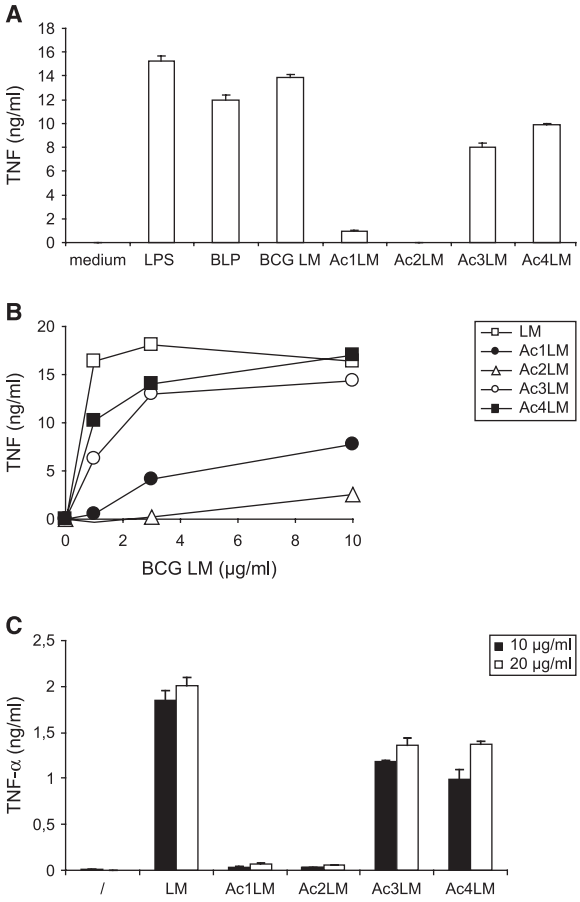


Figure 4. TNF-α Production by Primary Murine Macrophages and by Human Monocytic THP-1 Cell Line in Response to LM Acyl-Forms Mixture or Purified LM Acyl-Forms

TNF-α production by primary murine macrophages (A and B) and by human monocytic THP-1 cell line (C) in response to LM acyl-forms mixture or purified LM acyl-forms. (A and B). Murine bone marrow-derived macrophages were incubated with LPS (100 ng/ml), Pam_3CSK_4 (BLP; 0.5 μg/ml), BCG LM acyl-forms mixture, or purified LM acyl-forms at 3 μg/ml (A and C) or LM titrations from 1 to 10 μg/ml (B). After 24 hr of incubation, TNF-α (A and B) levels were measured in the supernatants. Results are mean ± SD from n = 2 mice, and are from one representative experiment of four independent experiments. (C) LM mixture or purified LM acyl-forms were tested at 10 (black bars) and 20 (white bars) μg/ml.

We next investigated whether the proinflammatory activity of the Ac_3LM and Ac_4LM was also observed on human monocytic cells, using the THP-1 cell line (Figure 4C). Likewise, we found that Ac_3LM and Ac_4LM induced elevated production of TNF-α, whereas Ac_1LM and Ac_2LM were essentially inactive, even at high concentrations (10 and 20 μg/ml) (Figure 4C). Therefore, LM proinflammatory activity on human monocytic cells was also dependent on the acylation degree and associated with tri- and tetra-acylated forms.

TLR2- and TLR4-Dependence Study of Macrophage Stimulation by the Major LM Acyl-Form, Ac_3LM

The TLR dependence of *M. bovis* BCG LM TNF-α-inducing activity was then investigated using specific anti-TLR2 and anti-TLR4 neutralizing antibodies on human THP-1 cells. The anti-TLR2 antibody significantly inhibited TNF-α production induced by LM, whereas the anti-TLR4 antibodies had no effect, as compared with an IgG2 isotype control (data not shown). These results document the role of TLR2 in mediating the stimulation of TNF-α production by human monocytic cells in response to *M. bovis* BCG LM. The results are in agreement with our results in macrophages from TLR2-deficient mice, where TNF-α release in response to *M. bovis* BCG LM was sharply decreased as compared with wild-type macrophages [7].

Furthermore, we analyzed the TLR-dependence of the major LM acyl-form-induced proinflammatory responses. Macrophages from mice deficient in TLR2, TLR4, or both were stimulated with the purified Ac_3LM acyl-forms (Figure 5A). TLR4 agonist LPS and TLR2 agonist BLP were used as controls of TLR2/4 specificity. Ac_3LM induced an elevated TNF-α release in the supernatant of wild-type macrophages, but this response was reduced by up to 95% in macrophages deficient in TLR2 in five independent experiments, whereas absence of TLR4 caused a sharp reduction in TNF-α, and no TNF-α could be detected in the supernatant of macrophages deficient in TLR2 and TLR4. Thus, Ac_3LM show a strong TLR2 agonist activity, as well as some TLR4 agonist activity. To evaluate a putative contribution of contaminating endotoxins to the TLR4-mediated activity of LM acyl-forms, endotoxin levels were quantified in the different LM acyl-forms by kinetic turbidimetric assays (see Experimental Procedures). LPS was downtitrated and tested side-by-side with Ac_3LM to evaluate the contribution of endotoxins in the induction of TNF-α. It appeared that some of the TLR4 agonist activity of Ac_3LM could be due to the level of endotoxin present in the preparation (18.9 ng per 10 ug of Ac_3LM).

Altogether, these results suggest that the fraction containing Ac_3LM induces TNF-α secretion through a TLR2-dependent pathway. No cytokine production was detected in the supernatants of macrophages isolated from the double TLR2- and TLR4-deficient mice, indicating that TLR2 and TLR4 represent the main receptors for the different *M. bovis* BCG LM acyl-forms.

TLR2 Heterodimerization for the Cytokine Stimulation by Ac_3LM

TLR2 is known to form heterodimers with TLR1 or TLR6 [15–17]. We have previously shown that the TLR2-dependent stimulatory activity of *M. bovis* BCG LM total fraction was independent of TLR6 [7]. Here we assessed whether TLR1 is involved in the immune response to Ac_3LM. Macrophages deficient in TLR1 or TLR2 showed a markedly reduced TNF-α production in response to stimulation with Ac_3LM, whereas, as expected, there was no decrease in the response of macrophages deficient in TLR6 (Figure 5B). Although less pronounced, the same trend was observed with the *M. bovis* BCG LM total fraction. Absence of either TLR1 or TLR6 had essentially no effect on the response to the TLR4 agonist LPS, or to the TLR9 agonist CpG. The TLR2 agonist BLP showed a reduced response in TLR1-deficient macrophages, although this was not as pronounced as in TLR2-deficient cells. Similar data were obtained for IL-12p40 secretion, suggesting that TLR1 is involved in the TLR2-dependent activation by Ac_3LM (data not shown).

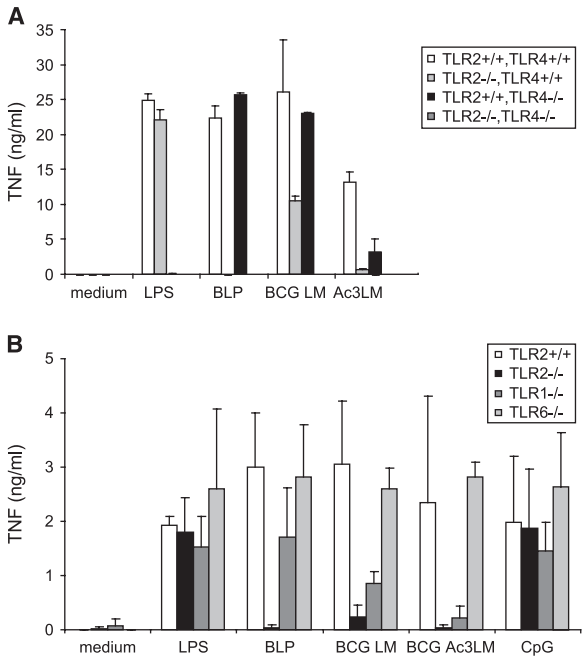


Figure 5. TLR-Dependence of the TNF-α Release Induced by LM Acyl-Forms

(A) TLR2- and TLR4-dependence of the TNF-α release induced by LM acyl-forms. Bone marrow-derived macrophages from mice deficient for TLR2, TLR4, or TLR2 and TLR4 were incubated with medium alone, with BCG LM, or the different LM acyl-forms at a concentration of 3 μg/ml, or with LPS (100 ng/ml) or Pam_3CSK_4 (BLP; 0.5 μg/ml) as TLR4 and TLR2 reference agonist, respectively. TNF was measured after 24 hr in the supernatants.

(B) TLR2/1-dependence of the TNF-α release induced by *M. bovis* BCG Ac_3LM. Bone marrow-derived macrophages from mice deficient in TLR1, TLR2, or TLR6 were incubated with medium alone, total LM from *M. bovis* BCG (BCG LM) or *M. bovis* BCG triacylated LM (BCG Ac_3LM) at a concentration of 3μg/ml, or with LPS (100 ng/ml) or CpG (0.125μM) for 24 hr. TNF was measured in the supernatants by ELISA.

Results are mean ± SD from n = 2 mice per genotype, and are representative of three independent experiments.

Thus, the release of proinflammatory cytokines by primary murine macrophages stimulated with Ac_3LM seems to be mediated through both TLR2 and TLR1, which may function as heterodimers, and not through TLR6.

Discussion

M. tuberculosis targets macrophages and dendritic cells in which it survives and multiplies. An efficient immune response against *M. tuberculosis* is critically dependent on its rapid detection through conserved molecular patterns by the innate immune system receptors, including TLR, that mediate the production of proinflammatory cytokines. Recently, a strategy has been evidenced by which *M. tuberculosis* might be able to escape immune surveillance. Indeed, binding of *M. tuberculosis* to the C-

type lectins, MR and DC-SIGN, expressed on the membrane of phagocytes, has been shown to alter TLR-mediated signaling [9, 10]. *M. tuberculosis* is recognized by TLR2 and, to a lesser extent, by TLR4 [2, 4, 18–20]. Purified mycobacterial LM have been shown in vitro to activate macrophages in a TLR2-dependent manner, leading to the production of proinflammatory cytokines [6, 7, 21]. Recent studies have revealed how soluble LM could function as TLR2 ligands in the context of a mycobacterial infection. Indeed, it is now clear that mycobacterial compounds, including LM, can be delivered from infected macrophages to noninfected bystander dendritic cells equipped with TLR receptors [22–25]. The release of LM from infected macrophages is mediated by at least two described mechanisms. On the one hand, lipid-containing moieties of the mycobacterial cell wall have been shown to be actively trafficked out of the mycobacterial vacuole, transported to endosomes/lysosomes, and ultimately found in extracellular vesicles isolated from the culture medium. On the other hand, it has been recently demonstrated that mycobacteria induce apoptosis of the macrophages that they infect, causing the release of apoptotic vesicles carrying TLR2 ligands, including LM.

In order to improve our insights into the structure/function relationships underlying the TLR-mediated proinflammatory activity of LM, we assessed the role of the degree of acylation of the molecule. A prerequisite of this work was the fractionation of LM acyl-forms and their chemical characterization using 2D NMR and MALDI MS.

The LM acyl-forms were first revealed by 1D ^{31}P NMR studies, and their structure was investigated in the total mixture using 2D ^{1}H-^{31}P NMR [11]. Using NMR, three sites of acylation were characterized, including two on the Gro and one at the *myo*-Ins at position C3. By analogy to PIM, an additional acylation site at the C6 of the Man*p* was postulated. This structural uncertainty was removed in the present study using negative MALDI-TOF MS. Indeed, MALDI-TOF MS appeared as an appropriate analytic tool to reveal and determine the molecular weight of the LM acyl-forms and, consequently, to define the number and nature of fatty acid residues and the number of Man*p* units present on the molecule. The LM acyl-forms mixture was fractionated using hydrophobic interaction chromatography [9, 26] and separated into four subfractions, namely A–D, from the most hydrophilic to the most hydrophobic LM. These fractions were characterized by MALDI-TOF MS and 2D ^{1}H-^{31}P NMR.

In summary, four major LM acyl-forms were characterized, differing in their degree of acylation, the most abundant ones being the diacylated and the triacylated forms. Fraction A contained Ac_1LM acylated on the C1 of Gro by C_{16} and, to a lesser extent, by C_{19}. Fraction B contained two isoforms of Ac_2LM, the major one containing a lyso-Gro and an acyl-Man*p* unit, the minor one containing a diacylated Gro residue. The fatty acids composition was $1C_{16}/1C_{19}$ or, to a lesser extent, $2C_{16}$. Fraction C corresponded to Ac_3LM, mainly composed of one isoform, with the Gro and the Man*p* being acylated almost exclusively by $2C_{16}/1C_{19}$. Finally, fraction D contained Ac_4LM esterified by $3C_{16}/1C_{19}$, $2C_{16}/2C_{19}$, and, to a lesser extent, $2C_{16}/1C_{18}/1C_{19}$. Whatever the acylation degree, the number of Man*p* units for each acyl-form varied between 15 and 45, with a systematic preponderance of the glyco-form containing 26 Man*p* units.

Tetra-acylated forms have been clearly demonstrated for PIM_2 [11, 12] and PIM_6 [5], but the corresponding acyl-forms of LM or LAM have only been suspected thus far [9, 11, 13, 27]. The present study reveals the existence of tetra-acylated LM. Interestingly, the abundance and fatty acid composition of mono-, di-, tri-, and tetra-acylated acyl-forms are similar for PIM_2, PIM_6, and LM, except that the relative abundance of the diacylated isoforms is different between PIM_2 and LM (see Table S1 in the Supplemental Data), supporting the concept of a biosynthetic linkage between PIM and LM, as previously hypothesized [12, 27]. Moreover, the heterogeneity of LM is here characterized in terms of glyco-forms. The exact number of mannose units present on each LM acyl-form was thus determined. To date, only an average number of Man*p* units per molecule was tentatively obtained by integrating the mannose and inositol signals on the gas chromatograms after total acid hydrolysis of LM mixture [28, 29]. In contrast, the MALDI-TOF mass spectra reveal the presence of the different glyco-forms that can contain up to 50 Man*p* units and, as such, highlight LM molecular complexity in terms of mass distribution and degree of mannosylation. MALDI-TOF results are in agreement with the previously published data [21], as they indicate a similar average number of Man*p* units per molecule, the glyco-forms with a very high number of Man*p* being present in small amounts. However, the present results reveal a large molecular distribution that was not suspected from previous analyses.

Having isolated and chemically characterized *M. bovis* BCG LM acyl-forms, we next determined their capacity to induce the proinflammatory cytokines TNF-α and IL-12, as well as their TLR usage. Ac_1LM showed a poor activity and Ac_2LM failed to induce cytokine production, whereas Ac_3LM and Ac_4LM strongly activated human monocytic cells and primary murine macrophages to release TNF-α and IL-12p40. As expected, TLR2 and TLR4 seemed to be the major TLR triggered by the LM acyl-forms, as no stimulation was obtained in the absence of both receptors. Ac_3LM, the most abundant acyl-form with Ac_2LM, induced a strong stimulatory signal, mostly mediated through TLR2, as observed for the original mixture of *M. bovis* BCG LM acyl-forms. Thus, LM proinflammatory activity requires at least three fatty acids on the molecule. However, the size of the mannan domain also modulates this activity. Indeed, PIM_2 and PIM_6 that have a shorter mannan domain, composed of two and six Man*p* units, respectively, also signal via TLR2, but their activity is dramatically reduced as compared with that of LM (at least 10-fold less) [5, 6].

Our data raised the question of the role of fatty acids for LM signaling via TLR2: why do Ac_3- and Ac_4LM show a proinflammatory activity, while Ac_1- and Ac_2LM do not? TLR2 are known to mediate the recognition of a variety of microbial components, such as bacterial lipopeptides and lipoproteins, porins, peptidoglycan, GPI-anchors, and lipotechoic acids [30]. Some of the ligands are recognized by heterodimers formed between TLR2 on the one hand, and TLR1 or TLR6 on the other. Interestingly, murine TLR2 discriminates between di- and

triacylated lipopeptides, in cooperation with TLR6 or TLR1, respectively [30]. Among these lipopeptides is the 2 kDa macrophage-activating lipopeptide, MALP-2, from mycoplasmas. Synthetic variants of MALP-2, sharing an identical peptide moiety but substituted with different lipid moieties, have helped to define the TLR system of recognition by using mouse fibroblasts bearing genetic deletions of various TLRs [31]. It has been shown that the removal of one or both ester-bound fatty acids lowered or deleted biological activity, indicating that esterification of the 3-deoxy-3-thio-Gro residue by two fatty acids is required to optimally stimulate innate immunity via TLR2 [3]. Ac_1LM and the major isoform of Ac_2LM, which are not active, carry a single acyl residue on the Gro. Thus, as for MALP-2, it seems that optimal TLR2 activation by LM requires two fatty acids on the Gro moiety, a structural feature observed in Ac_3LM. Moreover, as reported for triacylated lipopeptides [3], Ac_3LM signaling via TLR2 required dimerization of TLR2 with TLR1.

In conclusion, this study helps to refine our understanding of the molecular basis of TLR2-mediated LM proinflammatory activity, and defines Ac_3LM as an additional mycobacterial ligand of TLR2 aside from the 19 kDa lipoprotein, PIM_2, and PIM_6. The host defense against pathogenic microorganisms such as *M. tuberculosis* comprises innate and adaptive immunity. Studies in mice have shown that TLR2 contributes to the battle against *M. tuberculosis* infection. Indeed, $TLR2^{-/-}$ mice are more susceptible to infection with high doses of *M. tuberculosis* [20, 32, 33], while resistance to *M. tuberculosis* infection is marginally affected in $TLR4^{-/-}$ mice [19]. Moreover, absence of both TLR2 and TLR4 does not compromise resistance to *M. bovis* BCG infection [34]. In humans, a higher expression of TLR2 and TLR1 has been found in skin lesions from patients with the localized tuberculoid form of leprosy as compared with those with disseminated lepromatous disease [4]. An arginine-tryptophane mutation was also found in the cytoplasmic domain of TLR2 of a subset of lepromatous leprosy patients from Korea, and cells transfected with this mutated TLR2 were not able to mediate activation of NF-kB after stimulation with *Mycobacterium leprae* [35].

Altogether, these observations should prompt the testing of mycobacterial TLR2 ligands, such as PIM and Ac_3LM, as adjuvants together with immunogenic proteins for the development of an efficient subunit vaccine to prevent *M. tuberculosis* infection [36].

Significance

Toll-like receptors (TLR) are receptors of the innate immune system designed to rapidly detect invasion of microbial pathogens such as *Mycobacterium tuberculosis*. They control innate immunity response and subsequent activation of adaptive immunity. Lipomannans (LM) are major lipoglycans of the mycobacterial envelope and activate macrophages in a TLR2-dependent manner, leading to the production of proinflammatory cytokines. However, they are heterogeneous molecules composed of different acyl-forms and glyco-forms. To get better insights into the structure/function relationships, we investigated the role of LM acylation state in their ability to activate macrophage via TLR2. Four acyl-forms of LM (mono- to tetra-acylated: Ac_1- to Ac_4LM) were purified and structurally characterized. The heterogeneity of LM in terms of glyco-forms was also determined. The proinflammatory activity of LM was ascribed to the triacylated form essentially. Optimal TLR2 activation by LM seems to require two fatty acyl substituents on the glycerol unit, a structural feature observed in Ac_3LM, but not in Ac_1- or Ac_2LM.

Experimental Procedures

Mycobacterial LM Purification

LM from *M. bovis* BCG Pasteur strain were purified as previously described [11]. Briefly, the delipidated mycobacteria were extracted by refluxing in 50% ethanol. The resulting cells were then washed and disintegrated by sonication and by use of a French pressure cell, and extracted by refluxing in 50% ethanol. This latter extract, containing LAM, LM, and AM, was used and cleared from proteins and glucans by enzymatic digestion and hot aqueous phenol biphasic wash. Triton X-114 phase separation was used to separate glycans (AM) from amphipatic conjugates (LM and LAM) [37]. ManLAMs and LM were then separated by gel filtration. The purified LM, monitored by SDS-PAGE, as described by Venisse et al. [38], were observed as a broad band located around 20 kDa, indicating that LM were not contaminated by LAM.

MALDI-TOF MS

Analysis by MALDI-TOF MS was carried out on a Voyager DE-STR (PerSeptive Biosystems, Framingham, MA) using the linear or reflectron modes. Ionization was effected by irradiation with pulsed UV light (337 nm) from an N_2 laser. LM were analyzed by the instrument operating at 24,500 V in the negative ion mode using an extraction delay time set at 200 ns. Spectra from 250 to 500 laser shots were summed to obtain the final spectrum. The DHB matrix (Sigma Chemical, St. Louis, MO) was used at a concentration of ~10 mg/ml in water or ethanol/water (1/1, v/v), as indicated. Typically, 0.5 μl of LM sample (5–10 μg) in water and 0.5 μl of the matrix solution, were deposited on the target, mixed with a micropipette, and dried under a gentle stream of warm air. The laser intensity was set to 2500/2700.

Hydrophobic Interaction Chromatography of LM

A quantity of 26 mg of LM was loaded in 0.1 M ammonium acetate solution containing 20% (v/v) propanol-1 to an octyl-Sepharose CL-4B column (20 × 1.5 cm; Pharmacia, Orsay, France) preequilibrated with the same buffer. The column was first eluted with 25 ml of equilibration buffer and then with a linear propanol-1 gradient from 20% to 40% (v/v) (100 ml each) in 0.1 M ammonium acetate solution at a flow rate of 5 ml/hr. Each fraction was collected over 30 min. The column was finally eluted with 200 ml of 0.1 M ammonium acetate solution containing 40% (v/v) propanol-1.

A 20 μl sample of each fraction was dried and subjected to acidic hydrolysis (100 μl 2M trifluoroacetic acid for 2 hr at 110°C). The hydrolysates were dried, reconstituted in water, and then analyzed by high-pH anion exchange chromatography (HPAEC) for mannose content determination.

According to the mannose contents profile, the fractions were pooled and precipitated with ethanol. A total of 0.7 mg of Ac_1LM, 2.8 mg of Ac_2LM, 5.4 mg of Ac_3LM, and 0.8 mg of Ac_4LM were retrieved.

NMR Analysis

NMR spectra were recorded with an Avance DMX500 spectrometer (Bruker GmbH, Karlsruhe, Germany) equipped with an Origin 200 SGI using Xwinnmr 2.6. Samples were exchanged in ^2H_2O with intermediate freeze-drying, then dissolved in Me_2SO-d_6, and analyzed at 343 K. The 1D phosphorus (^{31}P) spectra were measured at 202.46 MHz using phosphoric acid (85%) as external standard (δ_P 0.0). Proton chemical shifts are expressed in ppm downfield from the signal of the methyl of Me_2SO-d_6 (δ_H/TMS 2.52 and δ_C/TMS 40.98). All the

details concerning NMR sequences used and experimental procedures were described in previous studies [11, 12].

Murine Primary Macrophages

Mice deficient for TLR1 [3], for TLR6 [39], or for TLR4 and/or TLR2 obtained by intercross from TLR4-deficient mice (from S. Akira [40]) and TLR2-deficient mice (from C. Kirsching [41]), and their control littermates were bred under specific pathogen-free conditions in the Transgenose Institute animal breeding facility (Orleans, France). Murine bone marrow cells were isolated from femurs and cultivated (10^6/ml) for 7 days in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine and 2×10^{-5} M β-mercapto-ethanol, 20% horse serum, and 30% L929 cell-conditioned medium (as source of M-CSF, as described in [42]). After resuspension in cold PBS and washing, the cells were further cultivated for 3 days in fresh medium to obtain a homogenous population of CD11b+ macrophages.

The bone marrow-derived macrophages were plated in 96-well microculture plates at a density of 10^5 cells/well in DMEM supplemented with L-glutamine and β-mercapto-ethanol, as above, and stimulated with 100 ng/ml of LPS (*Escherichia coli*, serotype O111:B4; Sigma), 0.5 μg/ml of synthetic bacterial lipopeptide Pam_3CSK_4 ([S-[2,3-bis-(palmitoyloxy)-(2-*RS*)-propyl]-N-palmitoyl-(*R*)-Cys-(*S*)-Ser-Lys_4-OH], trihydrochloride; EMC Microcollections, Tuebingen, Germany), or LM (at the concentrations indicated). The macrophages were activated with IFN-γ (500 U/ml) to study IL-12 expression. After 18–24 hr of stimulation, the supernatants were harvested and analyzed immediately, or stored at −20ºC until cytokine quantification using commercially available ELISA reagents for TNF and IL-12p40 (Duoset R&D Systems, Abingdon, UK).

THP-1 Cells

THP-1 monocyte/macrophage human cell lines were maintained as nonadherent cells in continuous culture with RPMI 1640 medium (Invitrogen, Cergy Pontoise, France), 10% fetal calf serum (Life Technologies), in an atmosphere of 5% CO_2 at 37ºC. LM acyl-forms were added in triplicate to monocyte/macrophage cells (5×10^5 cells/well) in 24-well culture plates and then incubated for 20 hr at 37ºC. In order to investigate the TLR dependence of TNF-α-inducing LM activity, anti-TLR2 (clone TL2.1; eBioscience, San Diego, CA) or anti-TLR4 (clone HTA125; Serotec, Cergy Saint-Christophe, France) monoclonal antibodies or an IgG2a isotype control (clone eBM2a; eBioscience) at a concentration of 5 or 15 μg/ml were added together with LM to THP-1 cells. Supernatants from THP-1 cells were assayed for TNF-α by sandwich ELISA using commercially available kits and according to manufacturer's instructions (R&D Systems, Minneapolis, MN).

Endotoxin Measurements

Endotoxin levels in the different LM samples were quantified by LAL kinetic turbidimetric assays (Cambrex, Verviers, Belgium).

Supplemental Data

Supplemental Data, including Table S1, are available at http://www.chembiol.com/cgi/content/full/13/1/39/DC1/.

Acknowledgments

We gratefully acknowledge Dr. B. Monsarrat (IPBS, CNRS, Toulouse) for MALDI-TOF MS facilities, Dr. P. Constant and Miss B. Sichi (IPBS, CNRS, Toulouse) for helping with THP1 experiments, and Dr. G. Tiraby (Société Cayla, Toulouse, France) for helpful discussions on TLR.

Received: March 9, 2005
Revised: September 7, 2005
Accepted: October 20, 2005
Published: January 20, 2006

References

1. Flynn, J.L. (2004). Immunology of tuberculosis and implications in vaccine development. Tuberculosis (Edinb.) *84*, 93–101.
2. Stenger, S., and Modlin, R.L. (2002). Control of *Mycobacterium tuberculosis* through mammalian Toll-like receptors. Curr. Opin. Immunol. *14*, 452–457.
3. Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R.L., and Akira, S. (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. *169*, 10–14.
4. Krutzik, S.R., and Modlin, R.L. (2004). The role of Toll-like receptors in combating mycobacteria. Semin. Immunol. *16*, 35–41.
5. Gilleron, M., Quesniaux, V.F., and Puzo, G. (2003). Acylation state of the phosphatidyl inositol hexamannosides from *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Rv and its implication in TLR response. J. Biol. Chem. *278*, 29880–29889.
6. Vignal, C., Guerardel, Y., Kremer, L., Masson, M., Legrand, D., Mazurier, J., and Elass, E. (2003). Lipomannans, but not lipoarabinomannans, purified from *Mycobacterium chelonae* and *Mycobacterium kansasii* induce TNF-alpha and IL-8 secretion by a CD14-Toll-like receptor 2-dependent mechanism. J. Immunol. *171*, 2014–2023.
7. Quesniaux, V.J., Nicolle, D.M., Torres, D., Kremer, L., Guerardel, Y., Nigou, J., Puzo, G., Erard, F., and Ryffel, B. (2004). Toll-like receptor 2 (TLR2)-dependent-positive and TLR2-independent-negative regulation of proinflammatory cytokines by mycobacterial lipomannans. J. Immunol. *172*, 4425–4434.
8. Nigou, J., Gilleron, M., Rojas, M., Garcia, L.F., Thurnher, M., and Puzo, G. (2002). Mycobacterial lipoarabinomannans: modulators of dendritic cell function and the apoptotic response. Microbes Infect. *4*, 945–953.
9. Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M., and Puzo, G. (2001). Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. J. Immunol. *166*, 7477–7485.
10. van Kooyk, Y., and Geijtenbeek, T.B. (2003). DC-SIGN: escape mechanism for pathogens. Nat. Rev. Immunol. *3*, 697–709.
11. Gilleron, M., Nigou, J., Cahuzac, B., and Puzo, G. (1999). Structural study of the lipomannans from *Mycobacterium bovis* BCG: characterisation of multiacylated forms of the phosphatidyl-myo-inositol anchor. J. Mol. Biol. *285*, 2147–2160.
12. Gilleron, M., Ronet, C., Mempel, M., Monsarrat, B., Gachelin, G., and Puzo, G. (2001). Acylation state of the phosphatidylinositol mannosides from *Mycobacterium bovis* bacillus Calmette Guerin and ability to induce granuloma and recruit natural killer T cells. J. Biol. Chem. *276*, 34896–34904.
13. Nigou, J., Gilleron, M., and Puzo, G. (1999). Lipoarabinomannans: characterization of the multiacylated forms of the phosphatidyl-myo-inositol anchor by NMR spectroscopy. Biochem. J. *337*, 453–460.
14. Gilleron, M., Bala, L., Brando, T., Vercellone, A., and Puzo, G. (2000). *Mycobacterium tuberculosis* H37Rv parietal and cellular lipoarabinomannans: characterization of the acyl- and glycoforms. J. Biol. Chem. *275*, 677–684.
15. Ozinsky, A., Smith, K.D., Hume, D., and Underhill, D.M. (2000). Co-operative induction of pro-inflammatory signaling by Toll-like receptors. J. Endotoxin Res. *6*, 393–396.
16. Bulut, Y., Faure, E., Thomas, L., Equils, O., and Arditi, M. (2001). Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. J. Immunol. *167*, 987–994.
17. Hajjar, A.M., O'Mahony, D.S., Ozinsky, A., Underhill, D.M., Aderem, A., Klebanoff, S.J., and Wilson, C.B. (2001). Cutting edge: functional interactions between Toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J. Immunol. *166*, 15–19.
18. Ottenhoff, T.H., and Bevan, M.J. (2004). Host-pathogen interactions. Curr. Opin. Immunol. *16*, 439–442.
19. Quesniaux, V., Fremond, C., Jacobs, M., Parida, S., Nicolle, D., Yeremeev, V., Bihl, F., Erard, F., Botha, T., Drennan, M., et al. (2004). Toll-like receptor pathways in the immune responses to mycobacteria. Microbes Infect. *6*, 946–959.

20. Drennan, M.B., Nicolle, D., Quesniaux, V.J., Jacobs, M., Allie, N., Mpagi, J., Fremond, C., Wagner, H., Kirschning, C., and Ryffel, B. (2004). Toll-like receptor 2-deficient mice succumb to *Mycobacterium tuberculosis* infection. Am. J. Pathol. *164*, 49–57.
21. Dao, D.N., Kremer, L., Guerardel, Y., Molano, A., Jacobs, W.R., Jr., Porcelli, S.A., and Briken, V. (2004). *Mycobacterium tuberculosis* lipomannan induces apoptosis and interleukin-12 production in macrophages. Infect. Immun. *72*, 2067–2074.
22. Beatty, W.L., Rhoades, E.R., Ullrich, H.J., Chatterjee, D., Heuser, J.E., and Russell, D.G. (2000). Trafficking and release of mycobacterial lipids from infected macrophages. Traffic *1*, 235–247.
23. Beatty, W.L., Ullrich, H.J., and Russell, D.G. (2001). Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. Eur. J. Cell Biol. *80*, 31–40.
24. Schaible, U.E., Winau, F., Sieling, P.A., Fischer, K., Collins, H.L., Hagens, K., Modlin, R.L., Brinkmann, V., and Kaufmann, S.H. (2003). Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nat. Med. *9*, 1039–1046.
25. Schaible, U.E., Hagens, K., Fischer, K., Collins, H.L., and Kaufmann, S.H. (2000). Intersection of group I CD1 molecules and mycobacteria in different intracellular compartments of dendritic cells. J. Immunol. *164*, 4843–4852.
26. Leopold, K., and Fischer, W. (1993). Molecular analysis of the lipoglycans of *Mycobacterium tuberculosis*. Anal. Biochem. *208*, 57–64.
27. Khoo, K.H., Dell, A., Morris, H.R., Brennan, P.J., and Chatterjee, D. (1995). Structural definition of acylated phosphatidylinositol mannosides from *Mycobacterium tuberculosis*: definition of a common anchor for lipomannan and lipoarabinomannan. Glycobiology *5*, 117–127.
28. Guerardel, Y., Maes, E., Briken, V., Chirat, F., Leroy, Y., Locht, C., Strecker, G., and Kremer, L. (2003). Lipomannan and lipoarabinomannan from a clinical isolate of *Mycobacterium kansasii*: novel structural features and apoptosis-inducing properties. J. Biol. Chem. *278*, 36637–36651.
29. Guerardel, Y., Maes, E., Elass, E., Leroy, Y., Timmerman, P., Besra, G.S., Locht, C., Strecker, G., and Kremer, L. (2002). Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*: presence of unusual components with alpha 1,3-mannopyranose side chains. J. Biol. Chem. *277*, 30635–30648.
30. Akira, S. (2003). Mammalian Toll-like receptors. Curr. Opin. Immunol. *15*, 5–11.
31. Morr, M., Takeuchi, O., Akira, S., Simon, M.M., and Muhlradt, P.F. (2002). Differential recognition of structural details of bacterial lipopeptides by toll-like receptors. Eur. J. Immunol. *32*, 3337–3347.
32. Reiling, N., Holscher, C., Fehrenbach, A., Kroger, S., Kirschning, C.J., Goyert, S., and Ehlers, S. (2002). Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. J. Immunol. *169*, 3480–3484.
33. Sugawara, I., Yamada, H., Li, C., Mizuno, S., Takeuchi, O., and Akira, S. (2003). Mycobacterial infection in TLR2 and TLR6 knockout mice. Microbiol. Immunol. *47*, 327–336.
34. Nicolle, D., Fremond, C., Pichon, X., Bouchot, A., Maillet, I., Ryffel, B., and Quesniaux, V.J. (2004). Long-term control of *Mycobacterium bovis* BCG infection in the absence of Toll-like receptors (TLRs): investigation of TLR2-, TLR6-, or TLR2-TLR4-deficient mice. Infect. Immun. *72*, 6994–7004.
35. Kang, T.J., Lee, S.B., and Chae, G.T. (2002). A polymorphism in the Toll-like receptor 2 is associated with IL-12 production from monocyte in lepromatous leprosy. Cytokine *20*, 56–62.
36. Finlay, B.B., and Hancock, R.E. (2004). Can innate immunity be enhanced to treat microbial infections? Nat. Rev. Microbiol. *2*, 497–504.
37. Nigou, J., Gilleron, M., Cahuzac, B., Bounery, J.D., Herold, M., Thurnher, M., and Puzo, G. (1997). The phosphatidyl-myo-inositol anchor of the lipoarabinomannans from *Mycobacterium bovis* bacillus Calmette Guerin: heterogeneity, structure, and role in the regulation of cytokine secretion. J. Biol. Chem. *272*, 23094–23103.
38. Venisse, A., Berjeaud, J.M., Chaurand, P., Gilleron, M., and Puzo, G. (1993). Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG: determination of molecular mass by laser desorption mass spectrometry. J. Biol. Chem. *268*, 12401–12411.
39. Takeuchi, O., Kawai, T., Muhlradt, P.F., Morr, M., Radolf, J.D., Zychlinsky, A., Takeda, K., and Akira, S. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. *13*, 933–940.
40. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. *162*, 3749–3752.
41. Michelsen, K.S., Aicher, A., Mohaupt, M., Hartung, T., Dimmeler, S., Kirschning, C.J., and Schumann, R.R. (2001). The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS): peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J. Biol. Chem. *276*, 25680–25686.
42. Muller, M., Eugster, H.P., Le Hir, M., Shakhov, A., Di Padova, F., Maurer, C., Quesniaux, V.F., and Ryffel, B. (1996). Correction or transfer of immunodeficiency due to TNF-LT alpha deletion by bone marrow transplantation. Mol. Med. *2*, 247–255.