

Mycobacteria use their surface-exposed glycolipids to infect human macrophages through a receptor-dependent process

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Abstract Two subfamilies of the polar glycopeptidolipids (GPLs) located on the surface of *Mycobacterium smegmatis*, along with unknown phospholipids, were recently shown to participate in the nonopsonic phagocytosis of mycobacteria by human macrophages (Villeneuve, C., G. Etienne, V. Abadie, H. Montrozier, C. Bordier, F. Laval, M. Daffe, I. Maridonneau-Parini, and C. Astarie-Dequeker. 2003. Surface-exposed glycopeptidolipids of *Mycobacterium smegmatis* specifically inhibit the phagocytosis of mycobacteria by human macrophages. Identification of a novel family of glycopeptidolipids. *J. Biol. Chem.* 278: 51291–51300). As demonstrated herein, a phospholipid mixture that derived from the methanol-insoluble fraction inhibited the phagocytosis of *M. smegmatis*. Inhibition was essentially attributable to phosphatidylinositol mannosides (PIMs), namely PIM₂ and PIM₆, because the purified phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol were inactive. This was further confirmed using purified PIM₂ and PIM₆ from *M. bovis* BCG that decreased by half the internalization of *M. smegmatis*. Both compounds also inhibited the uptake of *M. tuberculosis* and *M. avium* but had no effect on the internalization of zymosan used as a control particle of the phagocytic process. When coated on latex beads, PIM₂ and polar GPL (GPL III) favored the particle entry through complement receptor 3. GPL III, but not PIM₂, also directed particle entry through the mannose receptor. Therefore, surface-exposed mycobacterial PIM and polar GPL participate in the receptor-dependent internalization of mycobacteria in human macrophages.—Villeneuve, C., M. Gilleron, I. Maridonneau-Parini, M. Daffé, C. Astarie-Dequeker, and G. Etienne. **Mycobacteria use their surface-exposed glycolipids to infect human macrophages through a receptor-dependent process.** *J. Lipid Res.* 2005. 46: 475–483.

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The outcome of mycobacterial infections is thought to be critically dependent on the nonopsonic invasion and colonization of macrophages during primary infection of inhaled bacteria. This is why the initial step of mycobacteria phagocytosis by macrophages received considerable attention, including efforts to delineate the molecular mechanisms that underlie this process.

Nonopsonic internalization of mycobacteria in macrophages can be mediated by different kinds of receptors that specifically recognize ligands expressed at the surface of bacilli (1, 2). Glycophosphoinositol-anchored receptors such as CD14 (3, 4), scavenger receptor (5), and transferin receptor (6, 7) have been suggested as putative routes of entry for mycobacteria into macrophages. Additionally, complement receptor 3 (CR3) (8–10) and mannose receptor (MR) (8, 11) are both well-known receptors for the binding and internalization of mycobacteria in macrophages. Both receptors exhibited lectin-like properties. Indeed, MR is a monomeric transmembrane protein with an extracellular domain containing eight carbohydrate recognition domains with C-type lectin properties that recognize mannose, fucose, and *N*-acetylglucosamine residues present on the cell surface of pathogens, such as mycobacteria (12–14). CR3 is a heterodimeric type I transmembrane glycoprotein consisting of a CD18 β subunit noncovalently associated with the CD11b α chain. The extracellular portion of CD11b contained distinct functional binding domains. One of these, called the I domain, is essential for the recognition and internalization of C3bi op-

Abbreviations: CR3, complement receptor 3; GPL, glycopeptidolipid; MALDI-TOF, matrix-assisted laser-desorption/ionization time of flight; Man-BSA, mannosylated bovine serum albumin; MDM, macrophages derived from monocytes; MR, mannose receptor; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside.

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sonized particles (15, 16). Another one, located on the carboxyl-terminal end of the extracellular portion of CD11b, is a nonopsonic binding site with lectin properties (17). This latter domain is mainly involved in phagocytosis of particles containing β -glucan, such as zymosan (18), but also interacts with mannose, glucose, and *N*-acetyl-D-glucosamine (17). More recently, a second nonopsonic binding domain distinct from the lectin one has been proposed to bind and internalize mycobacteria (19).

We recently demonstrated that mycobacteria express molecules at their surface that play a role in their internalization by human macrophages (20). Using a biological test of phagocytosis inhibition, we found that lipids, but not proteins, isolated from the outermost layer of *Mycobacterium smegmatis* by a mechanical treatment decrease the nonopsonic phagocytosis of mycobacteria by human macrophages without affecting the internalization of particles such as zymosan or latex beads (20). A detailed analysis of the lipid fractions has indicated that the major inhibitory compounds belong to the glycopeptidolipid (GPL) and phospholipid families (20). When the former family was

chemically and structurally analyzed, several inhibitory C-type GPLs were identified, among which a novel class of succinylated GPLs (Fig. 1) passed unnoticed until now (20). We have also proposed that their capacity to inhibit the phagocytosis of mycobacteria is dependent on common structural features. Identification of these molecules and their putative role as surface-exposed ligands of phagocytic receptors would help us understand the host-pathogen interactions at the molecular level. The present study was designed first to identify the mycobacteria surface-exposed phospholipids that play a role in their internalization by human macrophages and second to examine the roles of GPLs and phospholipids as potential ligands of phagocytic receptors.

METHODS

Reagents and antibodies

Mannosylated BSA (Man-BSA), zymosan, soya phosphatidylinositol (PI), and fluorescein isothiocyanate were from Sigma

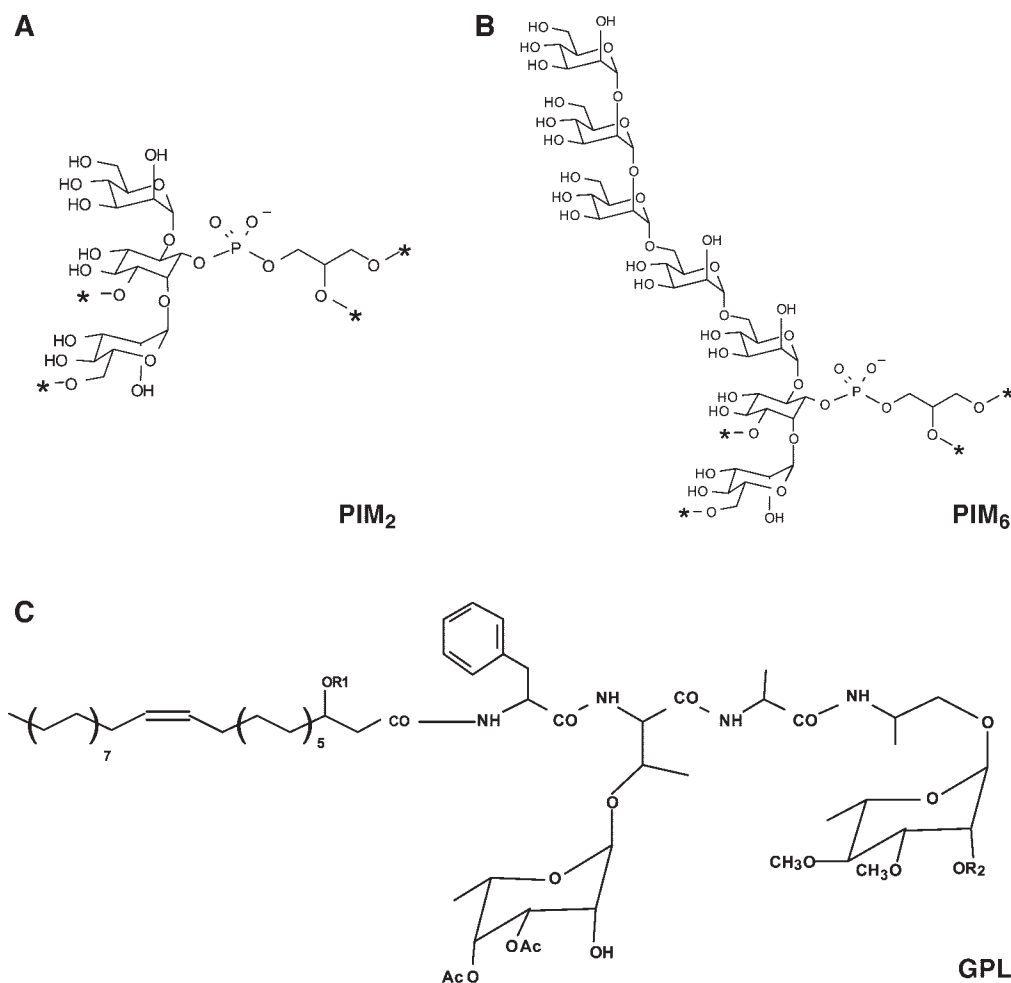


Fig. 1. Structures of the major glycopeptidolipid (GPL) and phosphatidylinositol mannoside (PIM) species found in *M. smegmatis*. R1, H or CH₃; R2, CH₃ in GPL I, succinyl in GPL IIIa, 2-*O*-succinyl-rhamnosyl in GPL IIIb. Asterisks indicate the positions of acylation in PIMs that are always diacylated on the glycerol moiety; they may also be diacylated at the indicated positions.

Chemical Co. (St Louis, MO). Polystyrene microspheres were from Polysciences, Inc. RPMI 1640, Ficoll-Hypaque, L-glutamine, and antibiotics were purchased from Eurobio (Les Ulisses, France). Mouse antibodies against the CD11b extracellular domain was used [2LPM (1:20); IgG1_(κ); Dako, Glostrup, Denmark]. Goat anti-human MR was a generous gift of P. Stahl (1:50). Anti-mycobacterium rabbit antibody Camelia (1:50) was obtained as previously described (19). Secondary Abs were purchased from Sigma.

Mycobacteria

M. smegmatis (ATCC 607) and *M. tuberculosis* H37Rv (ATCC 27294) were grown as surface pellicles on liquid Sauton's medium at 37°C without agitation; *M. avium* (serovar 4), known to form more clumps when grown as surface pellicles than the two former species, was cultured under shaking at 250 rpm in Middlebrook 7H9 medium supplemented with 10% albumin-dextrose-catalase enrichment (Difco). It is important to point out that the qualitative composition of the surface-exposed materials is independent of growth conditions (i.e., surface pellicles or shaken cultures) (21). Single cell suspensions were prepared with late log-phase cultures (6 days for *M. smegmatis*, 3 weeks for *M. tuberculosis* and *M. avium*) as previously described (20). Briefly, pellicles (*M. smegmatis* and *M. tuberculosis*) were harvested by pouring off the medium, dispersed by gentle shaking for 30 s with 5 g of glass beads (4 mm diameter), and resuspended in PBS, pH 7.4. In the case of *M. avium*, the culture was recovered by centrifugation at 10,000 g for 10 min. To remove the remaining clumps, the bacterial suspensions were centrifuged for 10 min at 200 g. Up to 90% of mycobacteria were individualized, and their viability averaged 85%. When required, mycobacteria were labeled with FITC, as previously described (22). We checked that their rate of internalization and their route of entry within macrophages derived from monocytes (MDM) were identical to those obtained by green fluorescent protein-expressing *M. smegmatis* (data not shown), ruling out a side effect of FITC labeling.

Extraction and purification of glycolipids

Glycolipids and phospholipids are present in both the outermost layer of the cell envelope and whole mycobacteria (21, 23). Therefore, the present study was performed on lipid extract from *M. smegmatis* whole cells to get enough material for further fractionation experiments (20). Mycobacterial wet cells were extracted with CHCl₃/CH₃OH (1:2, v/v) and then at last three times with CHCl₃/CH₃OH (1:1, v/v). The organic phases were pooled, extensively washed with water, and evaporated to dryness. Lipids were then resuspended in a minimal volume of chloroform and precipitated with trickling methanol. After standing for 2 h at 4°C, methanol-insoluble lipids were recovered by centrifugation at 4°C for 20 min (8,000 g). Lipids were further separated using an anion-exchange QMA-silica gel (Chromabond SB; Macherey-Nagel). Phospholipids were eluted using a stepwise gradient: 0.1 M ammonium acetate in 33% methanol in chloroform (fractions 9 and 10), 0.2 M ammonium acetate in 33% methanol in chloroform (fractions 11–13), and 0.2 M ammonium acetate in methanol (fraction 14). Purification of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) was achieved by preparative TLC. Plates were developed in CHCl₃/CH₃OH/H₂O (56:38:10, v/v/v); silica bands were scraped and extracted three times in the same solvent, adding trace amounts of acetic acid.

The purification of GPL has been described in detail (20). Briefly, the methanol-soluble lipids were chromatographed on a Florisil column irrigated with chloroform and then with a stepwise gradient of increasing concentrations of methanol and water in chloroform. GPL I was eluted with CHCl₃/CH₃OH (90:10, v/v), whereas GPL III was eluted in more polar fractions with a mixture of CHCl₃/CH₃OH/H₂O (65:25:4, v/v/v); the latter

were further separated from contaminating phospholipids using an anion-exchange QMA-silica gel (Chromabond SB; Macherey-Nagel).

All of the purification steps were monitored by TLC on silica gel 60-precoated plates (0.25 mm thickness; Merck) developed with CHCl₃/CH₃OH/H₂O (60:35:8) for the phospholipid-containing fractions. Sugar-containing compounds were visualized by spraying plates with 1.5% orcinol in 30% aqueous sulfuric acid, followed by heating, whereas the Dittmer-Lester reagent and ninhydrin were used to detect phosphorus- and amino group-containing substances. Phosphatidylinositol mannosides type 2 and 6 (PIM₂ and PIM₆) (Fig. 1) were purified on a QMA-Spherosil M (BioSeptra) column as previously described (24).

Mass spectrometry

Matrix-assisted laser-desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis of lipids was performed as previously described (25). MALDI-TOF spectra were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) equipped with a pulsed nitrogen laser emitting at 337 nm and were analyzed in the Reflectron mode using an extraction delay time set at 100 ns and an accelerating voltage operating in negative ion mode of 20 kV.

Human MDM

Human peripheral blood monocytes were isolated as previously described (11) and cultured for 7 days on sterile glass coverslips on 24-well tissue culture plates (5 × 10⁵ cells/well) containing RPMI medium with 10% heat-inactivated fetal calf serum in 5% CO₂. The culture medium was renewed on the third day. Before use, MDM were washed twice with fresh RPMI medium.

Coating of latex beads

Latex beads were coated as described by Schlesinger, Hull, and Kaufman (26). Briefly, 2 × 10⁸ polystyrene microspheres of 1 μm diameter were washed twice (10,000 g, 10 min) in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Beads were then incubated for 1 h at 37°C under gentle agitation with either 50 μg of various glycolipids, Man-BSA, or BSA in a reaction volume of 1 ml. The microspheres were washed twice, incubated in 5% BSA in PBS for 2 h at 37°C, resuspended in 5% BSA in PBS, and stored at 4°C until the experiments.

Infection of adherent macrophages and phagocytosis assay

When specified, MDM were first pretreated for 15 min at 37°C with the purified lipid fractions, which were previously resuspended in sterile apyrogenic water and sonicated for 10 min, and then added with particles at the appropriate multiplicity of infection. At the end of infection, cells were washed twice with fresh medium to remove unbound particles. Phagocytosis of mycobacteria, zymosan, and latex beads was assessed by immunofluorescence as previously described (11). For each set of conditions, duplicate experiments were performed, and at least 100 cells per slide were counted by fluorescence microscopy. The percentage of phagocytic cells having ingested at least one particle or bacterium was calculated as follows: number of phagocytic cells/number of total cells × 100. Data are expressed as the percentage of phagocytosis inhibition determined as follows: number of phagocytic cells in the control minus number of phagocytic cells under treated conditions/number of phagocytic cells in the control × 100.

Statistics

Data are presented as means ± SEM of the indicated number of experiments (n) performed in duplicate. The significance of the differences was determined by paired or unpaired Student's *t*-test.

Inhibitory activity of surface-exposed phospholipids on the phagocytosis of *M. smegmatis*

In the present study, we first attempted to identify the surface-exposed phospholipids implicated in the internalization of mycobacteria. For this purpose, lipid extract from *M. smegmatis* was first enriched in phospholipids by methanol precipitation. As depicted in Fig. 2, the methanol-insoluble fraction, analyzed by TLC, contained several compounds: PIMs, in particular PIM₂ and PIM₆ (Fig. 1) as the main subspecies, PI (Fig. 2A), PG (Fig. 2B), and PE (Fig. 2B). Examination of the blocking effect of the methanol-insoluble fraction on phagocytosis was the approach chosen to determine the role of phospholipids in the phagocytic process of mycobacteria. MDM were pretreated for 15 min with the phospholipid-enriched extract at concentrations ranging from 10 pg to 100 μg lipid/ml and then put into contact with *M. smegmatis* for 45 min. At the lowest tested concentration (i.e., 10 pg lipid/ml), treated and untreated macrophages equally internalized *M. smegmatis* (62 ± 6% and 61 ± 5%, respectively; n = 2). In contrast, increasing lipid concentration to 10 ng/ml resulted in a 30% decrease in the uptake of *M. smegmatis* (from 62 ± 6% to 45 ± 1%; n = 2). This inhibitory effect remained stable up to 100 μg/ml.

Purification and identification of the active phospholipid fractions of *M. smegmatis*

To establish the nature of the active phospholipids, we individually tested the effect of the addition of 10 μg/ml PI, PE, and PG on the phagocytosis of *M. smegmatis*. Preincubation of MDM for 15 min with 10 μg/ml commercial-

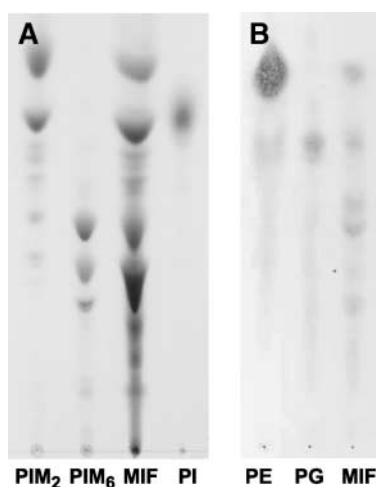


Fig. 2. Identification of phospholipids in lipids extracted from *M. smegmatis* whole cells. Phospholipids from *M. smegmatis* were enriched by methanol precipitation. Composition of the methanol-insoluble fraction (MIF) was analyzed by TLC using CHCl₃/CH₃OH/H₂O (60:35:8, v/v/v) as the solvent system. Different phospholipids [i.e., PIM₂, PIM₆, phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG)] were used as controls. Compounds were revealed by spraying with orcinol, following by heating (A) or by the Dittmer reagent (B).

ized PI did not influence the internalization of *M. smegmatis* compared with untreated cells (52 ± 7% vs. 50 ± 5%, respectively; n = 3). Similarly, 10 μg/ml PE or PG, purified from mycobacteria cell extracts by preparative TLC, did not affect the phagocytosis of *M. smegmatis* compared with untreated macrophages (43 ± 4% and 49 ± 3%, respectively, vs. 50 ± 5%; n = 3). These data strongly suggested that the observed inhibitory activity of the phospholipid-rich methanol-insoluble fraction was essentially attributable to PIM. This hypothesis was further confirmed using PIM-enriched fractions obtained from a *M. smegmatis* lipid extract as follows: methanol-insoluble lipids were fractionated by ion-exchange chromatography into 14 fractions eluted with increasing concentrations of ammonium acetate in chloroform-methanol. TLC and MALDI-TOF analysis showed that the first 8 fractions contained traces, if any, of phospholipids (data not shown), whereas fractions 9–14 contained essentially PIM and PI (Fig. 3A). Therefore, these last six fractions were tested on phagocytosis of *M. smegmatis* at 10 ng lipid/ml, the lowest active concentration for the phospholipid mixture. As shown in Fig. 3B, the tested fractions exhibited an inhibitory effect of ~50%. This set of experiments indicated that PIMs were the compounds responsible for the activity of the phospholipid mixture and therefore were the essential phospholipids involved in the phagocytic process of mycobacteria.

Inhibition of mycobacterial internalization by PIM₂ and PIM₆ subfamilies

PIM₂ and PIM₆ (Fig. 1) represent the main mycobacterial glycerophospholipids described to date (24, 27, 28). As expected, these two subclasses of PIM constituted the majority of glycerophospholipids of *M. smegmatis* and were differently distributed in fractions 9–14 (Fig. 3A). PIM₂ and triacylated PIM₂ (Ac₁PIM₂) were preferentially eluted in fractions 9–11. PIM₆ and triacylated PIM₆ (Ac₁PIM₆) appeared mainly in fractions 12–14. However, we failed to properly separate PIM₂ from PIM₆ by the purification protocol used. This prompted us to use purified PIM₂ and PIM₆ subfamilies isolated from *M. bovis* BCG by Gilleron et al. (24), which are structurally identical to those of *M. smegmatis* (27). As depicted in Fig. 4A, both PIM₂ and PIM₆ subfamilies reduced phagocytosis of *M. smegmatis*. Their inhibitory activity increased from 100 pg to 10 ng of lipid per milliliter, PIM₆ being slightly more efficient than PIM₂. A plateau of inhibition was then reached at 10 ng/ml up to 10 μg/ml. Importantly, 10 ng/ml PIM₂ and PIM₆ subfamilies did not affect zymosan internalization, used as a control of phagocytic process (33 ± 9% and 36 ± 11%, respectively, vs. 35 ± 9%; n = 3 and 4), indicating a specific inhibition of the MDM-particle interactions by structurally defined molecules. We then addressed the question of whether PIM₂ and PIM₆ interfered with the phagocytosis of the pathogenic mycobacteria (e.g., *M. avium* and *M. tuberculosis*). When tested at 10 ng/ml, both compounds reduced the internalization of bacteria (Fig. 4B). However, micromolar concentrations of PIM were required to achieve an inhibition of phagocytosis of the

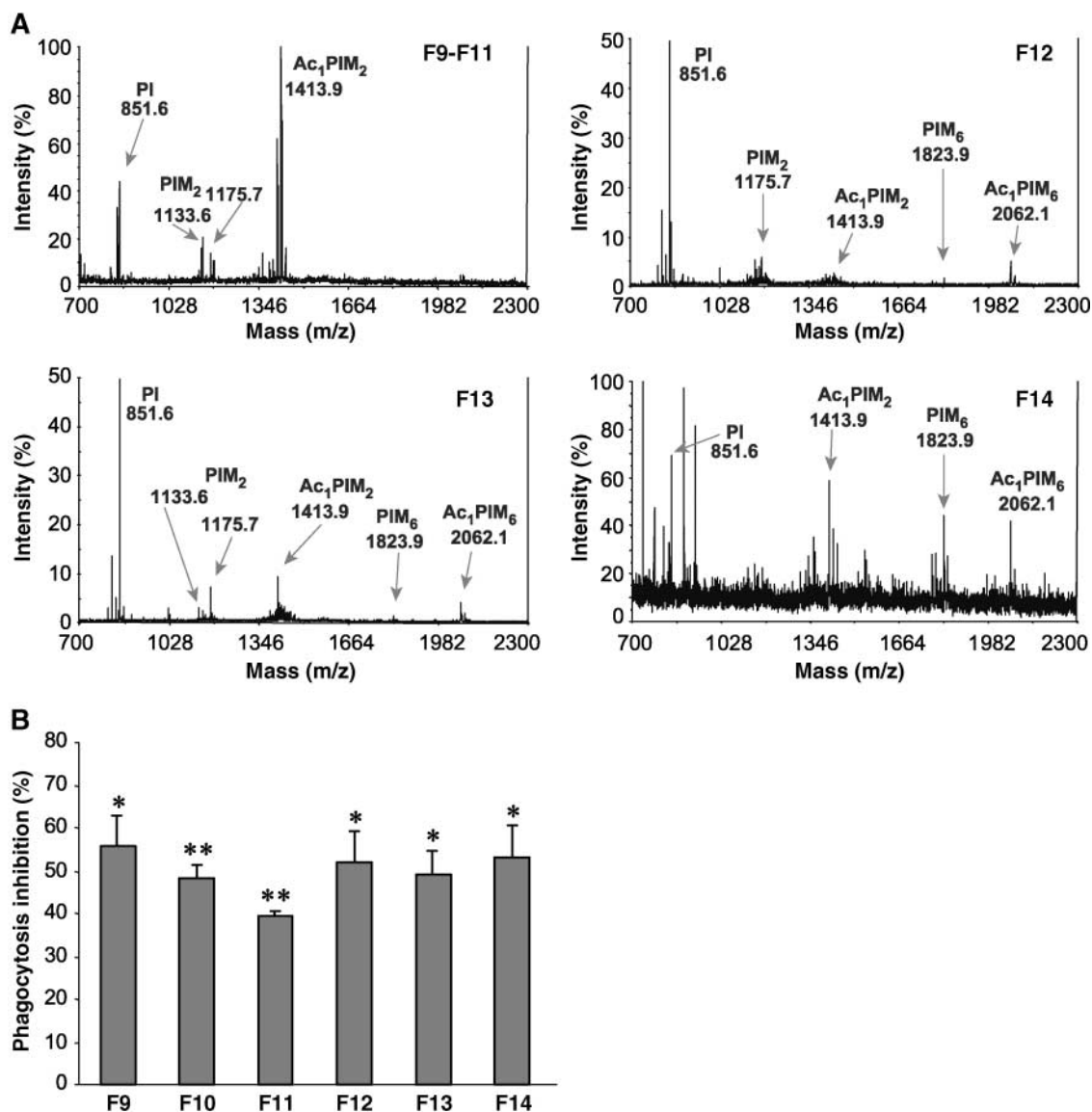


Fig. 3. The phospholipid-enriched fractions of *M. smegmatis* inhibit its phagocytosis. A: Methanol-insoluble lipids of *M. smegmatis* were separated by ion-exchange chromatography and eluted with a stepwise gradient of 0.1 M ammonium acetate in 33% methanol in chloroform [fractions 9 (F9) and F10], 0.2 M ammonium acetate in 33% methanol in chloroform (F11 to F13), and 0.2 M ammonium acetate in methanol (F14). The various PIM species and their relative abundances were determined by matrix-assisted laser-desorption/ionization time of flight mass spectrometry in the negative mode. PI was present in each fraction. PIM₂ and the triacylated form of PIM₂ (Ac₁PIM₂) were enriched in fractions 9–11 and to a lesser extent in fractions 11–14. PIM₆ and the triacylated form of PIM₆ (Ac₁PIM₆) appeared in fractions 12–14. B: Macrophages derived from monocytes (MDM) were preincubated with fractions 9–14 used at 10 µg lipid/ml for 15 min and then challenged with FITC-stained *M. smegmatis* for 45 min at 37°C (25 particles per cell). Phagocytosis was determined as the number of macrophages having engulfed at least one particle. Phagocytosis inhibition is expressed as the percentage effect compared with the control. Results are means ± SEM of three independent experiments performed in duplicate. Significance of the treatment effect was assessed by paired Student's *t*-test. * *P* < 0.05, ** *P* < 0.01 compared with untreated conditions.

pathogenic species (Fig. 4B) comparable to what was observed with nanomolar concentrations of *M. smegmatis* (Fig. 4A). Nevertheless, these observations clearly demonstrated that subfamilies of PIM are involved in the nonopsonic phagocytic process of mycobacteria.

Recognition of PIM and GPL by nonopsonic phagocytic receptors

Recently, we reported that subfamilies of another surface-exposed glycolipid of *M. smegmatis*, GPL (Fig.1), in-

hibit the phagocytosis of mycobacteria (20). We have also demonstrated that the nonopsonic phagocytosis of both pathogenic and nonpathogenic mycobacteria by human phagocytes is mediated at least in part by two major receptors, CR3 and MR (11, 29). Therefore, the ability of PIM and GPL to inhibit mycobacteria internalization by blocking their entry through these receptors was suspected and investigated. To this aim, latex beads were coated with purified PIM or GPL using the method previously described by Schlesinger, Hull, and Kaufman (26). As PIM₂ and

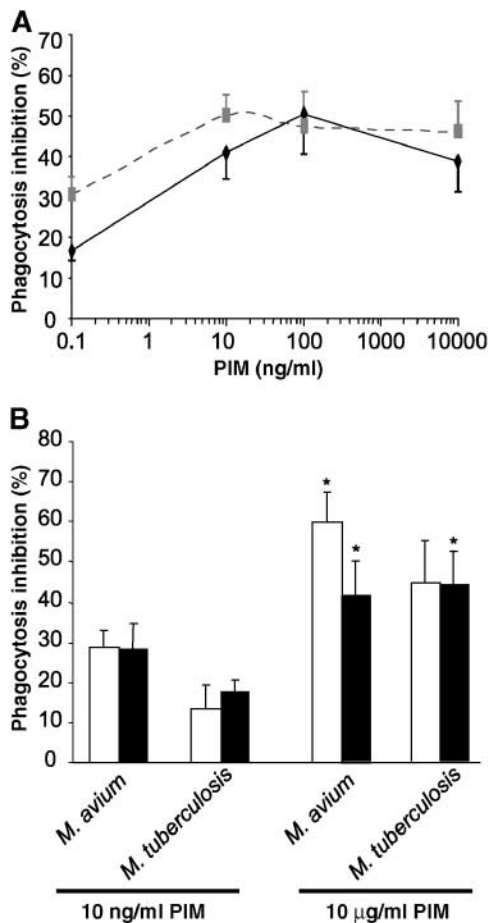


Fig. 4. PIM₂ and PIM₆ inhibit the phagocytosis of pathogenic and nonpathogenic mycobacteria. MDM were preincubated for 15 min at 37°C with or without purified PIM₂ (black diamonds and open bars) and PIM₆ (gray squares and closed bars) from *M. bovis* BCG at concentrations ranging from 100 pg lipid/ml to 10 µg lipid/ml and then challenged with FITC-*M. smegmatis* (25 particles/cell) for 45 min at 37°C (A) or from 10 ng lipid/ml to 10 µg lipid/ml and then put in contact with *M. avium* and *M. tuberculosis* (25 particles per cell) for 45 min at 37°C (B). Inhibition is expressed as the percentage effect compared with the control. Results are means ± SEM of three to four independent experiments in duplicate. Statistical differences were determined for cells treated with 10 µg lipid/ml compared with 10 ng lipid/ml by paired Student's *t*-test. * *P* < 0.05.

PIM₆ displayed comparable activities on the phagocytosis of mycobacteria, PIM₂, the quantitatively major constituent of PIM, was isolated from *M. bovis* BCG and used for the subsequent experiment as representative of the PIM family. Beads were also coated with either GPL III or GPL I from *M. smegmatis*, two classes of GPL that have been shown to exhibit different inhibitory activities on MDM (20).

The capacity of the coated beads to enter macrophages through MR or CR3 was then investigated using a competition approach with blocking antibodies directed against receptors or soluble ligands. Monoclonal antibody 2LPM was selected for its capacity to prevent both opsonic and nonopsonic internalization through CR3 (19, 30, 31). Serum-opsonized and nonopsonized zymosan were used as positive CR3 binding particles (19) and BSA-latex beads as a negative control. We did not observe any difference be-

tween the internalization of PIM₂- or GPL- and BSA-coated beads (data not shown). As expected, pretreatment of MDM with 2LPM (5 µg/ml) for 30 min at 37°C significantly decreased the internalization of serum-opsonized and nonopsonized zymosan but did not affect the phagocytosis of BSA-coated beads (Fig. 5A). Such pretreatment also reduced by approximately one-third the entry of both GPL III- and PIM₂-coated beads but not that of GPL I (Fig. 5A), indicating that entry of PIM₂- and GPL III-coated beads, but not that of beads coated with GPL I, involves CR3.

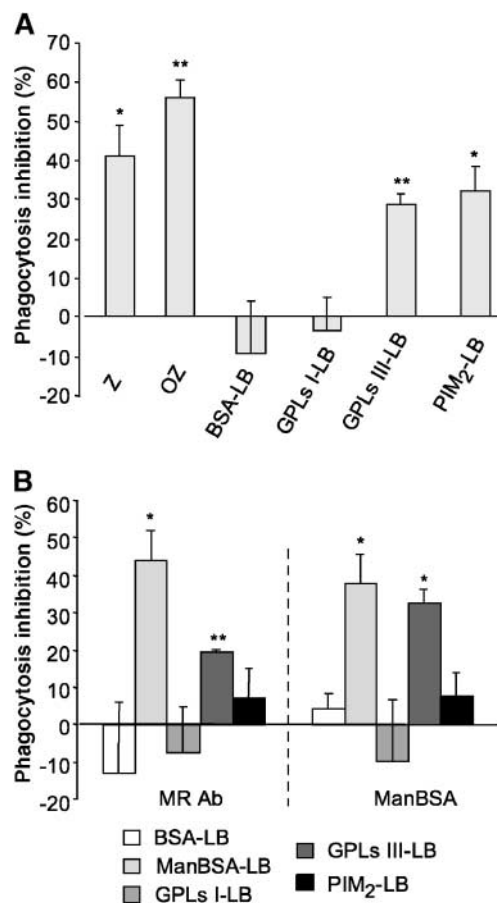


Fig. 5. Interaction of PIM₂ and GPL III with complement receptor 3 (CR3) and mannose receptor (MR). A: MDM were incubated for 15 min at 37°C with or without 5 µg/ml 2LPM Ab directed against CR3 and then allowed to ingest either serum-opsonized zymosan (OZ; 10 particles per cell) for 15 min or zymosan (Z; 25 particles per cell) and latex beads (LB; 50 particles per cell) coated with BSA, GPL I, GPL III, PIM₂, or mannosylated BSA (Man-BSA) for 45 min. B: MDM were pretreated at 37°C with or without anti-MR antiserum (1:50) for 30 min or 300 µg/ml Man-BSA for 15 min and then challenged with BSA-, GPL I-, GPL III-, PIM₂-, or Man-BSA-coated beads (50 particles per cell) for 45 min. Intracellular zymosan, opsonized zymosan, and coated latex beads were visualized by immunofluorescence after staining of the phagosomal membrane. Data are expressed as percentage of phagocytosis inhibition compared with untreated macrophages. Inhibition is expressed as the percentage effect compared with the control. Results are means ± SEM of three to four independent experiments in duplicate. Significance of the treatment effect was assessed by paired Student's *t*-test. * *P* < 0.05, ** *P* < 0.01 compared with untreated conditions.

The entry of beads through the MR was also evaluated using a competitive ligand, Man-BSA (11), or a goat anti-human MR antiserum selected for its capacity to block MR-dependent functions (32, 33). Man-BSA- and BSA-coated latex beads were used as positive and negative controls of MR-dependent phagocytosis, respectively (11). In this set of experiments, GPL III-coated beads were internalized as efficiently as beads coated with Man-BSA ($45 \pm 5\%$ vs. $33 \pm 7\%$; $n = 3$), whereas PIM₂-coated beads were slightly better internalized ($58 \pm 1\%$ vs. $33 \pm 7\%$; $n = 3$). As expected, pretreatment of MDM with anti-MR antiserum (1:50) or 300 $\mu\text{g}/\text{ml}$ Man-BSA for 30 min at 37°C dramatically reduced the internalization of Man-BSA-coated beads (Fig. 5B). Such pretreatments did not affect the phagocytosis of either GPL I- or PIM₂-coated beads. In contrast, both treatments slightly but significantly decreased the phagocytosis of GPL III-coated beads (Fig. 5B), indicating that only GPL III-coated beads enter MDM through the MR. Taken together, these data demonstrate that both PIM₂ and GPL III participate to the internalization of mycobacteria and thereby are potential ligands of phagocytic receptors expressed at the surface of MDM.

DISCUSSION

The outermost cell envelope layer, also called a capsule in the case of pathogenic mycobacteria, represents a privileged interface between mycobacteria and host cells. Some of its components, such as glucan (34) and GPL (20), have been implicated in the interaction with macrophages. Other constituents of the mycobacterial cell envelope have also been shown to induce various host cell responses (35), but their localization at the cell surface is still a matter of debate. We show here that the outermost layer of *M. smegmatis* contained PIMs that also participate in the nonopsonic phagocytosis of mycobacteria. The PIMs, described by Ballou and colleagues (36) in the 1960s, are based on PI and are formed of one to six mannosyl residues attached to the inositol moiety of the phospholipid. The PIMs usually occur as a mixture of compounds differing from one another by the number of mannosyl residues and fatty acyl groups. The nonpathogenic strain *M. smegmatis* ATCC 607 has been described to contain mainly two PIM subfamilies, PIM₂ and PIM₆ (24, 27), in agreement with the composition of the crude PIM fractions reported herein.


Although other forms of PIM have been reported in the literature (24), they were undetectable under our experimental conditions, probably because they occur in too small quantities. Therefore, the crude PIM-dependent inhibition of mycobacterial phagocytosis was essentially attributable to PIM₂ and PIM₆. This was further confirmed using enriched PIM₂ and PIM₆ fractions isolated from *M. bovis* BCG. Nanomolar concentrations of PIM decreased the internalization of *M. smegmatis* by MDM, whereas much higher concentrations of lipids were required to affect *M. avium* or *M. tuberculosis* phagocytosis to the same extent. PIMs are found on the bacterial surface of both patho-

genic and nonpathogenic mycobacteria (27, 37). However, there are strain differences in the surface exposure of PIMs that are inversely correlated with the abundant expression of capsular polysaccharides composed of α -glucan that can partially mask capsular PIM and directly act on phagocytic receptor, such as CR3 (30). If we assume that PIMs, exposed at the surface of *M. smegmatis*, have a lower affinity for the phagocytic receptors than the outermost polysaccharides of pathogenic species (38), this may explain why different PIM concentrations are required to inhibit to a comparable extent the phagocytosis of *M. smegmatis* and *M. avium* or *M. tuberculosis*.

It has to be noted that the occurrence of a carbohydrate portion on phospholipid is critical for the inhibitory activity, because PI displayed no effect but PIM₂ did. However, surprisingly, the length of the oligomannosyl chain does not appear to be important for the activity, because PIM₂ and PIM₆ equally inhibited phagocytosis. Previous studies have reported that soya PI and, to a lesser extent, mycobacterial PI inhibited the nonopsonic binding of *M. tuberculosis* to murine macrophages (39, 40). This is in apparent conflict with the data presented here. Such a discrepancy could be attributed to interspecies differences in the PI mode of action between murine and human macrophages. Our data, together with our previous report (20), collectively indicate that surface-exposed PIM and GPL participate in the internalization of mycobacteria by macrophages and, as such, are potential ligands of phagocytic receptors. It is possible, however, that other constituents of the mycobacterial envelope, such as glucan and unidentified molecules, could also be involved in the interactions of bacilli with macrophages, as illustrated by the variety of routes of entry of mycobacteria into host cells.

The molecular mechanisms by which surface-exposed GPLs and PIMs participate in the phagocytosis of mycobacteria deserve consideration. PIMs have been proposed to participate in the receptor-mediated uptake of mycobacteria (26, 41). For instance, they have been shown to act as an adhesin, allowing the binding of *M. tuberculosis* and *M. smegmatis* to hamster ovary fibroblasts, either directly or after opsonization with mannose binding protein (41). Here, we demonstrate that coating latex beads with PIM₂ favors their internalization through a CR3-dependent process, probably through the lectin binding site of CR3, which might recognize the mannosyl moiety of PIM, as mentioned above. Like other mannosylated conjugates, PIMs have been suspected to be ligands of the MR. We addressed this question and show, for the first time, that PIMs, at least PIM₂, do not in fact interact with this receptor in our cell model. As mentioned above, the length of the oligomannosyl chain was not critical for PIM inhibition of phagocytosis, suggesting that PIM₆ was not a partner of MR. The mode of action of GPL as a putative ligand of macrophages has been poorly investigated. We found that GPL III ensures the entry of particles through both CR3 and MR. Although all types of GPLs share the same peptidolipid core, GPL I, which is inactive, lacks the succinyl substituent that occurs in GPL III and is 3,4-*O*-acetylated on the 6-deoxytalosyl moiety (20). At present, these

moieties have not been proposed among those described to be recognized by MR and CR3, and their putative binding to these receptors need to be further investigated. Nonetheless, we propose that surface-exposed PIMs and GPLs coated on latex beads are potential ligands of CR3 and/or MR, thereby mimicking part of the entry process of mycobacteria in macrophages. When used as soluble molecules, PIMs (present study) as well as GPLs (20) affect the internalization of mycobacteria but not that of zymosan, whereas the two processes are well known to involve both MR and CR3. Concerning CR3, the fact that zymosan and mycobacteria use distinct nonopsonic binding sites of the receptor (19) suggests that the site involved in zymosan internalization does not recognize PIMs and GPLs.

In conclusion, we propose that surface-exposed GPLs and PIMs interact with the nonopsonic receptors CR3 and/or MR involved in the phagocytosis of mycobacteria. Characterization of their binding properties is beyond the scope of this report, but it would clearly help to further investigate their signaling pathways. Understanding the mycobacterial strategy to infect macrophages without triggering bactericidal activity is still a challenge. Finally, PIMs and GPLs, as inhibitors of mycobacteria phagocytosis, may also help to design new pharmacological drugs for the control of macrophage infection. 

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