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This information is current as of April 23, 2012

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J Immunol 2001;166:7477-7485

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Mannosylated Lipoarabinomannans Inhibit IL-12 Production by Human Dendritic Cells: Evidence for a Negative Signal Delivered Through the Mannose Receptor¹

Jérôme Nigou,* Claudia Zelle-Rieser,[†] Martine Gilleron,* Martin Thurnher,[†] and Germain Puzo^{2*}

IL-12 is a key cytokine in directing the development of type 1 Th cells, which are critical to eradicate intracellular pathogens such as *Mycobacterium tuberculosis*. Here, we report that mannose-capped lipoarabinomannans (ManLAMs) from *Mycobacterium bovis* bacillus Calmette-Guérin and *Mycobacterium tuberculosis* inhibited, in a dose-dependant manner, the LPS-induced IL-12 production by human dendritic cells. The inhibitory activity was abolished by the loss of the mannose caps or the GPI acyl residues. Mannan, which is a ligand for the mannose receptor (MR) as well as an mAb specific for the MR, also inhibited the LPS-induced IL-12 production by dendritic cells. Our results indicate that ManLAMs may act as virulence factors that contribute to the persistence of *M. bovis* bacillus Calmette-Guérin and *M. tuberculosis* within phagocytic cells by suppressing IL-12 responses. Our data also suggest that engagement of the MR by ManLAMs delivers a negative signal that interferes with the LPS-induced positive signals delivered by the Toll-like receptors. *The Journal of Immunology*, 2001, 166: 7477–8485.

Production of proinflammatory cytokines by APC is central to a successful immune response against intracellular pathogens such as *Mycobacterium tuberculosis*. Among the proinflammatory cytokines, IL-12 plays a critical role by orchestrating both innate and acquired immune responses (1). Indeed, IL-12 is a powerful signal for the generation of Th1 responses required to eliminate *M. tuberculosis*. Furthermore, individuals with mutations affecting the genes of IL-12 cytokine (2) or its receptor (3, 4) have increased susceptibility to mycobacterial infections (5). Further support for this concept comes from IL-12-deficient mice, which are susceptible to an infection by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG³; Ref. 6). To survive in the host, several intracellular pathogens astutely target the IL-12 signaling pathway to inhibit IL-12 production (7).

Recently, it was established that phosphoinositol-capped lipoarabinomannans (PILAMs; Ref. 3) from *Mycobacterium smeg-*

matis and *Mycobacterium species* activated murine macrophages in a Toll-like receptor (TLR)-2-dependent manner (8, 9), leading to the production of proinflammatory cytokines such as TNF- α (10–14) and IL-12 (15). In contrast, mannose-capped lipoarabinomannans (ManLAMs) isolated from *M. tuberculosis* and *M. bovis* BCG failed to induce a TLR-dependent activation of macrophages (9) and the production of proinflammatory cytokines (Refs. 12, 13, 15; for review, see Refs. 16–18). Conversely, *M. tuberculosis* ManLAMs were shown to attenuate TNF- α and IL-12 expression in human mononuclear phagocytes (19). Additionally, *M. bovis* BCG and *M. tuberculosis* ManLAMs were found to bind murine and human macrophages via the mannose receptor (MR; Ref. 20–22), suggesting a role for ManLAMs in the MR-mediated adhesion of these mycobacteria to macrophages. However, engagement of the MR, for example, by mannosylated particulate ligands was not sufficient to trigger a bactericidal response in macrophages (23). Furthermore, ManLAMs can effectively induce expression of the antiinflammatory cytokine TGF- β (13). All of these findings suggest that ManLAMs may contribute to the persistence of *M. tuberculosis* and *M. bovis* BCG within macrophages.

In the present study, we assessed whether purified ManLAMs from *M. bovis* BCG and *M. tuberculosis* were able to inhibit IL-12 production by human dendritic cells (DCs) stimulated with LPS. The relationship between the structure of ManLAM acyl forms and their capacity to inhibit IL-12 production as well as the role of signals transduced by the MR has been examined.

Materials and Methods

Mycobacterial glycoconjugate purification

ManLAMs and mannose-capped arabinomannans (ManAMs) from *M. bovis* BCG Pasteur strain were purified as described previously (24).

Hydrophobic interaction chromatography of ManLAMs

Cellular ManLAMs (cManLAMs; 10 mg) were loaded in 0.1 M ammonium acetate solution containing 15% (v/v) propanol-1 to a octyl-Sepharose CL-4B (Pharmacia, Orsay, France) column (20 \times 1.5 cm) preequilibrated with the same buffer. The column was first eluted with 50 ml of equilibration buffer and then with a linear propanol-1 gradient from 15 to 65% (v/v; 250 ml each) in 0.1 M ammonium acetate solution at a flow rate

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Received for publication August 31, 2000. Accepted for publication April 13, 2001.

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¹ This work was supported by grants from the Ministère de la Recherche (Programme de recherche fondamentale en microbiologie et maladies infectieuses et parasitaires; 1999) and from the European Community (TB vaccine cluster, shared costs, RTD actions; 2000).

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³ Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; PILAM, phosphoinositol-capped lipoarabinomannan; TLR, Toll-like receptor; ManLAM, mannose-capped lipoarabinomannan; MR, mannose receptor; DC, dendritic cell; ManAM, mannose-capped arabinomannan; cManLAM, cellular ManLAM; tManLAM, total fraction of ManLAM; α tManLAMs, α -mannosidase-treated tManLAMs; APTS, 1-aminopyrene-3,6,8-trisulfonate; CE, capillary electrophoresis; LIF, laser-induced fluorescence; NMR, nuclear magnetic resonance; GC, gas chromatography; MS, mass spectrometry; dtManLAM, deacylated tManLAM; pManLAM, parietal ManLAM; Man_p, mannopyranose; MALDI, matrix-assisted laser desorption-ionization; CRD, carbohydrate recognition domain.

of 5 ml/h. The fractions were collected every 15 min. A total of 20 μ l of each fraction was dried and submitted to acidic hydrolysis (100 μ l of trifluoroacetic acid 2 M, 2 h at 110°C). The hydrolysates were dried, reconstituted in water, and then analyzed by high pH anion exchange chromatography for arabinose and mannose content.

Preparation of deacylated ManLAMs

Total fraction of ManLAMs (tManLAMs; 200 μ g) was incubated in 200 μ l of NaOH 0.1 M for 2 h at 37°C. After neutralization with 200 μ l of HCl 0.1 M, the reaction products were dialyzed against water.

Preparation of α -mannosidase-treated ManLAMs (α tManLAMs)

A total of 200 μ g of tManLAMs was incubated for 6 h at 37°C in 30 μ l of α -mannosidase solution (Ref. 25) (2 mg/ml, 0.1 M sodium acetate buffer, pH 4.5, 1 mM ZnSO₄). After a second addition of 50 μ l of enzyme solution, the reaction was performed overnight. The reaction products then were dialyzed against 50 mM NH₄CO₃, pH 7.6. Elimination of α -mannosidase was achieved by denaturation (2 min at 110°C) followed by overnight tryptic digestion (37°C, trypsin/ α -mannosidase = 2% by weight). α tManLAMs were recovered after dialysis against water and analyzed for their cap contents by capillary electrophoresis (CE; see below).

CE and mannoooligosaccharide cap quantification

ManLAMs (30–60 pmol) were submitted to mild acidic hydrolysis (15 μ l of HCl 0.1 M for 20 min at 110°C; Ref. 24) in the presence of mannoheptose (100 pmol) as internal standard (26). After drying under vacuum, the reaction products were mixed with 0.4 μ l of 0.2 M 1-aminopyrene-3,6,8-trisulfonate (APTS; Interchim, Montluçon, France) in 15% acetic acid and 0.4 μ l of a 1 M sodium cyanoborohydrate solution dissolved in tetrahydrofuran (27). The reaction was performed for 90 min at 55°C and was quenched by addition of 20 μ l of water. From 1 to 5 μ l of the APTS derivatives solution was dissolved again in 20 μ l of total water before injection in CE. Analyses were performed on a P/ACE CE system (Beckman Instruments, Palo Alto, CA) with the cathode on the injection side and the anode on the detection side (reverse polarity). The electropherograms were acquired and stored on a Dell XPS P60 computer with the System Gold software package (Beckman Instruments).

APTS derivatives were loaded by applying 0.5 psi (3.45 kPa) vacuum for 5 s (6.5 nl injected). Separations were performed with an uncoated fused-silica capillary column (Sigma, Division Supelco, Saint-Quentin-Fallavier, France) with a 50- μ m internal diameter and a 40-cm effective length (47 cm total length). Analyses were conducted at a temperature of 25°C with an applied voltage of 20 kV and with acetic acid 1% (w/v), triethylamine 30 mM in water, pH 3.5 as running electrolyte. The detection system consisted of a Beckman laser-induced fluorescence (LIF) equipped with a 4-mW argon-ion laser with the excitation wavelength of 488 nm and emission wavelength filter of 520 nm. The number of each cap motif per ManLAM molecule was determined relative to the internal standard (26).

Nuclear magnetic resonance (NMR) analysis

NMR spectra were recorded on a Bruker AMX-500 spectrometer (Bruker, Billerica, MA) equipped with an Aspect X32 computer. Samples were exchanged in ²H₂O with intermediate freeze-drying, then dissolved in DMSO-²H₆ and analyzed in 200 \times 5-mm 535-PP NMR tubes at 343 K. The one-dimensional phosphorus (³¹P) spectra were measured at 202.46 MHz with phosphoric acid (85%) as the external standard (δ_p 0.0). The data were collected in 16,384 k complex data sets, and an exponential transformation (LB = 2 Hz) was applied prior the processing to 65,536 k real points in the frequency domain. The ManLAM concentration, scan number (ns), and spectral width (SW) are indicated in the figure legends.

Fatty acid quantification

ManLAMs (5–6 nmol) were deacylated by strong alkaline hydrolysis (200 μ l of NaOH 1 M for 2 h at 110°C) in the presence of pentadecanoic acid (8 nmol) as the internal standard. The reaction was stopped by neutralizing with HCl. Liberated fatty acids were extracted three times with 400 μ l of chloroform and after drying under nitrogen stream were methylated with three drops of 10% (w/w) BF₃ in methanol (Fluka, Buchs, Switzerland) at 60°C for 5 min. Reaction was stopped by addition of 400 μ l of water, and fatty acid methyl esters were extracted three times with 400 μ l of chloroform. After drying, fatty acid methyl esters were solubilized in 10 μ l of pyridine and trimethylsilylated with 10 μ l of hexamethyldisilazane and 5 μ l of trimethylchlorosilane at room temperature for 15 min. After drying under nitrogen stream, fatty acid derivatives were solubilized in cyclohexane before analysis by routine gas chromatography (GC) and GC/mass

spectrometry (MS) (24). The number of each fatty acid per ManLAM molecule was determined relative to the internal standard.

Generation of monocyte-derived DCs

PBMC were isolated from heparinized whole blood by standard density gradient centrifugation on Ficoll-Paque. Monocytes were isolated from PMBC by centrifugal elutriation (J-6 M centrifuge equipped with a JE-5.0 elutriation rotor; Beckman Instruments). Monocytes (1×10^6 cells) were cultured in 1 ml of RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, and 5×10^{-5} M 2-ME, as well as 800 IU/ml of each GM-CSF and IL-4. On day 2, 1 ml of fresh medium containing GM-CSF and IL-4 was added. Immature DCs were used on day 5. To induce maturation into CD83⁺ cells, DCs were cultured with LPS (2 ng/ml) for 48 h.

Measurement of IL-12

Day 5 DCs (3×10^5 /ml) were incubated with the stimuli indicated, and IL-12 levels were assessed in DC culture supernatants after 48 h with ELISA specific for either the p40 chain or the p70 heterodimer. IL-12 p70 was measured with matched Ab pairs (Endogen, Woburn, MA). Briefly, anti-p70 IL-12 (3 μ g/ml) was plated overnight at 4°C in PBS onto MaxiSorb plates (Nunc, Naperville, IL). Plates were blocked with PBS containing 4% BSA and washed with 0.05% Tween 20 in PBS, pH 7.4. DC culture supernatants were added to the coated wells and incubated for 1 h at room temperature. Wells were washed thoroughly before the addition of a biotinylated anti-IL-12 mAb (500 ng/ml). After 1 h, wells were washed again and then incubated with streptavidin-HRP (1:8000; Zymed Laboratories, San Francisco, CA) for another 60 min. Tetramethylbenzidine (BD Pharmingen, San Diego, CA) was used as a substrate, and 2 M H₂SO₄ was used to stop the enzyme reaction. IL-12 p40 was measured with a commercially available kit (Genzyme, Cambridge, MA). IL-12 was quantitated with a microtiter plate reader.

The capacity to produce cytokines can differ dramatically between individuals (IL-12 p70 production ranged from 0.2 to 5 ng/ml). Therefore, we decided to present the data as percentage of inhibition. The detection limit of the IL-12 p70 ELISA is 15 pg/ml.

Results

Effect of ManLAMs on IL-12 production by human DCs

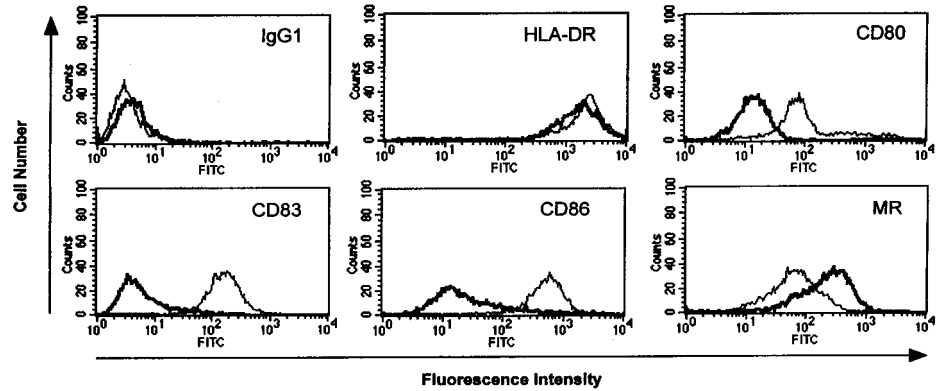
The total fraction of ManLAMs (tManLAMs) was obtained by ethanol/water extraction of the disrupted delipidated cells (16). We first tested the ability of tManLAMs from *M. bovis* BCG to stimulate the production of IL-12 by human monocyte-derived DCs. In addition to abundant MHC molecules, these cells express the MR (Fig. 1). In response to LPS treatment, DCs initiate expression of the CD83 Ag and up-regulate the T cell costimulator factor (Fig. 1). At 10 and 20 μ g/ml, tManLAMs were unable to stimulate the production of the IL-12 p70 heterodimer by DCs (data not shown), consistent with previous findings in murine macrophages (15).

Next, we tested whether tManLAMs can inhibit IL-12 production by DCs stimulated with 2 ng/ml LPS. Addition of tManLAMs to LPS stimulated-DCs resulted in a dose-dependant inhibition of IL-12 p70 production with 90% inhibition at 50 μ g/ml (around 3 μ M) tManLAMs (Fig. 2). The same concentration of tManLAMs induced an inhibition of IL-12 p40 production of ~80% (data not shown).

Determination of tManLAM domains involved in the inhibition of IL-12 production by DCs

ManLAMs present a tripartite structure composed of the GPI anchor, the polysaccharidic core, and the mannoooligosaccharide caps (16, 17). The caps of tManLAMs from *M. bovis* BCG and *M. tuberculosis* are mono-, $\alpha(1\rightarrow2)$ -di-, and $\alpha(1\rightarrow2)$ -trimannopyranosides, among which the dimannopyranoside is the most abundant motif (24, 26, 28). Their presence and their structure has been evidenced, after mild acidic hydrolysis, APTS derivatization, and analysis by CE-LIF (24, 26).

FIGURE 1. Phenotype of DCs. DCs were differentiated from monocytes as described in *Materials and Methods*. Day 5 DCs were recultured in the absence (bold lines) or presence of LPS (2 ng/ml; normal lines) for 48 h and analyzed by flow cytometry for the surface expression of the indicated Ags.



To determine whether specific tManLAM domains were involved in their inhibitory effect of IL-12 production by DCs, degraded tManLAMs were prepared. tManLAMs were treated with α -exomannosidase to obtain tManLAMs devoid of manno oligosaccharide caps (α tManLAMs). The reaction was controlled by CE-LIF analysis by using the procedure for cap analysis as described above (24, 26). A typical electropherogram obtained for α -exomannosidase-treated tManLAMs is presented in Fig. 3a. No significant peak corresponding to manno oligosaccharide caps was detected, revealing that α tManLAMs are actually devoid of manno oligosaccharide caps. In contrast to native tManLAMs, α tManLAMs at a dose of 10 μ g/ml failed to inhibit IL-12 production by DCs (Fig. 3b). Moreover, PILAMs from *M. smegmatis* containing phosphoinositol caps instead of manno oligosaccharide caps (14), previously described as inducing IL-12 secretion (15), also were unable to inhibit IL-12 secretion by DCs (Fig. 3b). Taken together, these results indicated that manno oligosaccharide caps were critical structural features for the IL-12 inhibitory effect of ManLAMs.

To test the role of the lipidic part of the GPI anchor, deacylated tManLAMs (dtManLAMs), were prepared by alkali treatment. In addition, the activity of *M. bovis* BCG ManAMs, which are distinguished from ManLAMs by the absence of the GPI anchor, was investigated (29). Both dtManLAMs and ManAMs (Fig. 4), at 10 μ g/ml, were unable to inhibit IL-12 production, indicating that a native acylated GPI anchor was required for the tManLAM inhibitory effect. In summary, both the manno oligosaccharide caps and the lipidic moiety of the GPI anchor were the crucial domains mediating the inhibitory activity of tManLAMs on DC IL-12 production.

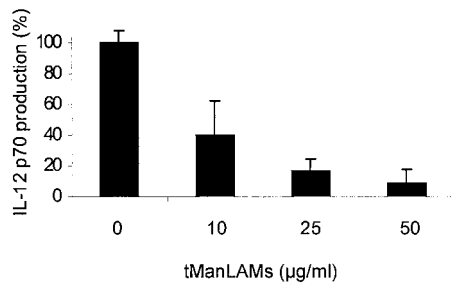


FIGURE 2. *M. bovis* BCG tManLAMs inhibit IL-12 production by DCs stimulated with LPS. DCs (3×10^5 /ml) were incubated with 2 ng/ml LPS and different amounts of tManLAMs. Supernatants were harvested for IL-12 p70 assays after 48 h. The capacity to produce cytokines can differ dramatically between individuals (IL-12 production ranged from 0.2 to 5 ng/ml). Therefore, the data will be presented along the present study as percentage of inhibition. The detection limit of the IL-12 p70 ELISA was 15 pg/ml. tManLAMs, total pool of ManLAMs.

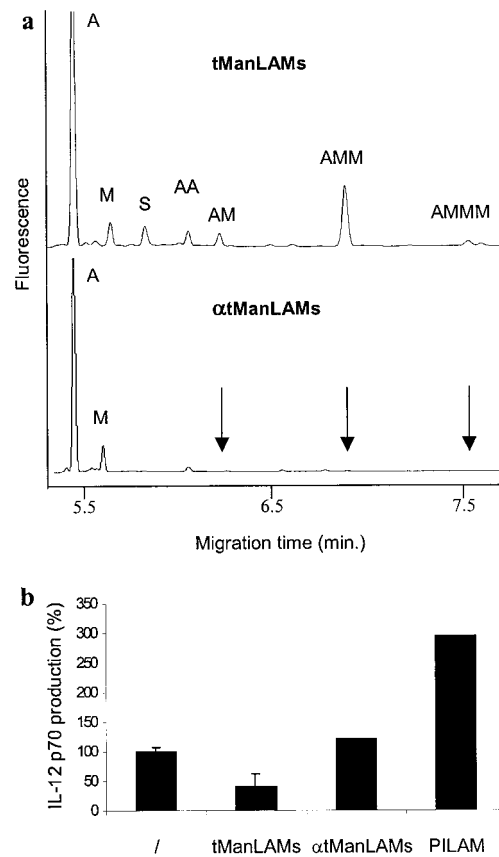


FIGURE 3. LAMs without manno oligosaccharide caps are unable to inhibit IL-12 production by DCs. *a*, CE-LIF analysis of manno oligosaccharide caps from tManLAMs (top) and α tManLAMs (bottom). One microgram of tManLAMs or α tManLAMs were hydrolyzed with 15 μ l of HCl 0.1 M for 20 min at 110°C, dried, APTS derivatized, and submitted to CE migration. CE analysis was conducted with a 470 mm \times 50 μ m capillary, at a temperature of 25°C with an applied voltage of -20 kV and monitored by LIF. Acetic acid 1% (w/v), triethylamine 30 mM in water pH 3.5 was used as running electrolyte. A, Ara-APTS; S, mannoheptose-APTS, internal standard; AA, Ara α -Ara-APTS; AM, Man α -Ara-APTS. AMM, Man α -Man α -Ara-APTS; AMMM, Man α -Man α -Man α -Ara-APTS. AM, AMM and AMMM arise from mono-, di-, and trimannoside caps, respectively. *b*, IL-12 p70 production by LPS-stimulated DCs in the presence of tManLAMs, α tManLAMs, or *M. smegmatis* PILAMs. DCs (3×10^5 /ml) were incubated with 2 ng/ml LPS and 10 μ g/ml tManLAMs, α tManLAMs, or *M. smegmatis* PILAM. Supernatants were harvested for IL-12 p70 assays after 48 h. /, LPS (2 ng/ml) alone.

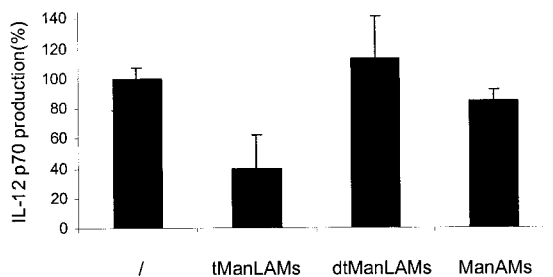


FIGURE 4. Role of acylation on ManLAM capacity to inhibit IL-12 production by DCs. DCs (3×10^5 /ml) were incubated with 2 ng/ml LPS and 10 μ g/ml of tManLAMs, dtManLAMs or ManAMs. Supernatants were harvested for IL-12 p70 assays after 48 h.

In previous studies, we have reported that the *M. bovis* BCG tManLAMs can be subdivided into a parietal pool and a cellular pool (pManLAMs and cManLAMs, respectively) based on their extraction mode (24, 30). It is noteworthy that the amount of cManLAMs represents >90% of tManLAMs. *M. bovis* BCG pManLAMs and cManLAMs mainly differ by the structure of the GPI anchor (24). pManLAMs contain a single acyl form bearing one nonconventional acyl group on the glycerol unit tentatively assigned to 12-*O*-methoxypropanoyl-12-hydroxystearic acid (24). Contrarily, cManLAMs are characterized by four multiacylated acyl forms, bearing at least one fatty acid for the less acylated form and at least three fatty acids for the most acylated form (31). These acyl groups are mainly palmitic and tuberculostearic acids and are located on the glycerol and the *myo*-inositol units (24, 31). Nevertheless, an additional acyl residue can be found on the mannose unit linked on O-2 of the *myo*-inositol (32). We then tested the ability of pManLAMs and cManLAMs to inhibit IL-12 production by DCs. Only cManLAMs were able to inhibit IL-12 production by DCs, suggesting that the acylation state influences the inhibitory activity of ManLAMs. To test this hypothesis, we tentatively fractionated the different cManLAM acyl forms by hydrophobic interaction chromatography.

Fractionation and identification of *M. bovis* BCG cManLAM acyl forms

cManLAMs were loaded on an octyl-Sepharose column and eluted with an increasing gradient of propanol-1 in ammonium acetate 0.1 M in water. The elution profile (Fig. 5b) showed four peaks, two of weak intensity, peaks A and B, eluting at ~27 and 28% propanol-1 respectively, and two of higher intensity, peaks C and D, eluting at ~31 and 35% propanol-1 respectively. The fractions corresponding to the different peaks were pooled, dialyzed, dried, and analyzed by one-dimensional ^{31}P NMR in DMSO (31). One-dimensional ^{31}P spectrum of cManLAMs from peak D (Fig. 5d) exhibited a single resonance at δ 1.66 corresponding to phosphate P1 previously evidenced in the cManLAM mixture (Fig. 5a; Ref. 31). Likewise, one-dimensional ^{31}P spectrum of ManLAMs from peak C (Fig. 5c) exhibited a single resonance at δ 1.83 corresponding to phosphate P3. As previously established by two-dimensional ^1H - ^{31}P NMR experiments, P1 and P3 typify ManLAM acyl forms containing at least three (two on the glycerol and one on O-3 of the *myo*-inositol) and two fatty acyl groups (both on the glycerol), respectively (see Fig. 6d, NMR data only). Unfortunately, the amount of cManLAMs collected in fractions A and B were not enough to record a one-dimensional ^{31}P NMR spectrum with significant ^{31}P signal. However, from the one-dimensional ^{31}P spectrum of the cManLAMs (Fig. 5a), it can be proposed that cManLAM A and B correspond to the remaining P5 and P4 phosphates. As noticed above for P1 and P3, we have previously es-

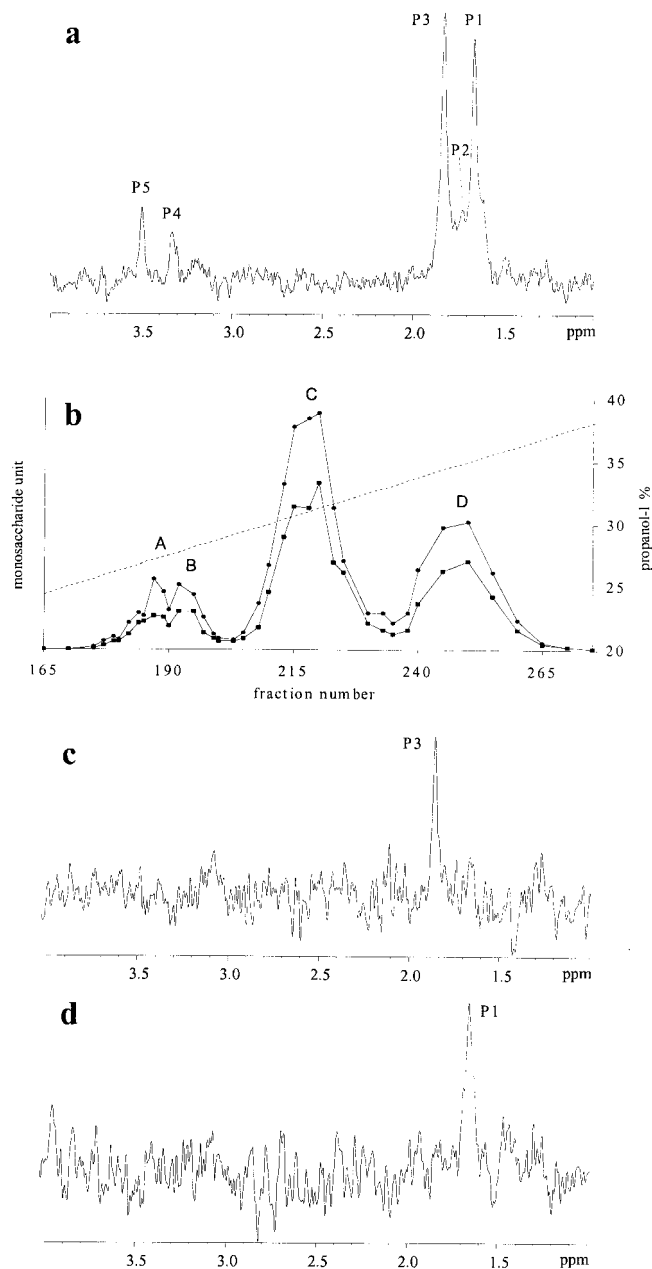
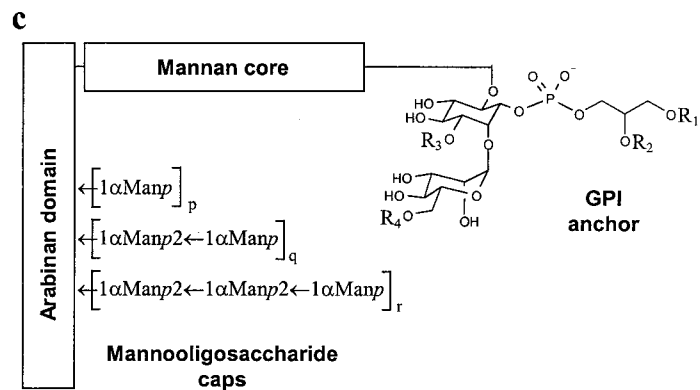
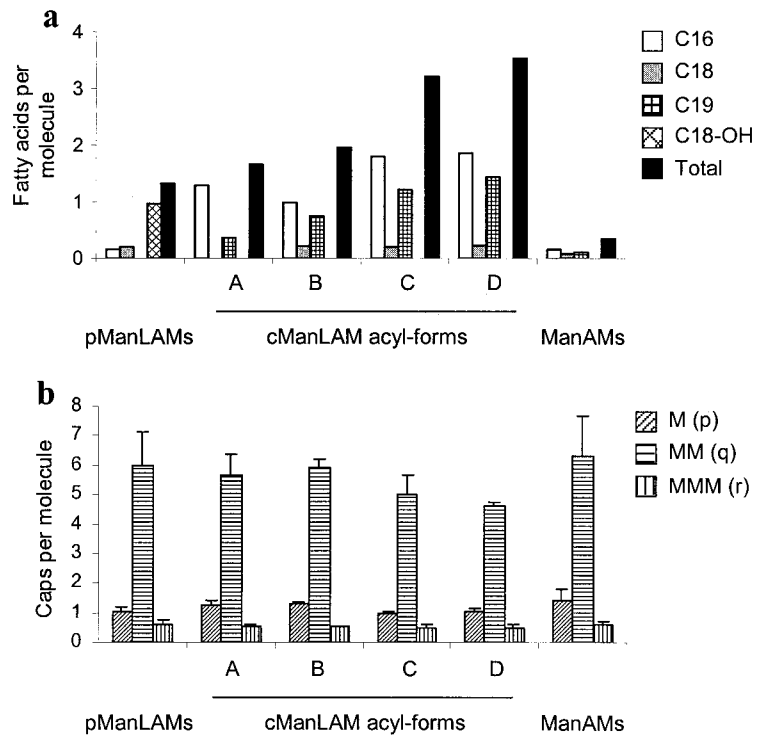


FIGURE 5. Purification of ManLAM acyl forms by hydrophobic interaction chromatography on octyl-Sepharose. One-dimensional ^{31}P spectrum ($\delta^{31}\text{P}$: 1.00–4.00) (a) and elution profile on octyl-Sepharose column (b) of cManLAMs from *M. bovis* BCG. One-dimensional ^{31}P spectrum ($\delta^{31}\text{P}$: 1.00–4.00) of ManLAMs recovered in fraction C (c) and D (d). ManLAMs (10 mg) were loaded in 0.1 M ammonium acetate buffer, pH 4.7, containing 15% (v/v) propanol-1 to an octyl-Sepharose column (20×1.5 cm) pre-equilibrated with the same buffer. The column was first eluted with 50 ml of equilibration buffer and then with a linear propanol-1 gradient (---) from 15 to 65% (v/v; 250 ml each) in 0.1 M ammonium acetate buffer, pH 4.7, at a flow rate of 5 ml/h. The fractions were collected every 15 min and analyzed after acidic hydrolysis by high pH anion exchange chromatography, for arabinose (●) and mannose (■) content (b). The ^{31}P NMR spectra were recorded at 343 K in DMSO- d_6 . An exponential multiplication (LB = 2 Hz) was applied before Fourier transformation. ManLAM concentration was 54 mg/ml (1662 ns; a), 10 mg/ml (5286 ns; c), 10 mg/ml (7346 ns; d).

tablished that cManLAMs characterized by P4 and P5 contain at least two and one fatty acids, respectively (see Fig. 6d, NMR data only). So, from the elution order of ManLAM A and B, it can be

FIGURE 6. Quantification of fatty acids (*a*) and manno oligosaccharide caps (*b*) of the different ManLAM acyl forms and ManAMs from *M. bovis* BCG. Proposed structural models (*c* and *d*). *a*, C16, palmitic; C18, stearic; C19, tuberculostearic (10-methyl-stearic); C18-OH, 12-hydroxy-stearic acids, respectively; Total, total number of fatty acids per molecule. The number of each fatty acid was quantified after strong alkaline hydrolysis (NaOH 1 M, 2 h at 110°C) by GC relative to pentadecanoic acid as internal standard. *b*, M, mono-; MM, di-; MMM, trimannoside caps, respectively. Letters p, q, r are relative to the structural model in *c*. The number of each cap motif was quantified, after mild acid hydrolysis (HCl 0.1 M, 20 min at 110°C), by CE-LIF, relative to mannoheptose as internal standard (26). *c*, Only the GPI anchor and the manno oligosaccharide caps are detailed. R₁ to R₄, potential fatty acids, the structure of which is tentatively defined in *d*. ManAMs are devoid of GPI anchor and consequently contain only the mannan core, the arabinan domain, and the manno oligosaccharide caps (29). *d*, The structure of the GPI anchor is tentatively detailed for the different ManLAM acyl forms. P, phosphate evidenced by ³¹P NMR analysis of cManLAMs (31); R₁ to R₄ are acyl groups relative to the structural model in *c*. NMR, GC, structural data obtained by ³¹P NMR and GC analysis of the anchor. +, Presence, -, absence (R = H) of the corresponding fatty acids. ?, Acylation state undetermined. +/-, presence suggested by the determination of the total number of fatty acids per ManLAM molecule in *a*. C16, palmitic; C19, tuberculostearic; C18-OR, 12-*O*-methoxypropanoyl-12-hydroxy-stearic acids, respectively; main fatty acids at the indicated position as determined by GC analysis of the acetolysis (24) and alkaline hydrolysis (the present study) products of ManLAMs.



d

| Acyl-forms | P | R ₁ | | R ₂ | | R ₃ | | R ₄ | |
|------------|---|------------------|-----------------|------------------|-----------------|------------------|-----------------|------------------|-----------------|
| | | NMR ^a | GC ^b | NMR ^a | GC ^b | NMR ^a | GC ^b | NMR ^a | GC ^b |
| pManLAMs | | + | C18-OR | - | / | - | / | ? | - |
| cManLAMs | D | P1 | + | C19 | + | C16 | + | ? | +/- |
| | C | P3 | + | C19 | + | C16 | - | ? | + |
| | B | P4 | + | C16 | - | / | + | ? | - |
| | A | P5 | + | C16 | - | / | - | ? | +/- |

^a from (31); ^b from the data in the present study and (24).

advanced that they correspond to the at least monoacylated (P5) and diacylated (P4) forms, respectively.

Structural characterization of M. bovis BCG ManLAM acyl forms

A supplementary acylation site, on position 6 of the mannopyranose (Man_p) unit linked to the O-2 of the myo-inositol, has been reported previously for *M. tuberculosis* ManLAMs (32). This data was established by degradative methods applied on the total ManLAM pool. Unfortunately, our ³¹P NMR approach did not

allow us to define the acylation state of this Man_p unit (see Fig. 6d, NMR data only). To tentatively gain access to this information, the four cManLAM acyl forms were analyzed by matrix-assisted laser desorption-ionization (MALDI)/MS (not shown). The mass spectra obtained were similar, all showing a broad peak centered around 17 kDa with a heterogeneity estimated at 5–6 kDa. This resulted from the large ManLAM molecular heterogeneity attributable to the presence of many glyco forms. As a consequence, MALDI/MS analysis was unsuccessful in determining precisely the presence of a supplementary acyl group on the purified

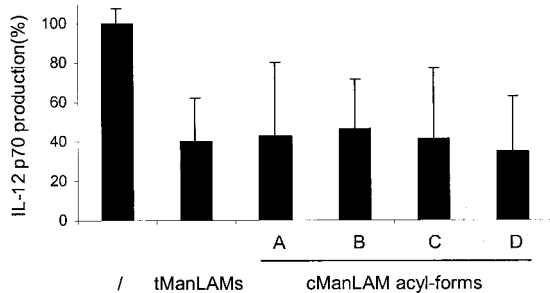


FIGURE 7. Effect of ManLAM acylation degree on IL-12 inhibition. DCs (3×10^5 /ml) were incubated with 2 ng/ml LPS and 10 μ g/ml of the different ManLAM variants. Supernatants were harvested for IL-12 p70 assays after 48 h. A, B, C, and D cManLAM acyl forms correspond to the ManLAMs purified, respectively, in the fractions A, B, C, and D by the hydrophobic interaction chromatography step on octyl-Sepharose (Fig. 5).

cManLAM acyl forms. We resorted to a classical degradative approach. This structural information was tentatively determined by the assay of the fatty acids liberated by alkaline hydrolysis and analyzed by GC. As indicated in Fig. 6a, we found 1.6, 2.0, 3.2, and 3.5 fatty acids on cManLAM A, B, C, and D respectively. So, cManLAM C may contain an acyl group on the mannosyl unit of the GPI anchor. cManLAMs A and D may also bear this extra acyl group. As expected, by using this approach, only one fatty acid was found for the pManLAMs (Fig. 6a). The presence and the structure of manno oligosaccharide caps on the purified acyl forms from *M. bovis* BCG cManLAMs and pManLAMs were investigated by CE-LIF as noticed above (24, 33, 34). The number of each manno oligosaccharide cap motif, i.e., mono-, $\alpha(1 \rightarrow 2)$ -di-, and $\alpha(1 \rightarrow 2)$ -trimannoside per ManLAM acyl form was determined relative to an internal standard (Ref. 26; Fig. 6b). All the acyl forms contained identical cap structures and similar numbers of each cap. Indeed, they shared 1 mannosyl unit, 5–6 dimannosyl units, and 0.5 trimannosyl units (Fig. 6b). It is noteworthy that these caps also were present in similar amounts in ManAMs (Fig. 6b).

In conclusion, from these structural studies, the five *M. bovis* BCG ManLAM acyl forms, including pManLAMs and cManLAMs A, B, C, and D, mainly differed by number and the structure of the acyl groups on the GPI anchor.

Effect of ManLAM acylation degree on IL-12 inhibition

The four cManLAM acyl forms were tested for their ability to inhibit IL-12 production by LPS-stimulated DCs. As shown in Fig. 7, all of the cManLAM acyl forms, at a concentration of 10 μ g/ml, exhibited the same IL-12 inhibitory effect than tManLAMs. To precisely determine the role of the acyl groups, dtManLAMs, de-

void of IL-12 inhibitory effect (Fig. 4), were randomly reacylated with palmitic anhydride in pyridine (35). Obviously, such an acylation is not selective and occurs on the accessible hydroxyl groups, leading to a large mixture of acyl forms. The mixture of reacylated ManLAMs showed an inhibitory effect in the same magnitude as that found for the native tManLAMs (not shown), suggesting that the fatty acyl residues probably play a key role in cManLAM clustering.

The following step was the identification of the molecular target of ManLAMs on DC membrane.

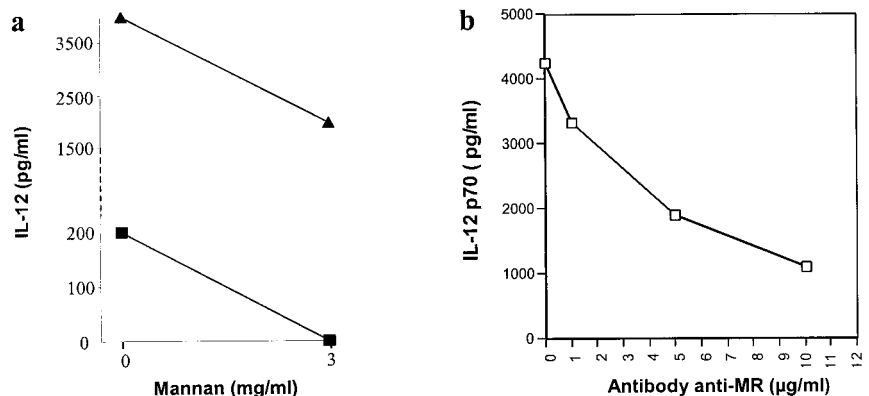
ManLAM inhibitory effect on IL-12 release by DCs is mediated by the MR

The data shown in Fig. 3b indicated that manno oligosaccharide caps were required for ManLAM IL-12 inhibitory activity, suggesting that the inhibitory signals were transduced by the MR, which is abundant on DC surface (Fig. 1). To test this hypothesis, we investigated whether *Saccharomyces cerevisiae* mannan, which is known to be a ligand for the MR, was able to inhibit IL-12 production by DCs. At a concentration of 3 mg/ml, *S. cerevisiae* mannan completely abolished IL-12 p70 and >50% IL-12 p40 secretion by DCs (Fig. 8a). In addition, to definitively prove that MR ligation was involved in the inhibition of IL-12 production, an mAb directed against the MR was tested. Fig. 8b shows that the anti-MR Ab inhibited IL-12 production by LPS-stimulated DCs in a dose-dependant manner. An isotype-matched control Ab (IgG1) had absolutely no effect on the LPS-induced IL-12 production (not shown). Because it has been established that *M. bovis* BCG and *M. tuberculosis* ManLAMs bind murine and human macrophages via the MR (20–22), these data strongly indicated that ManLAMs inhibited DC IL-12 production via binding and cross-linking to the MR.

Discussion

IL-12 is a dominant factor in directing the development of Th1 cells, which produce high levels of IFN- γ (1). Th1 cells together with cytolytic CD8 T cells are critical to eradicate intracellular pathogens such as *M. tuberculosis* (36, 37). Indeed, in humans, IL-12 deficiency leads to a predisposition to mycobacterial infections despite the formation of mature granulomas (3). Surprisingly, *M. tuberculosis*, the causative agent of human tuberculosis, induces IL-12 production in vitro from murine phagocytic cells (38) and human DCs (39) and in vivo at the human infection site (40). The *M. tuberculosis* 19-kDa lipoprotein was found to be the potent stimulator of TLR-2-dependent IL-12 production by human macrophages (41). However, in vivo, administration of IL-12 to mice enhances their resistance to *M. tuberculosis* infection, suggesting that the level of IL-12 is critical to the control of *M. tuberculosis* infection (42, 43).

FIGURE 8. Inhibition of IL-12 production by *S. cerevisiae* mannan (a) and an anti-MR Ab (b). a, DCs (3×10^5 /ml) were incubated with 2 ng/ml LPS and with or without 3 mg/ml *S. cerevisiae* mannan. Supernatants were harvested for IL-12 p70 (■) and IL-12 p40 (▲) assays after 48 h. b, DCs (3×10^5 /ml) were incubated with 2 ng/ml LPS and different amounts of the 19.2 IgG1 anti-MR Ab (BD Pharmingen). Supernatants were harvested for IL-12 p70 assays after 48 h. An isotype-matched control Ab (IgG1) had absolutely no effect on the LPS-induced IL-12 production.



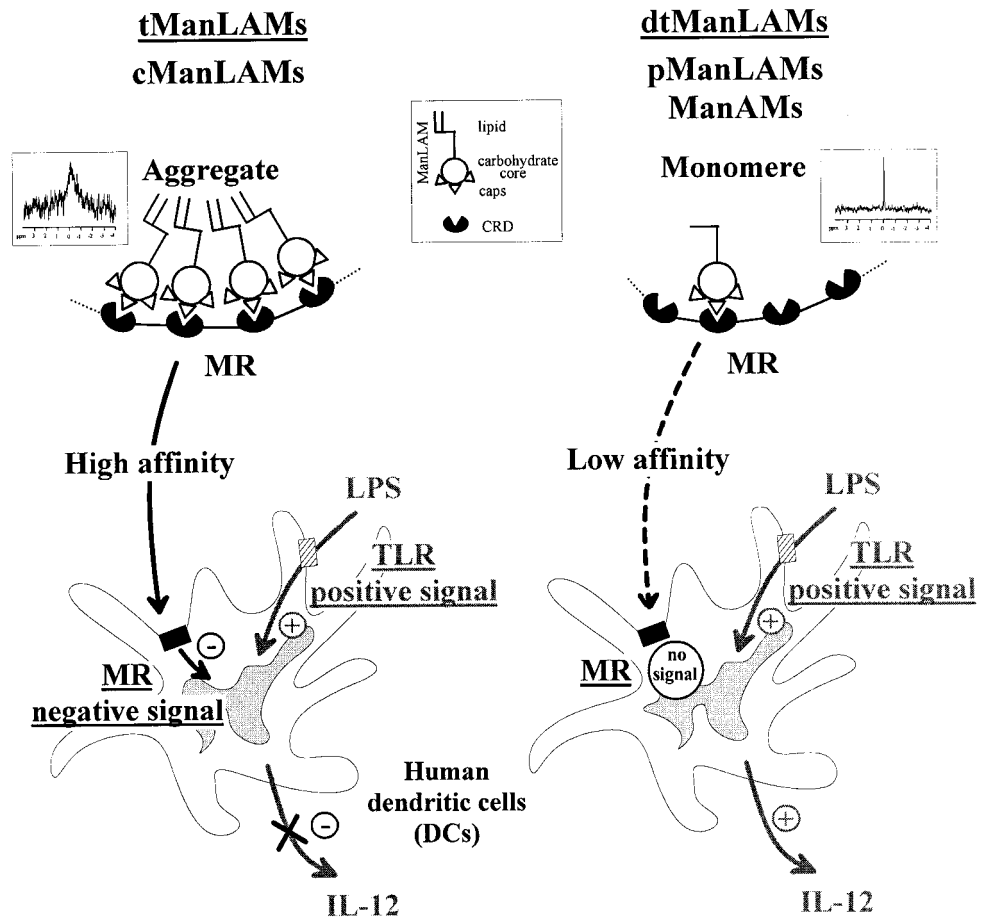


FIGURE 9. A model of IL-12 inhibition by ManLAMs. The structure of the GPI anchor modulates ManLAM clustering in water, as demonstrated by ^{31}P NMR spectra. An efficient clustering allows mannooligosaccharide caps to be efficiently presented to CRDs of the MR (25). This results in high-affinity binding to the MR and MR signaling activation. MR signaling interferes with TLR signaling resulting in IL-12 inhibition.

In the present study, we demonstrate that *M. bovis* BCG tManLAMs inhibit IL-12 production in human monocyte-derived DCs, most likely via MR ligation. This property also was shared by *M. tuberculosis* H37Rv tManLAMs (not shown). In contrast, PILAMs from *M. smegmatis*, a nonpathogenic mycobacterial strain, did not inhibit the production of IL-12. ManLAMs and PILAMs share the same tripartite structure composed of the polysaccharidic core, the GPI anchor, and the caps (16, 17). To date, the cap structure, assigned to mannooligosaccharide and phospho-*myo*-inositol residues, typify ManLAMs and PILAMs, respectively. Thus, mannooligosaccharide caps appear to play a critical role in the ability of *M. bovis* BCG and *M. tuberculosis* tManLAMs to inhibit IL-12 production by DCs. This assumption was further supported by the finding that *M. bovis* BCG uncapped tManLAMs, obtained by an α -mannosidase treatment, failed to inhibit IL-12 production. The acyl groups of the GPI anchor appear to play a crucial role because: 1) *M. bovis* BCG tManLAM deacylation abolished IL-12 inhibitory effect; 2) this activity was restored after reacylation by palmitic anhydride; 3) *M. bovis* BCG ManAMs, corresponding to ManLAMs devoid of the GPI anchor (29), failed to inhibit IL-12 production; and finally, 4) *M. bovis* BCG pManLAMs were found to be devoid of inhibitory activity. It is noteworthy that dtManLAMs as well as pManLAMs and ManAMs contained the same caps and in similar number as tManLAMs (Fig. 6*b*). The abundance of pManLAM acyl form in the BCG envelope represent only less than 10% of tManLAMs (24). This is in agreement with the fact that *M. bovis* BCG tManLAMs inhibited IL-12 production. *M. bovis* BCG pManLAMs (24) differ from *M. bovis* BCG cManLAM acyl forms (29) and from *M. tuberculosis* H37Rv ManLAM acyl forms (28) by the GPI anchor bearing a single acyl group tentatively assigned to 12-*O*-me-

thoxypropanoyl-12-hydroxystearic acid (Ref. 24; Fig. 6*d*). Collectively, these findings suggested that GPI acyl group structure and GPI acylation degree modulate ManLAM activity. We previously have established, based on one-dimensional ^{31}P and two-dimensional ^1H - ^{31}P NMR experiments, that *M. bovis* BCG cManLAMs are composed by a mixture of four acyl forms, namely A (at least one acyl group, on the glycerol), B (at least two acyl groups, one on the glycerol and the second one on O-3 of the *myo*-inositol unit), C (also at least two acyl groups but both on glycerol), and D (at least three acyl groups, two on glycerol and one on O-3 of the *myo*-inositol unit; Ref. 31; Fig. 6*d*). In this report, the four *M. bovis* BCG cManLAM acyl forms A, B, C, and D were successfully fractionated by hydrophobic interaction chromatography and their structure characterized by the chemical shift of the ^{31}P resonance. In addition to the three acylation sites accessible by our ^{31}P NMR experiments, another potential position has been described in *M. tuberculosis* ManLAMs, by Brennan and coworkers (32), on the O-6 of the mannosyl unit linked on O-2 of the *myo*-inositol. Despite the use of nondegradative techniques, such as NMR or MALDI/MS, our efforts to determine the presence of a supplementary fatty acid on the purified cManLAM acyl forms were not successful. As a consequence, this structural information was assessed, with less accuracy, by fatty acid quantification by GC analysis after alkaline hydrolysis. Palmitic and tuberculostearic acids were the major fatty acids liberated, but stearic acid also was recovered in small amount (Fig. 6*a*). The determination of the total number of fatty acids per ManLAM molecule allowed us to propose the final structures for the cManLAM acyl forms A, B, C, and D as presented in Fig. 6*d*. The four purified BCG cManLAM acyl forms A, B, C, and D, at a concentration of 10 $\mu\text{g}/\text{ml}$, exhibited the same IL-12 inhibitory effect as tManLAMs. The dominant acyl

form of H37Rv pManLAMs and cManLAMs is ManLAM B (28). Likewise, *M. tuberculosis* H37Rv pManLAMs and cManLAMs were found to inhibit IL-12 production. Because the position of the acyl groups seems to be irrelevant for the inhibitory activity (Fig. 7), it can be concluded that fatty acid residues are probably involved in the ManLAM clustering, allowing the mannooligosaccharide caps to be presented efficiently to DC membrane receptors. This is in agreement with the finding that the inhibitory effects on IL-12 were mediated by the MR. Indeed, the involvement of MR in IL-12 inhibition was supported by 1) the abolition of tManLAM inhibitory effect after the removing of mannooligosaccharide caps (Fig. 3); 2) the inhibitory effect of the *S. cerevisiae* mannan, known to be a ligand of the MR (Fig. 8a); and 3) the inhibitory effect of an anti-MR Ab (Fig. 8b). The MR is a type I transmembrane protein containing eight carbohydrate recognition domains (CRDs) associated in tandem. It has been established that high-affinity binding to C-type lectins, such as the MR, requires the cooperation of the multiple CRDs to bind different terminal mannose residues of the same multivalent ligand (44). It may be reasonable to speculate that the differences between *M. bovis* BCG pManLAMs and cManLAMs are attributable to differential clustering capacities. An efficient cManLAM clustering would allow mannooligosaccharide caps from different cManLAM molecules to bind the MR via multiple CRDs resulting in high-affinity binding, or more precisely, in high-avidity binding. In contrast, *M. bovis* BCG pManLAMs, containing a single-branched fatty acid, might not cluster, leading to low-affinity binding to the MR (Fig. 9). Indeed, pManLAMs and cManLAMs acyl forms have the same mannooligosaccharide caps and in similar number per molecule, suggesting that the cap structure was not responsible for the differences in biological activity. Moreover, differences in binding affinity between *M. bovis* BCG pManLAMs and cManLAMs to C-type lectins have recently been established by Sidobre et al. (25). Indeed, *M. bovis* BCG cManLAMs were found to bind to the human surfactant protein A with a higher affinity than *M. bovis* BCG pManLAMs (25). Surfactant protein A is a C-type lectin containing 18 CRD with the same carbohydrate recognition specificity as the MR (45). From these data, it can be concluded that the ManLAM capacity to inhibit IL-12 production by DCs is controlled by the binding affinity to the MR and, consequently, by their capacity to cluster, i.e., to form large aggregates in water. The capacity of ManLAMs to form aggregates is evidenced by the line width of their ^{31}P NMR resonance in water. Indeed, in contrast to *M. bovis* BCG cManLAMs typified by a large ^{31}P resonance, pManLAMs and dtManLAMs show well resolved signals indicating that these compounds are poorly aggregated in water (Ref. 24; Fig. 9). Thus, ManLAM clustering seems to require at least two fatty acyl appendages on the GPI (assumption corroborated by S. Sidobre, G. Puzo, and M. Rivière, unpublished results), and the inhibitory effect of cManLAM acyl form A can be explained by the presence of an extra fatty acid on the GPI mannosyl unit (Fig. 6, a and d).

Mechanisms other than the binding to the MR also could be envisaged for the inhibition of IL-12 production in response to ManLAMs. Indeed, it has been established that the binding to the complement receptor type 3 (CR3, CD11b/CD18) of human monocytes suppresses IL-12 production (46). However, β -glucans but not the α -mannans are able to bind CR3, via a cation-independent lectin site (47), suggesting that CR3 does not mediate ManLAM IL-12 inhibitory effect. The ManLAM effect also could result from a negative regulation of IL-12 synthesis by the production of anti-inflammatory cytokines such as IL-10 and TGF- β . However, PILAMs and ManLAMs have been shown to elicit TGF- β in similar amount from human monocytes, whereas PILAMs are more potent inducers of IL-10 than ManLAMs (13).

Therefore, we favor the hypothesis that the inhibition of IL-12 production by DCs in response to ManLAMs is mediated by the MR. The mechanism by which the MR signaling antagonizes CD14/TLR signaling remains an interesting question. In all systems where IL-12 suppression has been molecularly defined, it was found predominantly at the transcriptional level (7). In fact, IL-12 inhibition mediated by MR appears to be able to proceed by more than one mechanism. As noticed above, the signaling pathway of IL-12 stimulation by LPS and PILAMs (8) requires TLR-4 and TLR-2 respectively. TLRs activate intracellular signaling, most notably via the transcription factor NF- κ B, which results in the induction of genes coding for effectors of innate immunity such as TNF- α and chemokines (48). The signaling pathway requires the MyD88 adaptor proteins that interact with the cytoplasmic domain of TLR and recruits IL-1R-associated serine/threonine kinase (49, 50). At present, it is unclear how ManLAMs can interfere with this signaling pathway through MR ligation. ManLAMs were found to alter signaling pathway activation of human mononuclear phagocytes by promoting the tyrosine phosphatase 1 (SHP-1), a tyrosine phosphatase known to be important for attenuating activation signals (19). It is conceivable that ManLAMs exert their inhibitory effects by inducing the dephosphorylation of multiple proteins, including mitogen-activated protein kinase, involved in the IL-12 signaling pathway. It also has been reported that ManLAMs induced nuclear translocation of the transcription factor KBF-1 in murine macrophages (51). KBF-1 is a homodimer of the NF- κ B subunit protein p50 that can function as a transcriptional repressor by blocking the binding of the NF- κ B p50-p65 heterodimer to DNA (52) and consequently the production of IL-12. Finally, MR also serves as a signaling receptor to elicit transient rise in intracellular free Ca^{2+} concentration in macrophages by a mechanism requiring coligation of both CD14 and MR (53). Consequently, ManLAM binding to MR and ligation to CD14 could prevent TLR signaling.

In summary, the present study provides support that ManLAMs are virulence factors that contribute to the persistence of *M. bovis* BCG and *M. tuberculosis* in the host and to their maintenance in the human reservoir. This study highlights that the inhibition of IL-12 production by DCs is modulated by the structure of the ManLAM acyl forms. Thus, structural modifications of ManLAM GPI anchor may represent a mechanism for mycobacteria to gain advantage within host tissues.

The activation of macrophages and DCs depends on the net balance between positive and negative signals. Although it is established that the CD14/TLR pathway activates macrophages and DCs, the present study demonstrates that MR ligation negatively regulates DC function and that the MR can transduce inhibitory signals that interfere with TLR-mediated stimulatory signals.

Acknowledgments

We gratefully acknowledge Thérèse Brando for technical assistance with CE-LIF and Drs. Stéphane Sidobre and Michel Rivière for stimulating discussions.

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