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Identification by surface plasmon resonance of the mycobacterial lipomannan and lipoarabinomannan domains involved in binding to CD14 and LPS-binding protein

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Abstract The mycobacterial lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), regulate host defence mechanisms through their interaction with pattern recognition receptors such as Toll-like receptors (TLRs). We have developed a surface plasmon resonance assay to analyse the molecular basis for the recognition of *Mycobacterium kansasii* LM or LAM, by immobilized CD14 and LPS-binding protein (LBP) both being capable to promote presentation of bacterial glycolipids to TLRs. The affinity of either LM/LAM was higher to CD14 than to LBP. Kinetic and Scatchard analyses were consistent with a model involving a single class of binding sites. These interactions required the lipidic anchor, but not the carbohydrate domains, of LM or LAM. We also provide evidence that addition of recombinant LBP enhanced the stimulatory effect of LM or LAM on matrix metalloproteinase-9 expression and secretion in macrophages, through a TLR1/TLR2-dependent mechanism.

Keywords: Lipomannan; Lipoarabinomannan; Mycobacteria; LPS-binding protein; CD14; Matrix metalloproteinase 9

1. Introduction

Phosphatidyl-*myo*-inositol mannosides (PIMs) and their multiglycosylated counterparts, lipomannan (LM) and lipoarabinomannan (LAM), are complex lipoglycans that are ubiquitously found in the envelopes of all mycobacterial species. PIMs, LM, and LAM all share a conserved mannosyl-phosphatidyl-*myo*-inositol (MPI) domain that presumably anchors these structures into the plasma membrane. In addition to the MPI, LAM possesses a mannan core with a branched arabinan polymer and, in some cases, capping motifs decorate the termini of the branched arabinan [1,2]. LAM can be classified into three major structural families according to the capping motifs present on the non-reducing termini of the arabinosyl side-chains. The arabinan termini in the pathogenic strains *Mycobacterium tuberculosis*, *M. leprae*, *M. avium*, *M. bovis* BCG and *M. kansasii* are modified with mannose caps [3–8] resulting in molecules designated ManLAM, whereas in the fast-growing non-pathogenic species branches of the terminal arabinan are terminated by inositol phosphate-caps [2,7], characterising the PILAM family. A third LAM family, designated AraLAM, identified in *M. chelonae* comprises a LAM molecule devoid of both the manno-oligosaccharide and inositol phosphate caps [9].

These lipoglycans play important roles in the physiology of the bacterium and modulate the innate immune system through their binding to various pattern recognition receptors (PRRs), including Toll-like receptor-2 (TLR2), CD14, mannose receptor and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) [10–16].

LM and PILAM induce, via a TLR2-dependent signalling mechanism, the expression of proinflammatory cytokines [17–19] and the synthesis of matrix metalloproteinase protein (MMP-9) [20]. MMP-9 is involved in extracellular matrix remodelling, leukocyte migration at infectious sites and the granuloma formation during *M. tuberculosis* infection [21]. Recent work also demonstrated that LM induces granuloma macrophages fusion through a mechanism involving TLR2 [22]. Furthermore, both DC-SIGN and mannose receptor were shown to participate in the anti-inflammatory response elicited by ManLAM [14,15,23,24]**.** Although it is now admitted that TLR2 is required for cell activation by LM and LAM, numerous studies suggested that CD14 and LPS-binding protein (LBP) may be associated with TLR to increase the responsiveness of cells to these mycobacterial glycolipids [25–29]. LBP is an acute-phase serum protein which disaggregates and transfers bacterial glycolipids to CD14 [29,30]. CD14 is a glycoprotein which exists in two forms, either bound to the membrane of monocytes/macrophages by a GPI anchor (mCD14) or soluble in plasma (sCD14) [31]. Anti-CD14 antibodies decreased

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Abbreviations: LM, lipomannan; LAM, lipoarabinomannan; LBP, LPS-binding protein; MMP, matrix metalloproteinase; LPS, lipopolysaccharide; MPI, mannosyl-phosphatidyl-*myo*-inositol; TLR, Toll-like receptor; PIM_2, phosphatidyl-*myo*-inositol dimannoside; PIM, phosphatidyl-*myo*-inositol mannoside; DC-SIGN, dendritic cell-specific ICAM-3 grabbing non-integrin; AMannan, arabinomannan; P-AMannan, phosphorylated arabinomannan; Mannan, mannan; P-Mannan, phosphorylated mannan; PRR, pattern recognition receptor; SPR, surface plasmon resonance technology; RU, resonance unit; R*equ*, equilibrium response values

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the TLR2-dependent activities of LM and PILAM on macrophages [17,20], whereas a CD14/TLR2 cotransfection or the addition of sCD14 to cells, enhanced the biological responses [25–27]. However, despite the suggested role of CD14 and LBP in the presentation of LM/LAM to TLR2, no information is available with regard to the binding parameters and the nature of the structural domains of these lipoglycans that interact with CD14 and LBP.

Herein, we determined, through the use of surface plasmon resonance technology (SPR), the binding affinity and kinetic parameters of LM and ManLAM isolated from *M. kansasii*, to immobilized human recombinant CD14 and LBP. In order to define the relative contribution of the lipidic or carbohydrate domains in the interactions with CD14 or LBP, we also analysed the binding properties of various structurally related lipoglycans isolated from *M. kansasii*. These include PIM_2 (Fig. 1a), which is a direct biosynthetic precursors of LM, as well as as a representative panel of non-acylated derivatives of LM and LAM such as phosphorylated arabinomannan (P-AMannan), phosphorylated mannan (P-Mannan), arabinomannan (AMannan) and mannan (Mannan) (Fig. 1b). Our work not only suggests that the lipidic aglycone anchor of LM or ManLAM is essential for interacting with CD14 and LBP, but also that these interactions trigger TLR1/TLR2-dependent biological effects, as demonstrated by MMP-9 expression in the THP-1 macrophagic cell line.

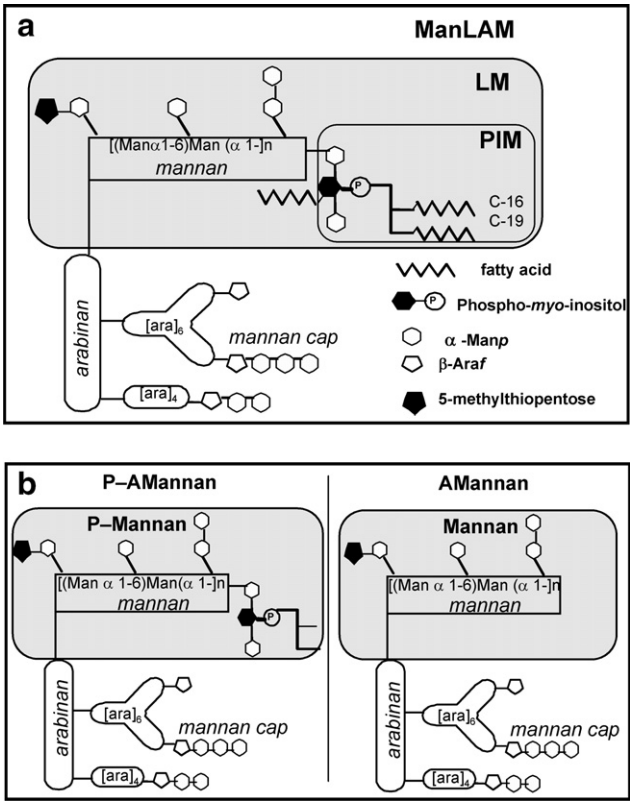

Fig. 1. Schematic representation of the mycobacterial lipoglycans and carbohydrate molecules purified from *M. kansasii* and used in this study. (a) Acylated glycoconjugates: ManLAM, LM, PIM_2 and (b) non-acylated variants of LM/LAM: P-AMannan, P-Mannan, AMannan and Mannan.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium and L-glutamine were purchased from Invitrogen (Cergy Pontoise, France) and foetal calf serum (FCS) from Eurobio (Les Ulis, France). Dulbecco's phosphate buffered saline with calcium and magnesium (PBS) was from Sigma (St. Louis, MO). 1,25-Dihydroxy-vitamin D3 was purchased from Calbiochem (Darmstadt, Germany) and the apyrogen water was from Cooper (Melun, France). Human recombinant LBP and CD14 were from Biometec (Greifswald, Germany). Various neutralizing anti-receptor mouse monoclonal antibodies, known to inhibit biological activities of microbial products, were provided without azide: IgG1 anti-CD14 (clone MEM-18) and IgG2a anti-TLR2 (clone TL2-1) were from HBT (Uden, The Netherlands), IgG1κ anti-TLR1 (clone GD2.F4), IgG2aκ anti-TLR4 (clone HTA 125) and isotype control antibodies were from BD Biosciences (San Diego, CA), whereas the IgG1κ anti-mannose receptor (clone 19) was purchased from BD Pharmingen (San Diego, CA). Quantikine human MMP-9 protein detection kit was purchased from R&D systems (Minneapolis, MN).

2.2. Extraction and purification of lipoglycans

LM, LAM and PIM_2 (phosphatidyl-*myo*-inositol dimannoside) from *M. kansasii* (PHRI 901, a clinical strain isolated from a HIV-positive patient) were purified by successive detergent and phenol extractions, separated by gel filtration on a Sephacryl 200 column in Tris/deoxycholate buffer and extensively dialyzed as described previously [3,9]. The average molecular masses of these molecules were estimated at approximately 16000 and 7000 Da for ManLAM and LM, respectively [3]. Purity of all preparations was assessed through gas chromatography/mass spectrometry (GC/MS) routine experiments, NMR spectra and SDS–PAGE following silver nitrate staining. No contaminating lipoproteins lipopeptides could be detected. The endotoxin content of each preparation was <20 pg LPS/10 μg, as determined by the chromogenic Limulus lysate assay (QCL1000; BioWhittaker, Walkersville, MD).

Mannan, P-Mannan, AMannan and P-AMannan were extracted from detergent depleted phase of *M. kansasii* after phase partitioning with Triton X-114 and separated by successive gel chromatographies as already described [31].

2.3. Direct binding assays by surface plasmon resonance

Binding analyses were performed on a BIAcore 3000 instrument (Biacore Ab, Uppsala, Sweden). This methodology is based on SPR, which enables a real-time detection of molecular interactions by monitoring refractive index changes caused by alteration in mass on the optical sensor. Human recombinant CD14 (or LBP) was covalently coupled to a CM5 (carboxymethylated) sensor chip, using a standard Amine Immobilization kit supplied by the manufacturer (Biacore). Briefly, after activation of the chip surface with *N*-hydroxysuccinimide and dimethylaminopropyl-*N*′-ethylcarbodiimide, 65 μl of CD14 or LBP at 20 μg/ml, diluted in 10 mM sodium acetate, pH 4.8, were injected at 5 μl/min. The remaining free sites were inactivated by 1 M ethanolamine, pH 8.5. Amounts of 3800–4000 RU (resonance unit) and 1800–2000 RU of LBP and CD14, respectively, were immobilized under these conditions, with 1 RU corresponding to an immobilized protein concentration of 1 pg/mm^2. Immobilized proteins were used within 48 h for providing maximal reproducibility of the results.

Increasing concentrations of each mycobacterial glycoconjugate (PIM_2, LM, LAM, Mannan, AMannan, P-Mannan, P-AMannan), ranging from 20 μg/ml to 160 μg/ml in PBS containing calcium and magnesium (Gibco) were injected at a flow rate of 10 μl/min. Following injection of 65 μl of sample at 25 °C, the formed complexes were allowed to dissociate in PBS. Regeneration of the sensor chip surface was performed by short pulses of 90% ethylene-glycol, at a flow rate of 30 μl/min and repeated washes with PBS. Control flow-cells were obtained by injection of the respective analyte solutions on a chip submitted to the activation-deactivation coupling protocol. Results are represented as sensorgrams, expressed as a response in resonance units, which is proportional to the quantity of analyte bound to immobilized CD14 or LBP. Data were analysed using the Biacore 3.0 evaluation software. The measured association (k_{on}) and dissociation (k_{off}) rate constants allowed the determination of the equilibrium dissociation constant K_D, through the ratio k_{off}/k_{on}. K_D was also determined from

Scatchard analysis. Since binding equilibrium was not reached during the time of injection, the equilibrium response values (R*equ*) were calculated from the association phases obtained with increasing concentrations of analytes.

2.4. Cells

Human pro-monocytic leukaemia THP-1 cells (ECACC no. 88081201) were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 2×10^{-5} M β-mercaptoethanol in a 5% CO_2-air humidified atmosphere at 37 °C. Viability of cells was over 96% as determined by trypan blue dye exclusion.

2.5. Effect of LBP on the MMP-9 gene expression

5×10^6 differentiated THP-1 cells were incubated in 12-well plates in RPMI/glutamine serum-free medium with various lipoglycan concentrations in the absence or presence of 0.2 μg/ml of recombinant human LBP. After 24 h of incubation at 37 °C, total RNA was extracted and purified from activated cells using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Reverse transcription (RT) and PCR amplification were performed as described previously [20] by using primer pairs (Eurogentec) designed for the specific detection of human *MMP-9* [5′-GCGGAGATTGGGAACCAGCTGTA-3′ (forward primer) and 5′-GACG- CGCCTGTGTACACCCACA-3′ (reverse primer)] at 68 °C for 23 cycles. *GAPDH* was used as an internal reference, since this gene is constitutively expressed in differentiated THP-1 cells [5′-CTCTGCC- CCCTCTGCTGATGC-3′ (forward primer) and 5′-CCATCACGCCACAGTTTCCCG-3′ (reverse primer) (69 °C, 17 cycles)]. PCR products (208 and 256 bp for *MMP-9* and *GAPDH*, respectively) were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining under ultraviolet light illumination. Gels were then analysed by computerized densitometric imaging with the Bio-Rad GelDoc analysis system and Quantity One software, version 4.1.0 (Bio-Rad, Italy).

In order to identify receptors which mediate MMP-9 expression induced by LAM, differentiated THP-1 cells were pre-treated for 30 min at 37 °C with 10 μg/ml of cell surface neutralizing monoclonal antibodies (anti-CD14, anti-TLR1, anti-TLR2, anti-TLR4, and anti-mannose receptor). Cells were then stimulated for 24 h to allow MMP-9 expression by adding 1 μg/ml of ManLAM in the presence of 0.2 μg/ml of LBP. The corresponding isotype controls were used as negative controls.

2.6. Quantification of MMP-9 secretion by ELISA

2×10^5 differentiated THP-1 cells were grown in 96-well plastic culture plates for 48 h, in RPMI supplemented with glutamine, in the presence of increasing concentrations of purified LM or ManLAM, in the absence or presence of 0.2 μg/ml of human recombinant LBP. Quantification of total MMP-9 (pro and active forms) released in the cell culture supernatant was performed by enzyme-linked immunosorbent assay (ELISA), as previously described [20]. Statistical significance of differences was calculated by using the Student's *t* test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. SPR analyses of LM and ManLAM binding to LBP and CD14

The ability of LM and ManLAM purified from *M. kansasii* to bind to immobilized human recombinant LBP (Fig. 2a and c) and sCD14 (Fig. 2b and d) was investigated using the SPR technology, as described in Section 2. The molecular association and dissociation phases were illustrated by sensorgrams obtained for each concentration of glycolipids, after substraction of the non-specific binding to a sensor chip control cell. As shown in Fig. 2, injection of increasing concentrations of LM (Fig. 2a and b) and ManLAM (Fig. 2c and d) provided a dose-dependent response, consistent with a specific interaction with CD14 or LBP. Specificity of the interaction of glycolipids with LBP was also confirmed by using the properties of denaturation of the protein by rapid changes of pH, as recommended by the manufacturer. After passage of 30 μl of HCl 0.1 M (pH 2) for 3 min on the LBP-containing flow cell, no binding of glycolipids to the protein could be observed (not shown). With regard to CD14, the binding specificity was assessed by its inability to interact with glycolipids after 4 days of SPR experiments at 20 °C (data not shown). Additionally, a chip cell coated with bovine serum albumin was used as a control. Analysis of the sensorgrams with the BIA evaluation 3.0 software indicated that binding of LM and ManLAM to either CD14 or LBP were monophasic and fitted with the langmuir binding model. The association rate constants (k_{on}) calculated from the binding of LM and ManLAM to CD14 were similar to the k_{on} measured for the binding to LBP (Table 1). The dissociation of analytes from all the complexes in the presence of PBS was very slow. Various buffers were tested to regenerate sensor chip. Whereas saline buffer (pH 7.2) containing 1 M of NaCl with EDTA or Glycine/HCl (pH 5) were unefficient, ethylene glycol (90% (v/v) in PBS) was required to release bound LM and ManLAM, suggesting occurrence of hydrophobic interactions. Furthermore, the equilibrium dissociation constants (K_D) calculated from kinetic rate constants (k_{off}/k_{on} ratio) revealed higher binding affinity of these glycolipids to CD14 than to LBP: K_D were 2.89×10^{-8} M and 7.52×10^{-9} M for LM and ManLAM with CD14, as compared to 1.84×10^{-7} M and 4.25×10^{-8} M, respectively, with LBP. The K_D were also determined by Scatchard plot analysis (Fig. 2, insets) and are consistent with the kinetically-determined values, as illustrated in Table 1. The linear Scatchard representations are suggestive of a single class of binding site in CD14 or LBP for both LM and ManLAM.

3.2. Identification of the lipoglycan structural motifs required for the interaction with CD14 and LBP

To decipher the motifs of LM/ManLAM important for ligation to CD14 and LBP, we took advantage of using a vast panel of structural variants of LM and LAM which were identified and isolated during the LM/LAM purification process. These natural cell wall-associated glycoconjugates only differ from the parental LM and LAM molecules by lacking the fatty acids attached to the MPI anchor, hence their names P-Mannan and P-AMannan (Fig. 1b) [31]. Mannan and AMannan molecules, respectively corresponding to LM and LAM lacking the entire MPI anchors, were also added in our experiments.

First, ligation of PIM_2, known as direct precursors of LM, to the glycoproteins has also been evaluated, as shown in Fig. 3. PIM_2 bound to LBP (a) and CD14 (b) with similar K_D of 4.38×10^{-7} M and 7.54×10^{-7} M (Table 1), respectively. However, the affinity of PIM_2 was lower than to LM or ManLAM with respect to CD14 binding. PIM_2 appeared to dissociate more rapidly from CD14 or LBP complexes than LM and ManLAM.

In contrast, no significant interactions of AMannan or Mannan were detected with respect to LBP or CD14 binding (Fig. 3c and d), even when higher concentrations were used (up to 80 μg/ml). Because these polysaccharides correspond to LAM and LM devoid of MPI anchors, the lack of interactions could be attributed either to the absence of phosphatidylinositol or to the absence of fatty acids bound to the MPI

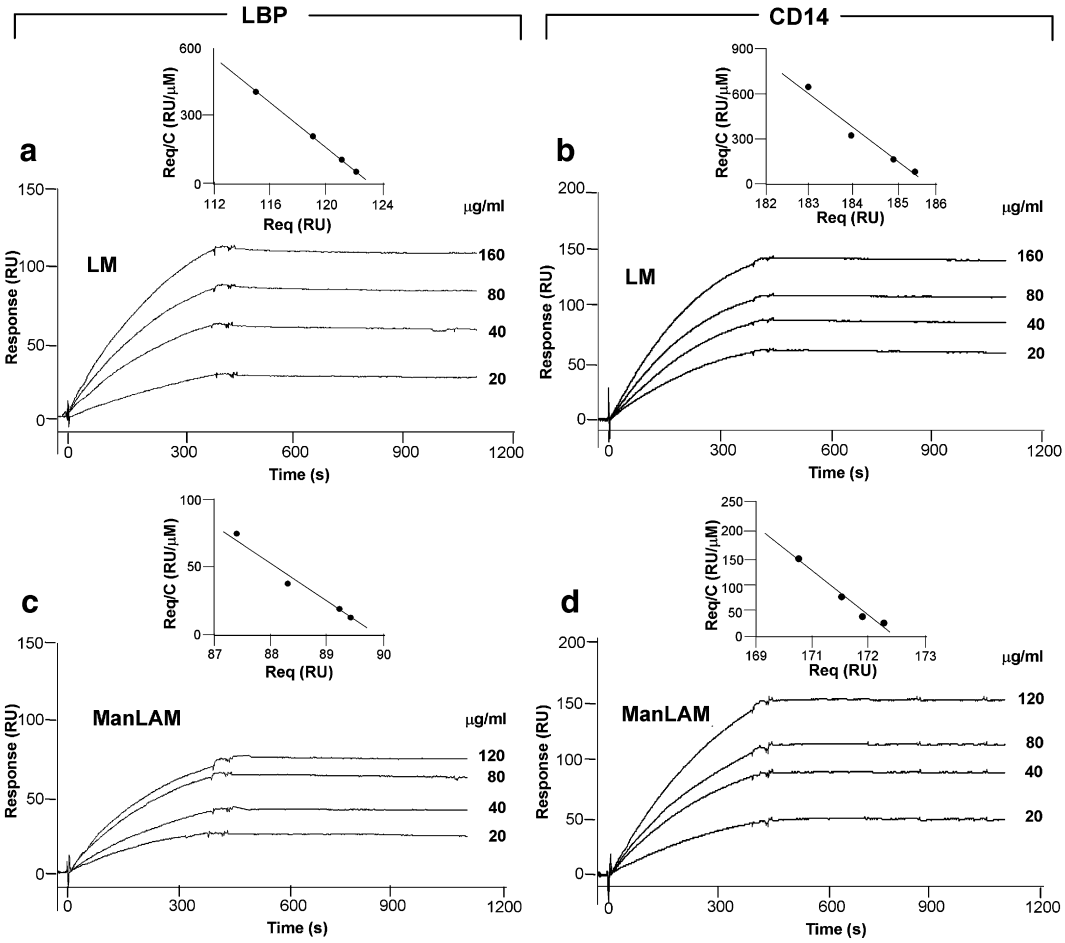

Fig. 2. Binding of LM and ManLAM to LBP and CD14 by SPR analysis. LBP (a and c) and CD14 (b and d) were immobilized on a CM5 sensor chip and 65 μl of LM (a and b) or ManLAM (c and d) were injected at the indicated concentrations at a flow rate of 10 μl/min. Dissociation of complexes was then performed in PBS. Each sensorgram was obtained by substracting unspecific binding of lipoglycans to the sensor chip control cell. Scatchard plot analyses of the sensorgram data are shown in insets. These results are representative of at least four independent experiments with similar results.

Table 1
Binding kinetic parameters for the interaction of mycobacterial lipoglycans with LBP and CD14

	k_{on} (1/Ms)	k_{off} (1/s)	K_D (M) (k_{off}/k_{on})	K_D (M)$^{(Scatchard)}$
Binding to LBP				
LM	263	4.83e−5	1.84e−7	1.99e−7
ManLAM	680	2.89e−5	4.25e−8	3.27e−8
PIM_2	542	2.37e−4	4.38e−7	1.07e−7
Binding to CD14				
LM	318	9.17e−6	2.89e−8	4.39e−8
ManLAM	627	4.73e−6	7.52e−9	1.56e−8
PIM_2	839	6.33e−4	7.54e−7	7.50e−7

anchor. In order to discriminate between the two possibilities, we analysed the binding properties of P-AMannan and P-Mannan (only differing from LAM and LM by the absence of the two fatty acids of the MPI anchor [31]). As shown in Fig. 3, P-AMannan/AMannan and P-Mannan/Mannan behave similarly, with no significant binding to LBP or CD14.

Together, these results indicate that the fatty acyl chains of the MPI anchor, but neither the carbohydrate domains nor the phosphatidyl inositol group of LM/LAM, are required for ligation to CD14 or LBP.

3.3. Effect of LBP on MMP-9 expression induced by LM and LAM in macrophages

LM has been described as a potent pro-inflammatory-inducing factor [2,11,17–19] through induction of pro-inflammatory cytokines such as IL-8, TNF-α or IL-12 [17–19] or MMP-9 expression [20]. LM-dependent expression of MMP-9 has been demonstrated in macrophage-like differentiated THP-1 cells that co-express both TLR2 and CD14 [20]. However, this effect has not been reported yet for ManLAM. Since the biological activity of various bacterial glycolipids is enhanced in presence of LBP [29,30], and because our results indicate that LM and ManLAM physically interact with LBP, we investigated the ability of LBP to modulate the activity of LM and ManLAM in relation with MMP-9 expression, both at a transcriptional and a translational level.

As shown in Fig. 4b, incubation of differentiated THP-1 cells with increasing concentrations of LM stimulates the *MMP-9* gene expression. Addition of 0.2 μg/ml of LBP resulted in a reproducibly two-fold increase in MMP-9 secretion, as compared to cells exposed to LM alone. Incubation with LBP

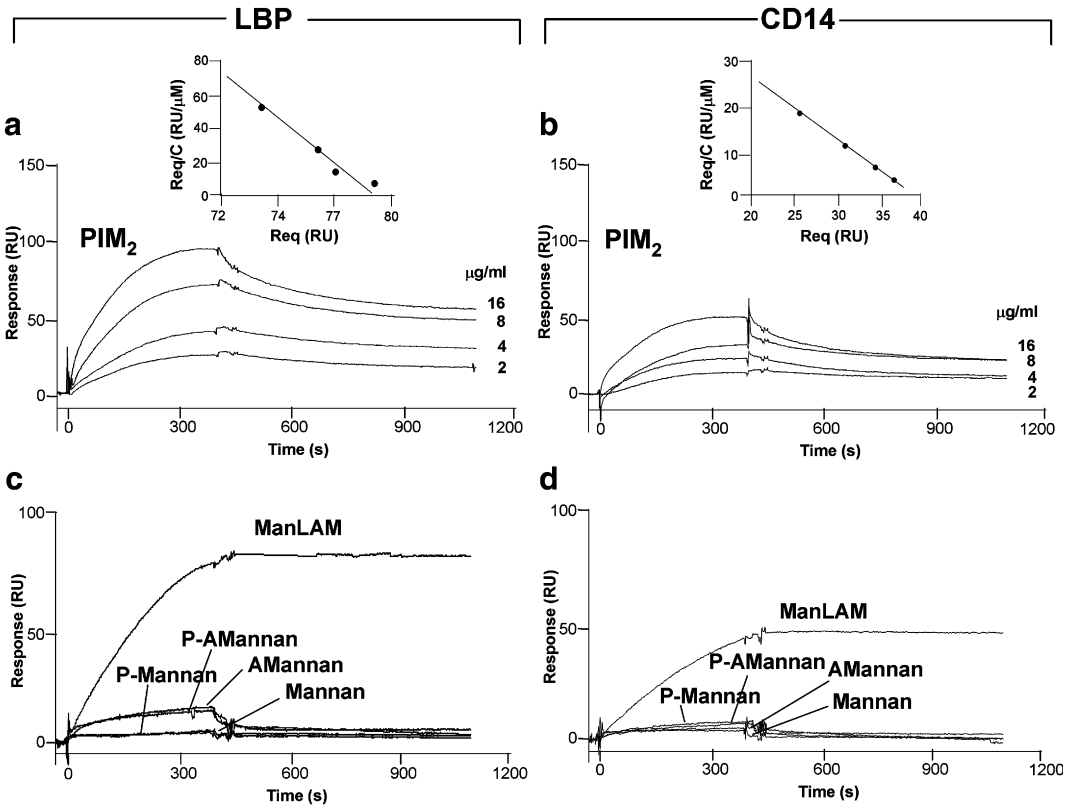

Fig. 3. Identification of the structural motifs of LM and ManLAM required for interaction with LBP and CD14. Increasing concentrations of PIM_2, LM, ManLAM, as well as their non-acylated structural variants were passed over immobilized LBP (a) or CD14 (b), in conditions similar to those described in Fig. 2. Binding properties of P-AMannan, P-Mannan, AMannan and Mannan (40 μg/ml) to LBP (c) and CD14 (d) were analysed by SPR and compared to those of ManLAM (20 μg/ml). Data are representative of two independent experiments with similar results.

alone did not further increase MMP-9 release. Together, these results suggest that, while LBP is not required for the MMP-9-inducing activity of LM, it potentializes its effect, particularly at low concentrations. Similar results were also obtained with PIM_2 (data not shown). Indeed, expression of *MMP9* mRNA is induced in a dose-dependent manner by PIM_2 and increased in the presence of LBP.

In contrast to LM, ManLAM alone was not found to trigger MMP-9 transcription (Fig. 4a). Only a slight increase of MMP-9 secretion could be detected when cells were exposed to 10 μg/ml of ManLAM (Fig. 4b). Nevertheless, LBP strongly enhanced the capacity of ManLAM to induce both *MMP-9* mRNA expression (Fig. 4a) and MMP-9 secretion (Fig. 4b). These results clearly emphasize the importance of LBP in the MMP-9 expression by ManLAM. However, it is noteworthy than the amount of MMP-9 measured following LAM-LBP stimulation was reproducibly lower than that obtained upon stimulation with LM-LBP.

Taken together, these results underscore the potency of LBP to enhance *MMP-9* gene expression and MMP-9 secretion induced by LM and LAM, presumably due to its role as a lipoglycan-presenting molecule to the macrophage cell surface receptors.

The involvement of TLR2/TLR1 and CD14, but not TLR4, in MMP-9 synthesis after stimulation of macrophages with LM from *M. kansasii* has been previously described [20]. Rivera-Marrero et al. [24] also showed that a specific anti-mannose receptor antibody blocked the induction of MMP-9 by ManLAM from *M. tuberculosis*. Therefore, in order to identify receptors recognizing ManLAM-LBP complexes and participating in signalling events leading to MMP-9 expression, we evaluated the inhibitory effect of various neutralizing anti-receptor antibodies on MMP-9 synthesis. As illustrated in Fig. 5, *MMP-9* gene expression induced by ManLAM plus LBP was inhibited by 72% with anti-TLR1 (clone GD2.F4), 34% with anti-TLR2 (clone TL2-1) and 64% with anti-CD14 (clone MEM-18) antibodies, whereas anti-TLR4 (clone HTA 125) appeared inefficient. The contribution of anti-mannose receptor antibodies (clone 19) to reduce MMP-9 expression (16%) was not significant ($P > 0.05$). The corresponding control isotype antibodies were also included in the experiment and no significant inhibition was detected (not shown). Altogether, these results suggest the major participation of TLR1/TLR2 and CD14 in the signalling pathway leading to *MMP-9* induction from differentiated THP-1 cells activated by ManLAM-LBP complexes.

4. Discussion

This study defines, for the first time, the binding kinetics of LM and ManLAM to immobilized CD14 and LBP and identifies the structural motifs of LM/LAM involved in the recognition.

CD14 and LBP were reported to interact with a very wide range of bacterial compounds which are, for the majority,

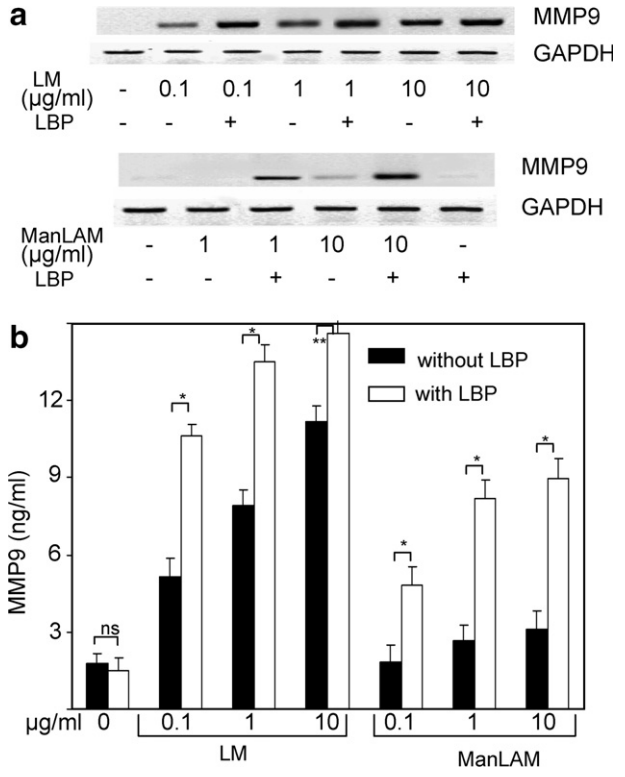

Fig. 4. Effect of LBP on MMP-9 expression induced by LM or ManLAM. Various concentrations of LM or ManLAM were added alone or supplemented with 0.2 μg/ml of purified human recombinant LBP in serum-free medium. (a) *MMP-9* gene expression was determined by RT-PCR analysis after 24 h of induction. RT-PCR data are representative of three independent experiments with similar results. *GAPDH* was used as a constitutively-expressed marker. (b) MMP-9 secretion levels in supernatants of activated cells were determined by ELISA after 48 h of induction. ELISA data are expressed as the means ± S.D. of triplicate wells and are representative of three independent experiments with similar results. The statistical significance of differences in comparison with untreated cells by LBP was calculated by using Student's *t* test (*indicates $P < 0.001$, **$P < 0.005$ and *ns* non-significant value $P > 0.05$).

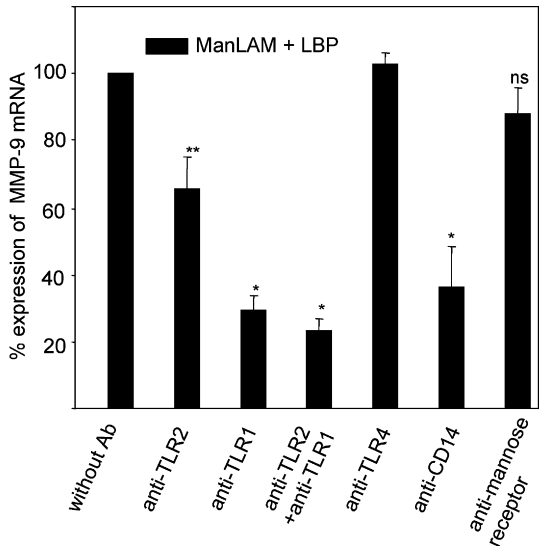

Fig. 5. Inhibition of MMP-9 expression induced by ManLAM-LBP complexes by specific PRRs. Differentiated THP-1 cells were pre-treated with 10 μg/ml of either anti-CD14, anti-TLR1, anti-TLR2, anti-mannose receptor or control isotype antibodies for 30 min at 37 °C, prior to the addition of ManLAM (1 μg/ml) plus LBP (0.2 μg/ml). Control cells were incubated in the absence of antibodies. After 24 h of incubation, expression levels of *MMP-9* and *GAPDH* mRNA were determined by RT-PCR analysis as described in Section 2. The ratios of fluorescence intensities of *MMP-9* and *GAPDH* PCR products were calculated and results are expressed as percentages of the *MMP-9*/*GAPDH* ratio obtained with ManLAM in the presence of LBP. The ratio values obtained in the absence of antibodies were arbitrarily placed at 100%. Data represent the mean values ± S.E.M. of three independent experiments (Student's *t* test to compare anti-receptor antibodies treated cells with non-treated cells: *$P < 0.001$, **$P < 0.005$ and *ns* non-significant value $P > 0.05$).

amphiphilic molecules such as lipoglycans (LPS, lipoteïchoïc acid), lipopeptides [29] and glycerophospholipids [32]. While the hydrophobic region of these various molecules is essential in these interactions, LBP can also bind to heparin anionic glycosaminoglycans [33] and CD14 exhibits lectin-like properties [34] by interacting with peptidoglycan [35] or uronic acid polymer [36]. In this study, the dissociation constants (K_D) determined by SPR for the binding of LM and ManLAM to CD14 and LBP, were close to the value previously reported for LPS-CD14 (2.9×10^{-8} M) and LPS-LBP (3.5×10^{-9} M) complexes [30]. We show here that these high-affinity interactions result from very slow association and dissociation kinetics. Furthermore, structure/activity relationship studies using natural non-acylated forms of LM and LAM (P-Mannan, P-AMannan, Mannan and AMannan) indicated that the major fatty acids in the glycerophosphoinositol headgroup (palmitic acid (C_{16}) and tuberculostearic acid (C_{19})) are essential for ligation of LM or ManLAM to CD14 and LBP. The total dissociation of LM/ManLAM-CD14 and LM/ManLAM-LBP complexes in presence of detergent also supports the involvement of hydrophobic interactions. This is in agreement with a recent SPR study demonstrating a direct interaction between a triacylated lipopeptide and CM5-immobilized CD14 [37]. Furthermore, since PIM_2 (that only contain a limited number of mannose residues compared to LM or LAM) is recognized by both CD14 and LBP glycoproteins, this suggests that the arabinosyl and mannosyl carbohydrate domains of LM or LAM are not required for interaction. Nevertheless, it is noticeable that PIM_2 bound to CD14 with a lower affinity than LM and ManLAM and dissociated much faster from LBP and CD14, suggesting that whereas the carbohydrate domain is not required for recognition, the mannan core could participate in the stability of these complexes. Thus, the presence of additional mannose residues in LM/LAM participate in the slow dissociation of the complexes with CD14 or LBP. Additionally, the heterogeneity of the fatty acids attached to PIM_2 may influence their binding to CD14 or LBP. Indeed, LM, LAM and PIM_2 contain a mixture of di- and triacyls. Interestingly, triacylated, but not diacylated, lipopeptides were shown to bind to CD14 [37]. In a previous SPR study conducted to analyse the interaction of ManLAM from *M. tuberculosis* with human pulmonary surfactant lectinic protein A (SP-A), it was shown that both the terminal mannose capping and the fatty acids of ManLAM play a critical role for binding to SP-A [38]. In this particular case, the lipid moiety of ManLAM does not directly interact with SP-A, but is rather involved in the formation of supramolecular assemblies necessary for the multivalent binding of the 18 carbohydrate-recognition-domains (CRD) present

in this lectin. Whereas CD14 has been reported to exhibit lectin-like properties [34], no multivalent carbohydrates binding sites have been described. Thus, CD14 and SP-A receptors not only differ by their structures and biological functions, but also by their physical interactions with LM and LAM.

LBP is predominantly produced in the liver but is also found in the lung alveolar epithelial cells [39] and seems to play, *in vivo,* an essential role in pulmonary innate immune responses against bacteria [40]. The serum concentration of LBP is elevated during bacterial infections such as in tuberculosis [41] and patients with various inflammatory lung diseases displayed elevated LBP and sCD14 concentrations in their bronchoalveolar lavage fluid [42,43]. Moreover, a recent study demonstrated that LBP-deficient mice have reduced lymphocyte recruitment and activation during the early stages of *M. tuberculosis* infection [44]. Our results suggest that the addition of recombinant human LBP to macrophagic THP-1 cells potentializes MMP-9 expression/secretion induced by low concentrations of LM. Since mycobacterial lipoglycans are complex amphipatic molecules forming micelles in aqueous solution, we propose that LBP disaggregates these glycolipidic complexes and promotes the transfer of monomers to TLR1/TLR2 and CD14 receptors, thereby amplifying the cell activation signal, as reported for LPS with TLR4 [45]. Recently, Gilleron et al. [13] demonstrated that triacylated LM is the best agonist of TLR2/TLR1 heterodimers, suggesting that the degree of LM acylation is critical in the interaction with these PRR and the proinflammatory activity of LM.

The capacity of ManLAM to upregulate *MMP-9* gene expression and inducing MMP-9 secretion was highly dependent on the presence of LBP. In the absence of LBP, ManLAM from *M. kansasii* displayed no eliciting effect, as it was previously shown for the production of pro-inflammatory cytokines in differentiated THP-1 cells [17,18]. Nevertheless, MMP-9 secretion induced by ManLAM-LBP complexes was lower than with LM-LBP complexes. The use of neutralizing antibodies demonstrated that this stimulatory effect of ManLAM-LBP complexes is dependent on TLR1/TLR2 and CD14-signalling pathways. Together, these observations lead us to hypothesize that the physical interaction between ManLAM and LBP induces conformational changes of ManLAM molecules that would allow the binding of this lipoglycan to TLR1/TLR2. LBP may dissociate LAM molecules from bulky aggregates, thus promoting binding to TLR and CD14 receptors. This is in agreement with a recent work demonstrating self-aggregation of ManLAM by transmission electron microscopy and revealing a supra-molecular structural organization [46]. These authors proposed than the critical micellar concentration may modulate the biological activities of these lipoglycans.

In conclusion, our results clearly indicate that LM and LAM from *M. kansasii* are high affinity ligands for both CD14 and LBP, which in turn can present these lipoglycans to TLR1/TLR2 receptors. Although their lipidic anchors seem to represent the major recognition sites for binding CD14 or LBP, the glycan domains may represent modulatory domains. A complete understanding of the molecular mechanisms of host recognition is required and may help to improve therapeutical strategies against mycobacterial infections.

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