Fine Discrimination in the Recognition of Individual Species of Phosphatidyl-\textit{myo}\textit{-inositol Mannosides from} \textit{Mycobacterium tuberculosis} \textit{by C-Type Lectin Pattern Recognition Receptors}

Jordi B. Torrelles, Abul K. Azad and Larry S. Schlesinger

\textit{J Immunol} 2006; 177:1805-1816; doi: 10.4049/jimmunol.177.3.1805
http://www.jimmunol.org/content/177/3/1805

\textbf{References} This article \textbf{cites 50 articles}, 37 of which you can access for free at: http://www.jimmunol.org/content/177/3/1805.full#ref-list-1

\textbf{Subscription} Information about subscribing to \textit{The Journal of Immunology} is online at: http://jimmunol.org/subscription

\textbf{Permissions} Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

\textbf{Email Alerts} Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

\textit{The Journal of Immunology} is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2006 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Fine Discrimination in the Recognition of Individual Species of
Phosphatidyl-myoo-Inositol Mannosides from Mycobacterium
tuberculosis by C-Type Lectin Pattern Recognition Receptors

Jordi B. Torrelles, Abul K. Azad, and Larry S. Schlesinger

The Mycobacterium tuberculosis (M.tb) envelope is highly mannosylated with phosphatidyl-myoo-inositol mannosides (PIMs), lipomannan, and mannosyl-capped lipoarabinomannan (ManLAM). Little is known regarding the interaction between specific PIM types and cell and host C-type lectin pattern recognition receptors. The macrophage mannose receptor (MR) and dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells engage ManLAM mannose caps and regulate several host responses. In this study, we analyzed the association of purified PIM families (f, separated by carbohydrate number) and individual PIM species (further separated by fatty acid number) from M.tb H37Rv, with human monocyte-derived macrophages (MDMs) and lectin-expressing cell lines using an established bead model. Higher-order PIMs preferentially associated with the MR as demonstrated by their reduced association with MDMs upon MR blockade and increased binding to COS-1-MR. In contrast, the lower-order PIM2f associated poorly with MDMs and did not bind to COS-1-MR. Triacylated PIM species were recognized by MDM lectins better than tetra-acylated species and the degree of acylation influenced higher-order PIM association with the MR. Moreover, only higher-order PIMs that bind the MR showed a significant increase in phagosome-lysosome fusion upon MR blockade. In contrast with the MR, the PIM2f and lipomannan were recognized by DC-SIGN comparable to higher-order PIMs and ManLAM, and the association was independent of their degree of acylation. Thus, recognition of M.tb PIMs by host cell C-type lectins is dependent on both the nature of the terminal carbohydrates and degree of acylation. Subtle structural differences among the PIMs impact host cell recognition and response and are predicted to influence the intracellular fate of M.tb. The Journal of Immunology, 2006, 177: 1805–1816.

Mycobacterium tuberculosis (M.tb) is an intracellular pathogen that survives within the macrophage. It has developed multiple strategies to enhance its entry into human mononuclear phagocytes by interaction of its surface-exposed cell wall components with specific host pattern recognition receptors (PRRs) and through these interactions, modulates diverse biological functions (reviewed in Refs. 1 and 2). The complex outermost components of the mycobacterial cell wall, comprised predominantly of lipids and carbohydrates, are the first to contact host cellular constituents, and therefore play a major role in facilitating host cell entry and modulating the host cell response (2, 3). The M.tb cell wall is dominated by a group of biosynthetically related mannosylated lipoglycoconjugates (3, 4) including phosphatidylinositol mannosides (PIMs) and their hypermannosylated derivatives, lipomannan (LM) and the mannosyl-capped lipoarabinomannan (ManLAM) (Fig. 1) (5, 6). They are thought to be both incorporated into the M.tb membrane and also exposed on its cell surface (7–9). In this regard, M.tb ManLAM with its well-defined structure comprised of a carbohydrate core (i.e., β-mannan and α-arabinan), a mannosyl-phosphatidyl-myoo-inositol anchor and various terminal mannosyl-capping motifs (Fig. 1), has been shown to bind to the macrophage mannose receptor (MR) mediating phagocytosis of M.tb by human macrophages (10). ManLAMs from different M.tb strains vary in the degree to which they bind to the MR pointing to a potential relationship between the length and/or presentation of the mannose caps and their affinity for the MR (11). Importantly, our recent studies showed that engagement of the MR by M.tb ManLAM during the phagocytic process directs M.tb to its initial phagosomal niche (12). ManLAM caps also bind to dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) on dendritic cells (13, 14). Thus, terminal components of ManLAM are very important in host cell recognition. The role of ManLAM in MR- and DC-SIGN-dependent cytokine and Ca2+ responses has also been reported (14–16).

The PIMs are the major phospholipid components of the mycobacterial cell wall. They are a heterogeneous mixture of families (Fig. 1), which differ by the number of mannosyl residues in their structure (PIMx,y, x = number of mannoses). Each family is composed of several species that differ in their fatty acid content (AcyPIMx,y, y = number of fatty acids where 0, 1, or 2 denotes di-, tri-, or tetra-acylated species, respectively). As shown in Fig. 1, PIM structures consist of a phosphatidylinositol anchor with a mannosyl unit attached to position C2 of the myo-inositol. Additional substitutions at the C6 position of the myo-inositol by one, two, or three mannosyl units define the PIMxf, PIMxf, and PIMxf, respectively. The terminal mannose of the PIMxf may be further substituted at position C2 by one or two mannosyl units leading to...
the formation of PIM5f or PIM6f, respectively. The latter two families are the highest ones present in M.tb (Fig. 1) and their nonreducing termini resemble the mono- and dimannosyl cap present in ManLAM, respectively, raising the possibility that along with ManLAM, PIMs participate in the phagocytic process. Multiacylated forms (di- to tetra-acylated) of PIMs exist in M.tb. Diacylated forms have been reported to have both fatty acids located in their ns-glycerol (denoted PIMx), triacylated forms have an additional fatty acid at the C6 position of their 2-mannosyl unit attached to the myo-inositol (Ac1PIMx), and tetra-acylated forms have the fourth fatty acid located in the C3 position of their myo-inositol (denoted Ac2PIMx) (17) (Fig. 1).

Mixtures of PIMs have been found to regulate cytokine, oxidant, and T cell responses (18–20), and to stimulate early endosomal fusion (21). In all of these studies, the nature of the PIMs used was not clearly defined particularly at the level of acylation. There are very few studies regarding the immunologic responses of macrophages to individual families of highly purified PIMs (22–24) or to purified species, i.e., PIMs separated by the degree of acylation (19, 22). In all these studies, PIMs were used free in solution with murine bone marrow-derived macrophages. In this study, we evaluate the association of highly purified M.tb PIM families and species with the human macrophage MR. In addition, by comparing the PIM association pattern between the MR and DC-SIGN using mammalian cell lines expressing each of these C-type lectins, we establish the importance of PIM composition in host cell recognition by these lectins that play a role in tuberculosis pathogenesis. Finally, we determine the degree to which the MR is involved in phagosome-lysosome (P-L) fusion events for phagosomes containing PIM-coated beads. These data provide evidence that higher-order PIMs with mannose cap-like structures participate along with ManLAM in the phagocytic and trafficking process mediated by the macrophage MR.

Materials and Methods

Bacteria, media, and Abs

M.tb H37Rv, M.tb H37Ra, and M.tb Erdman were grown on Middlebrook 7H11 (Difco) with 5% oleic acid, albumin, dextrose, and catalase enrichment for 14 days at 37°C. Mycobacterium smegmatis mc²155 was grown under identical conditions described above for M.tb strains but for 5 days. RPMI 1640 medium with L-glutamine was purchased from Invitrogen Life Technologies. RPMI 1640 medium was used alone or with 20 mM HEPES
(Difco) and 1 mg/ml human serum albumin (HSA; ZLB Bioplasma; pH 7.2). Anti-MR mAb (CD206, clone no. 19.2) used for immunofluorescence studies was purchased from BD Pharmingen. For receptor blocking, CD206 (clone no. 15-2) mAb was purchased from Serotec.

Purification of M.tb PIMs, LM, and ManLAM

For experiments with monocyte-derived macrophages (MDMs) and COS-1 cells, M.tb H37Rv cells were harvested and then delipidated at 37°C for 12 h using CHCl3:CH3OH (2:1, v/v) followed by CHCl3:CH3OH:H2O (10:10:3, v/v/v) for an additional 12 h. Total lipid extracts were further precipitated in cold acetone for 24 h at −20°C, resulting in a pellet that contained a mixture of phospholipids. PIMs were separated by silica column chromatography using a gradient from 100% chloroform to 100% methanol and elution by 60% methanol fraction. PIM families and species were further purified by preparative TLC and purified components were visualized as described below for one-dimensional TLC. A mixture of PIMs, PIM families (separated by their mannose content) and PIM species (separated by their mannose content and the degree of acylation) were used to coat 1 or 6 μm in diameter beads (Polysciences) to determine their association with MDMs, COS-1-MR, and COS-1-DC-SIGN.

ManLAM and LM from M.tb H37Rv were extracted and purified from the delipidated cells after PIM extraction as previously described (25). Briefly, the dried biomass was suspended in breaking buffer containing protease inhibitor mixture (pepsatin A, PMSF, leupeptin), DNase, and RNase in PBS and disrupted mechanically using a bead beater. Triton X-114 (Sigma-Aldrich) was added to the lysed cells to a final concentration of 8% (v/v), and after cooling on ice, the solution was mixed at 4°C overnight. The cell wall was removed by centrifugation at 27,000 × g for 1 h at 4°C, and the supernatant was incubated at 37°C to induce biphasic separation (26). The upper aqueous layer was mixed with the cellular debris and re-extracted as described above. The detergent layers were combined, and the lipoglycans were precipitated by the addition of 9 volumes of cold ethanol (98%, −20°C). The precipitate was collected and treated with proteinase K for 2 h at 60°C. The solution containing ManLAM and LM was dialyzed and lyophilized. For purification of ManLAM, HPLC was performed on a Beckman liquid chromatography system fitted with a Sephacryl S-200 HiPrep 16/60 column in tandem with a HiPrep 16/60 Sephacryl S-100 column (Amersham Biosciences) equilibrated with 0.2 M NaCl, 0.25% deoxycholate, 0.1 mM EDTA, 0.02% sodium azide, and 10 mM Tris (pH 8.0) at a flow rate of 1 ml/min. SDS-PAGE and periodic acid-silver staining (27) were used to monitor the elution profile of the fractions containing ManLAM and LM, which were then pooled and dialyzed at 37°C without detergent followed by 1 M NaCl and water for several purifications. ManLAM and LM fractions recovered were reanalyzed by SDS-PAGE, sugar analysis and [3H]nuclear magnetic resonance to confirm purity. The purified detailed analysis of the two-dimensional TLC performed were done using endotoxin-free water (Hospira). Endotoxin levels were calculated for all Ags in this study and was <18 pg/sample.

One- and two-dimensional TLC analyses of total lipids from different mycobacterial strains

For analysis of mannose-containing lipids located in the cell wall of different mycobacterial strains, M.tb strains and M. smegmatis were harvested from Middlebrook 7H11 plates and lysed. Protein quantification was performed by the bicinchoninic acid method following the manufacturer’s instructions (Bio-Rad). Lysates totaling 100 mg of protein from each strain were delipidated at 37°C for 12 h using CHCl3:CH3OH (2:1, v/v) followed by CHCl3:CH3OH:H2O (10:10:3, v/v/v) for an additional 12 h. Lipids were analyzed by one-dimensional TLC. For total lipids and phospholipids, respectively, and heated at 110°C until lipid bands appeared. For two-dimensional TLC analysis, total crude lipids from each strain were loaded based on equal amounts of protein content (100 μg) from the bacterial lysates and run in the first dimension using CHCl3:CH3OH:H2O (60:30:6, v/v/v) as a solvent system. The TLC plate was then rotated 90° to the left and run in the second dimension using CHCl3:CH3OH:CH2O:H2O (40:25:3:6, v/v/v/v) as a solvent system. TLC plates were developed as described above. Spots were identified as PIMs when positively reacted for Dittmer reagent (specific for phospholipids) and α-naphthol (specific for glycolipids).

Sample preparation and MALDI-TOF mass spectrometry

Analyses by MALDI-TOF were conducted on a Bruker Daltonic Reflex III (Bruker Daltonic) mass spectrometer using DE-reflectron mode. Ionization was effected by irradiation with pulsed UV light (337 nm) from a Nd:YAG laser. PIMs and PIM species were analyzed by the instrument operating at 22.5 kV in the positive ion mode using an extraction delay time set at 200 ns. Typically, spectra from 100 to 250 laser shots were summed to obtain the final spectrum. Saturated α-cyano-4-hydroxycinnamic acid (Bruker Daltonik) in 50% acetonitrile/0.1% aqueous trifluoroacetic acid (50:50) was used for the matrix. Typically, 1 μl of PIM sample (mixture, families or species) (10 μg) in a CHCl3:CH3OH:H2O (10:10:3, v/v/v) solution and 5 μl of the matrix solution were deposited on the stainless steel target, mixed with a micropipet, and allowed to air-dry, forming a cocrystalline sample/matrix complex. The measurements were externally calibrated at two points with PIMs.

Monocyte-derived macrophages

PBMC were isolated from healthy donors (using an approved Institutional Review Board protocol for the human subjects at the Ohio State University) and day 5 MDMs were cultivated as previously described (29). Monolayers on glass coverslips in 24-well tissue culture plates contained ~2 × 105 MDMs.

MR and DC-SIGN-transfected COS-1 cell lines

COS-1-transfected cells in this study were obtained as previously described (12). Briefly, full-length MR cDNA, derived from MDM mRNA, was cloned into the mammalian expression vector, pcDNA3.1/V5-His-TOPO (Invitrogen Life Technologies). This expression construct was transfected into COS-1 cells. Stable clones of COS-1 expressing the MR were selected by limiting dilution and expanded. An MR-positive clone was further sorted by flow cytometry (designated COS-1-MR) using a FITC-conjugated MR mAb (BD Pharmingen). Full-length DC-SIGN cDNA was PCR amplified from Human Placenta QUICK-Clone cDNA mix (BD Biosciences/BD Clontech) and cloned into the mammalian expression vector pSeCtag2 (Invitrogen Life Technologies). The vector was transfected into COS-1 cells and cell surface expression of DC-SIGN was confirmed by flow cytometry using an anti-DC-SIGN mAb. Further functional activity of the MR and DC-SIGN was assessed by flow cytometry using FITC-labeled fucose-BSA and zymosan in the absence or presence of mannan to measure mannan-inhibitable pinocytosis and phagocytosis, respectively.

Preparation of HSA- and lipoglycoconjugate-coated polystyrene beads

Beads coated with HSA (sham control), ManLAM, LM, mixture of PIMs, PIM families and PIM species were prepared as previously described (10) using Polybead polystyrene beads (1 or 6 μm). Briefly, 1.5 × 109 Polybead polystyrene beads were washed twice in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and then incubated with 50 μg of various purified M. H37Rv, lipoglycoconjugates or buffer alone for 1 h at 37°C. Beads were then blocked with 5% HSA, washed repeatedly in 0.5% HSA, and finally adjusted to 4.0 × 108/ml in 0.5% HSA before being used in the MDM or COS-1 cell association assays.

Association assay using MDMs and COS-1 cells

Before incubation with mannosylated lipoglycoconjugate-coated beads, MDM monolayers were washed and repleted with RPMI 1640 + 10% M. H37Rv, 1 mg/ml HSA (UnizLB Bioplasma). M.tb H37Rv lipoglycoconjugate-coated beads were added to cells at a ratio of 100:1 and monolayers were incubated for 3 h with shaking at 100 rpm as previously described (10). In some cases, MDMs were preincubated with mannan (Sigma-Aldrich) at 2.5 mg/ml for 30 min at 37°C or with anti-MR mAb (Serotec) at 10 μg/ml for 20 min at 37°C to block the activity of the MR. These conditions have been shown to effectively inhibit binding to the MR in the assay (12, 30). MDMs were then incubated in 10% formalin for 15 min and then washed three times in Dulbecco’s PBS before coverslips being mounted on glass slides. The average number of beads per cell was determined by counting 200–300 cells per coverslip using a ×100 oil-immersion objective with a wide bandwidth 570 nm dichroic mirror on a BX51 Olympus fluorescence microscope. The mean ± SEM was determined for triplicate coverslips in each test group. For each experiment, the mean ratio was tested for a significant difference from 1 using T statistics. The number of experiments cited were independent experiments performed using MDMs derived from different donors.
For COS-1 cell association assays, freshly grown COS-1-wild-type (WT), COS-1-MR, or COS-1-DC-SIGN cells were seeded onto coverslips (1 x 10^5 cells/well, triplicate wells/test group) in wells of a 24-well tissue culture plate and incubated overnight to form monolayers. Cell monolayers were washed and repleted with PBS-HHG medium (PBS + 10 mM HEPES + 1 mg/ml HSA + 0.1% glucose) to which HSA- or lipoglycoconjugate-beads were added (ratios of 50–100:1) in the presence of absence of mannan (Sigma-Aldrich) as described for the MDM assays. The cells were incubated at 37°C for 3 h without shaking. After washing, the cells were fixed with 2% paraformaldehyde for 10 min followed by several washes using Dulbecco’s PBS before the coverslips being mounted on glass slides. Bead association analysis was performed as described for the MDM assays.

Quantitative P-L fusion assay in MDMs using confocal microscopy

MDM monolayers (2 x 10^5 cells/well) on coverslips were incubated with isotype control IgG1 or anti-MR mAb at 37°C for 20 min as described for the binding experiments. Then, M.tb H37Rv lipoglycoconjugate-coated green fluorescent beads (4 x 10^7/well) were added to the monolayers at 37°C for 2 h (12). Cell monolayers on coverslips were washed with PBS buffer, fixed with 2% paraformaldehyde, and permeabilized with 100% methanol for 5 min at room temperature. MDMs were blocked overnight at 4°C in blocking buffer (Dulbecco’s PBS + 5 mg/ml BSA + 10% heat-inactivated FBS), stained with mAb against late endosomal/lysosomal marker CD63 (0.5 g/ml final concentration), and next stained with Alexa Fluor 647-conjugated goat anti-mouse IgG secondary Ab (4 g/ml final concentration). After extensive washing in blocking buffer, the coverslips were dried, mounted on glass slides, and examined by confocal microscopy (Zeiss LSM 510).

The percentage of bead phagosomes that colocalized with the lysosomal marker was quantified by counting over 1000 consecutive bead phagosomes in each test group in ≥2 independent experiments using a minimum of two different donors.

Statistical and three-dimensional prediction analyses

Statistical analyses were performed using GraphPad Prism version 4.0 (www.graphpad.com). Three dimensional predictions of PIM structures depending on the number and nature of their fatty acids were performed by using ACD/ChemSketch version 8.0 (Advanced Chemistry Development, www.acdlabs.com).

Results

Purification of ManLAM, LM, and PIM families and species from virulent M.tb H37Rv

To assess the association profile of individual PIM types with the human macrophage MR, we purified PIMs based on their mannose content as well as degree of acylation. M.tb H37Rv cells were harvested and delipidated to obtain total lipids. An enriched phospholipid fraction containing mainly PIMs was obtained after total lipid precipitation in cold acetone (17). To separate PIMs from other mycobacterial phospholipids further purification was necessary by silica column chromatography using a chloroform/methanol gradient. A mixture of PIM families was obtained in the 60% methanol in chloroform fraction. PIM families and species were finally purified by TLC, visualized by one-dimensional TLC (Fig. 2A) and identified by MALDI-TOF for each individual PIM species purified (Fig. 2C). The molecular ions observed for Ac1PIM2 (m/z 1475.9), Ac2PIM2 (m/z 1756.2), Ac1PIM5 (m/z 1985.9), Ac2PIM5 (m/z 2154.4), Ac1PIM6 (m/z 2108.2), and Ac2PIM6 (m/z 2404.5) were confirmed by MALDI/mass spectrometry in a positive ion mode.

FIGURE 2. Purification of PIMs, LM, and ManLAM from M.tb. A, TLC showing the indicated purified PIM species and (B) a 15% SDS-PAGE showing the purified ManLAM and LM used in this study. C, Identification of each PIM species and its fatty acid composition by MALDI/mass spectrometry in a positive ion mode.

<table>
<thead>
<tr>
<th>PIM species</th>
<th>m/z [M-H+2Na]^*</th>
<th>Fatty acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac1PIM2</td>
<td>1475.9</td>
<td>2 x palmitic acids, TBST^*</td>
</tr>
<tr>
<td>Ac2PIM2</td>
<td>1756.2</td>
<td>2 x palmitic acids, oleic acid, TBST</td>
</tr>
<tr>
<td>Ac1PIM5</td>
<td>1985.9</td>
<td>2 x palmitic acids, TBST</td>
</tr>
<tr>
<td>Ac2PIM5</td>
<td>2154.4</td>
<td>2 x palmitic acids, oleic acid, TBST</td>
</tr>
<tr>
<td>Ac1PIM6</td>
<td>2108.2</td>
<td>2 x palmitic acids, TBST</td>
</tr>
<tr>
<td>Ac2PIM6</td>
<td>2404.5</td>
<td>2 x palmitic acids, oleic acid, TBST</td>
</tr>
</tbody>
</table>

^* TBST, tuberculostearic acid
were attributed to their sodiated forms \([M-H+2Na]^-\) with an additional sodium atom, presumably on the phosphate moiety. Other mannosylated components of the \(M.tb\) \(H_37R_v\) cell wall, ManLAM and LM, were extracted and purified from the delipidated cells as previously described (25) (Fig. 2B).

**Recognition of different PIM families by the MR on human macrophages**

To establish the association profile of PIM families with the macrophage MR, we used our established bead model (10). MDM monolayers \((2 \times 10^5)\) on glass cover slips were incubated with \(2 \times 10^7\) beads coated with a mixture of PIMs or individual PIM families (PIM, PIM, and PIM) as well as ManLAM (positive control) and LM or HSA (negative controls (10)). The total number of beads associated with the MDMs was enumerated by microscopy (Fig. 3, A and B) and MR-dependent association was defined as the percent reduction in the presence of mannan (2.5 mg/ml), a soluble carbohydrate inhibitor of MR activity on macrophages (30) (Fig. 3C). As in previous studies, ManLAM beads bound well to macrophages \((p < 0.01)\) in a highly mannan-inhibitable manner (63% inhibition, \(p < 0.0005)\). In contrast, LM bead association was low and not mannan-inhibitable when compared with HSA beads, as previously reported (10). The magnitude of association of PIM-coated beads with MDMs was comparable to that observed with ManLAM beads \((p < 0.05)\), followed in magnitude by beads coated with a mixture of PIM families (PIMs) and PIM. The association of PIM-coated beads was poor, pointing toward the importance of the nonreducing termini of the PIM and PIM for association with MDMs.

Mannan inhibition indicative of MR involvement was greatest for the PIM-coated beads (61.3% inhibition, \(p < 0.0005)\) followed by the mixture of PIMs (43.2% inhibition, \(p < 0.005)\) and PIM (42.4% inhibition, \(p < 0.05)\) indicating that in addition to ManLAM, higher-order PIMs preferentially associate with macrophages.

**FIGURE 3.** Mannan-inhibitable association of PIM beads with MDMs. MDM monolayers \((2 \times 10^5)\) on glass coverslips were incubated with \(2 \times 10^7\) PIM-, LM-, ManLAM-, or HSA (control)-coated beads. The total number of beads associated with the cells was enumerated by microscopy. Specific association of coated beads was measured in the presence of mannan \((2.5\, mg/ml)\), a soluble carbohydrate inhibitor of MR activity on macrophages. A, Representative phase-contrast microscopy images of MDM monolayers after incubation with control HSA- or \(M.tb\) lipoglycoconjugate-coated beads in the presence or absence of mannan. Beads associated with MDM monolayers (arrows) were visualized at \(\times 1000\) as round refractive objects. B, Association of PIM with MDMs is shown as fold increase relative to control HSA beads (Student \(t\) test; *, \(p < 0.05)\), **, \(p < 0.01\). C, Association of PIM with the MDM MR as indicated by the percent mannan inhibition (Student \(t\) test; *, \(p < 0.05)\), **, \(p < 0.005)\). The data shown are from four separate experiments (mean \(\pm\) SEM). PIMs: mixture of PIM, PIM, and PIM; PIM: mixture of Ac, Ac, and Ac; PIM: mixture of Ac, Ac, and Ac.
the MR on macrophages (Fig. 3C). In this regard, the nonreducing termini of the PIM5f and PIM6f resemble the mono- and dimannoside cap of ManLAM, respectively. The low level of PIM5f association was also mannan-inhibitable but to a lesser degree (12.6% inhibition) suggesting that a small proportion of PIM5f association with the macrophage surface is C-type lectin dependent.

To complement the results we obtained with mannan, we further evaluated involvement of the macrophage MR in PIM association using an anti-MR mAb (12). MDMs were preincubated with anti-MR mAb or IgG1 isotype control before incubation with lipoglycoconjugate-coated beads. Results in Fig. 4A show a representative experiment in the absence (■) or presence (●) of anti-MR mAb and Fig. 4, B and C, show combined data from three independent experiments. In this set of experiments, bead association with MDM monolayers was greatest when beads were coated with ManLAM (p < 0.05) followed by a mixture of PIMs and the PIM5f, and this association was markedly inhibited when MDMs were pretreated with MR mAb (Fig. 4C). ManLAM association was inhibited by 76.2% (p < 0.0001) comparable to the level of inhibition observed in the presence of mannan (Fig. 3C). Similarly, the association of beads coated with higher-order PIM families was MR dependent, with a reduction in association between 40 and 45% in the presence of anti-MR mAb (Fig. 4C). In contrast, MR-dependent association of PIM5f beads was low (17.5% inhibition in the presence of anti-MR mAb) (Fig. 4C). Together, these data provide evidence that the higher-order PIM families (PIM5f and PIM6f) along with ManLAM preferentially associate with the macrophage MR, whereas the PIM2f does not.

**The degree of acylation of PIMs impacts their recognition by the macrophage MR**

PIMs vary in the degree and nature of their fatty acids. How this impacts recognition by PRRs such as the MR is unknown. Studies have shown that the type and degree of acylation play a key role in regulating the bioactivity of LPSs from other bacteria (31–35).

We purified individual PIM species (based on their degree of acylation) and assessed their association with macrophages and the MR. Fig. 5A shows a representative experiment for the association assay in the absence (□) or presence (■) of mannan. Data combined from four independent experiments shown in Fig. 5B provide evidence that Ac1PIM6 beads (triacylated) associate with MDMs to a much greater degree than Ac2PIM6 (tetra-acylated) beads (p = 0.001). Similarly, triacylated forms of other PIM species associated with MDMs to a greater degree than their respective tetra-acylated forms (Fig. 5B). For example, higher association was also observed for Ac2PIM5 followed by Ac1PIM6, and Ac2PIM2 association was higher than Ac1PIM2; however, in both of the latter cases, the baseline association with MDMs was poorer as was seen with the PIM2f (Fig. 4B).

Mannan-inhibitable association indicative of the MR involvement (Fig. 5C) was high for Ac1PIM6 (55.3% inhibition, p < 0.001) and Ac2PIM6 (56.7% inhibition, p < 0.05), at the same level as that seen with ManLAM (56.3% inhibition, p < 0.005) followed by Ac2PIM5 (37.8% inhibition, p < 0.005). Ac1PIM6 has three fatty acids (two in the glycerol and the third in the C6 position of the mannose attached to the C2 position of its myo-inositol) and Ac2PIM6 has four fatty acids (the fourth located in the C3 position of its myo-inositol). Our data suggest that the addition of the fourth fatty acid bound to beads alters the conformation of Ac2PIM6 and results in lower association of this lipoglycan with the MR. This fact may account for the variability of the macrophage association seen with the PIM6f which contains a mixture of Ac1PIM6 and Ac2PIM6. This variability is also observed to a lesser degree with the PIM5f (Ac1PIM5 and Ac2PIM5), but not with the PIM2f (Ac1PIM2 and Ac2PIM2) suggesting that in the case of the

**Figure 4.** MR-dependent association of higher-order PIMs with MDMs. A, A representative experiment of MR-mediated association of lipoglycoconjugate beads with MDMs. MDM monolayers in triplicate were preincubated with 10 μg/ml anti-MR mAb (●) or IgG1 isotype control (□) before incubation with lipoglycoconjugate-coated or control HSA beads. Results from three independent experiments (mean ± SEM) show the fold increase (relative to HSA beads) in association of ManLAM and PIM beads with MDM monolayers; and C, MR-dependent association of lipoglycoconjugate beads with MDMs (represented by the percent inhibition in the presence of anti-MR mAb relative to the subtype control mAb). In A, Student t test, *p < 0.005; in B and C, *p < 0.05; ***p < 0.001) when compared with control HSA beads. PIMs: mixture of PIM2f, PIM5f and PIM6f; PIM2f: mixture of Ac1PIM2, Ac2PIM2; PIM5f: mixture of Ac1PIM5, Ac2PIM5; PIM6f: mixture of Ac1PIM6, Ac2PIM6.
PIM₂f, the fatty acids do not play a role in the low association of this family with the MR.

Mannan-inhibitable association of ManLAM and PIM beads with COS-1-MR or COS-1-DC-SIGN cell lines

To confirm the direct involvement of the MR in PIM association with MDMs, we assayed for PIM association using a mammalian cell line expressing the MR (COS-1-MR) and compared the level of association to that seen with a cell line expressing DC-SIGN (COS-1-DC-SIGN), another C-type lectin known to bind to the terminal mannose caps of ManLAM (13). DC-SIGN is expressed in abundance on DCs (36). In contrast, recent studies using flow cytometry and Western blotting (37–39) together with our own work (our unpublished data) indicate that there is little or no DC-SIGN expression on the surface of MDMs. Lipoglycoconjugate-coated beads were added to COS-1-MR or COS-1-DC-SIGN cell monolayers and specific association of coated beads with the MR or DC-SIGN was measured in the absence (Fig. 6, A and B, respectively) or presence (Fig. 6, C and D, respectively) of mannan (2.5 mg/ml). Similar to the data with MDMs, Ac₁PIM₅ and Ac₂PIM₂ beads associated poorly with the COS-1-MR cells (Fig. 6A) and the association was not mannan-inhibitable (Fig. 6C). Ac₂PIM₆ beads also associated poorly with the cells (Fig. 6A) as was seen with the MDMs (Fig. 5B); however in this case, the association was mannan-inhibitable (43.5% inhibition) (Fig. 6C). In contrast, Ac₁PIM₆ beads associated with COS-1-MR cells to a high degree (equivalent to that seen with ManLAM) and the association was reduced to control levels in the presence of mannan (62.4% inhibition), also equivalent to the degree of mannan inhibition seen with ManLAM (66.3% inhibition) (Fig. 6, A and C). Association profiles for Ac₁PIM₅, Ac₂PIM₅, and Ac₁PIM₆ with the COS-1-MR cells were also high and significantly inhibited in the presence of mannan (36.5% inhibition for Ac₁PIM₅ and 58% inhibition for Ac₁PIM₆, p < 0.05 and p < 0.01, respectively).

In contrast to the results obtained with the COS-1-MR cells, beads coated with ManLAM, LM, and all PIM types (especially Ac₁PIM₅, Ac₂PIM₅, and Ac₁PIM₆) associated with the COS-1-DC-SIGN cells (Fig. 6B) and in all cases, the association was significantly reduced in the presence of mannan (Fig. 6D). Association of lipoglycoconjugate-coated beads with COS-1 WT cells was negligible and not mannan-inhibitable (data not shown). Thus, both carbohydrate and fatty acid composition play important roles in enabling the recognition of PIMs by the MR, whereas specific recognition of PIMs by DC-SIGN appears to be relatively independent of the nature of the carbohydrates and fatty acids.

The role of the MR in directing P-L fusion events in macrophages for higher-order PIM-coated beads

ManLAM is a critical regulator of phagosome maturation in murine macrophages and a human monocytic cell line (40, 41). Recently, we have shown that engagement of the MR by ManLAM through its mannose caps during the phagocytic process directs M.tb to its initial phagosomal niche in human macrophages by limiting P-L fusion (12). Blockade of the MR with a mAb reversed the inhibition of P-L fusion. The fact that Ac₁PIM₅, Ac₂PIM₅, and Ac₁PIM₆ have greater association with the MR when compared
with the other *M. tb* PIMs, and that the nonreducing termini of these PIMs resemble the mono- and dimannoside caps of Man-LAM, directed us to examine the degree to which MR blockade on macrophages by anti-MR Ab affects P-L fusion of beads coated with several PIM species.

MDM monolayers were preincubated with anti-MR or control IgG1 mAbs followed by the addition of either ManLAM-, Ac2PIM2- (as a representative of the PIM2f), Ac2PIM5- (as a representative of the PIM5f), Ac1PIM6- or Ac2PIM6-coated fluorescent beads and subsequent assessment for P-L fusion using confocal microscopy. Results (Fig. 7) showed that when the MR was blocked, ManLAM- (used as positive control; \( p < 0.0001 \)), Ac2PIM5- (\( p < 0.05 \)), and Ac1PIM6- (\( p < 0.05 \)) coated beads demonstrated a significant increase in P-L fusion when compared with the sham control HSA beads. In contrast, blockade of the MR had minimal effects on P-L fusion for Ac2PIM2- and Ac2PIM6-coated beads (Fig. 7B). Thus, these results indicate that the MR regulates P-L fusion events for higher-order PIMs but not lower-order PIMs and therefore correlate with MR recognition by the former PIM types.

![FIGURE 6. Mannan-inhibitable association of ManLAM and PIM beads with COS-1-MR or COS-1-DC-SIGN cell lines. Cells (2 \( \times 10^5 \)) were adhered to glass coverslips in 24-well plates and washed. Lipoglycoconjugate-coated beads were added to cell monolayers (multiplicity of infection 50–100:1). Specific association of coated beads with the MR (n = 3) (A) or DC-SIGN (representative experiment) (B) was measured in the absence or presence of mannan (2.5 mg/ml). Percent mannan inhibition was calculated and is shown in C for COS-1-MR (n = 3) and D for COS-1-DC-SIGN (n = 3) (mean ± SEM). In A, Student t test, *, \( p < 0.05 \); **, \( p < 0.005 \); ***, \( p < 0.0001 \); in B, Student t test, *, \( p < 0.05 \); **, \( p < 0.005 \); in C, Student t test, *, \( p < 0.05 \); **, \( p < 0.005 \); and in D, Student t test, *, \( p < 0.05 \); **, \( p < 0.005 \).](http://www.jimmunol.org/)

![FIGURE 7. Higher-order PIMs that associate with the macrophage MR demonstrate increased P-L fusion upon MR blockade. MDM monolayers on coverslips were incubated with isotype control IgG1 or anti-MR mAb at 37°C for 20 min before adding *M. tb* H37Rv lipoglycoconjugate-coated green fluorescent beads (4 \( \times 10^7 \)/well) and allowing for phagocytosis to occur at 37°C for 2 h. MDM monolayers were fixed, permeabilized, and then immunostained with the late endosomal/lysosomal marker CD63. Coverslips were mounted on glass slides and examined by confocal microscopy. A, Representative fluorescence microscopy images of MDM monolayers after incubation with control HSA- or *M. tb* lipoglycoconjugate-coated beads in the presence or absence of anti-MR mAb. Departments that stain positive for CD63 are red, beads in unfused phagosomes are green, and beads in fused phagolysosomes are yellow. B, Percent increase in P-L fusion of HSA- or *M. tb* lipoglycoconjugate-coated beads in the presence of anti-MR mAb is shown as analyzed by confocal microscopy. P-L fusion was enumerated by counting over 1000 consecutive phagosomes in each test group (mean ± SEM, n = 3, except for Ac2PIM6, n = 2), Student t test, *, \( p < 0.05 \); ***, \( p < 0.0001 \).](http://www.jimmunol.org/)
M. tb strains contain greater amounts of higher-order PIMs in their cell envelope than the nonpathogenic M. smegmatis mc²155 strain

To further explore the importance of higher-order PIMs in M. tb recognition by the MR and their potential involvement in M. tb pathogenesis, we purified cell envelope PIMs from the virulent M. tb Erdman and M. tb H₃₇R₇ strains, the attenuated M. tb H₃₇R₅ strain and the saprophytic M. smegmatis mc²155 strain, and compared the relative amounts of higher-order and lower-order PIMs. Bacteria were grown under identical conditions (except 12 days for M. tb strains and 5 days for the M. smegmatis strain due to the different growth rate between both species), homogenized, and PIMs resolved by two-dimensional TLCs for each strain (loading was based on protein equivalents of cell lysates). A representative experiment (Fig. 8) shows a clear difference in the amounts of higher-order PIMs between strains. In all cases, M. tb strains have significantly more Ac₁PIM₆, Ac₁PIM₅, and Ac₂PIM₅ (Fig. 8, spots 1, 2, and 3, respectively) when compared with M. smegmatis mc²155, while lower-order PIMs (Ac₁PIM₂ and Ac₂PIM₂) are much less predominant in M. tb than in M. smegmatis (Fig. 8 spots 4 and 5, respectively). In addition, virulent M. tb strains (M. tb Erdman and M. tb H₃₇R₇) have quantitatively more higher-order PIMs (especially Ac₁PIM₆) than the attenuated M. tb H₃₇R₅ strain.

The abundance of exposed higher-order PIMs along with ManLAM by macrophages (10) and den- sylated lipoglycoconjugates between these C-type lectins. In this study, we evaluated the lectin association pattern of the PIMs, major phospholipids present in the M. tb cell wall. To accomplish this, we purified PIM families based on their mannose content followed by purification of PIM species based on the number of fatty acids. This proved to be a powerful approach in enabling us to demonstrate for the first time that there is preferential engagement of the MR by higher-order PIMs in contrast to the engagement of DC-SIGN by both higher-order and lower-order PIMs and LM. Preferential engagement of the MR by higher-order PIMs was found to regulate P-L fusion events in macrophages. Thus, this work makes the fundamental observation that recognition of PIMs by the MR and DC-SIGN is dependent on both the nature of their mannosyl units and the fatty acid content which impacts the host response.

The human macrophage has high MR activity and negligible DC-SIGN activity (37, 43). To demonstrate specific interaction between PIMs and the MR, we used a model established in this laboratory in which M. tb lipoglycoconjugates are bound to poly-styrene beads by their lipid tails with the carbohydrates preferentially exposed for engagement of macrophage C-type lectins (10). This technique has been used successfully in several studies with mycobacterial lipoglycoconjugates (23, 29, 44). MR activity is blocked with mannan (10) and an anti-MR mAb (12). Using these approaches with human macrophages and an MR-expressing mammalian cell line, we demonstrate that higher-order PIM families (PIM₁f and PIM₆f) associate well with the MR, whereas the lower-order PIM₂f does not. We confirmed that ManLAM is a

**FIGURE 8.** Two-dimensional TLC analysis of the total lipid from virulent and attenuated M. tb strains shows increased production of higher-order PIMs when compared with the saprophytic M. smegmatis. PIMs from the indicated strains were extracted from 10 mg of whole cell mycobacterial lysates by organic extraction before being dried and resuspended in CHCl₃:CH₃OH (2:1, v/v) for two-dimensional TLC analysis. Following separation, plates were sprayed with α-naphthol or Dittmer reagent (data not shown) and charred to visualize glycolipids and phospholipids, respectively. Spots were identified as PIMs went positive for α-naphthol and Dittmer reagent. Results are representative of three independent experiments. Equal amounts of samples were loaded per TLC according to total protein content as described in Materials and Methods. Spot 1, Ac₁PIM₆; 2, Ac₁PIM₅; 3, Ac₂PIM₅; 4, Ac₁PIM₂; 5, Ac₂PIM₂.
major ligand for the MR (via binding of its mannose caps) whereas LM demonstrates negligible binding to this receptor (10), a finding which indicates that the MR recognizes the branches [1-Manp-α(1→2)-Manp-α(1→6)-] and the linear terminus [1-Manp-α(1→6)-Manp-α(1→6)-] present in LM poorly. The fact that PIMf and PIMf termini resemble the mono- and dimannoside caps of ManLAM [1-2-Manp and t-Manp(1→2)-α-Manp, respectively] together with their location on the cell wall surface, allows us to postulate that both families play an important role in the interaction of the bacillus with human macrophages through recognition by the MR. The PIMf accumulates as a final biosynthetic product on the Mtb cell wall (4, 17, 45), and as such, is likely to play a particularly important role in bacterial recognition by this receptor.

Important differences exist in the carbohydrate recognition domain (CRD) structural motifs between the MR and DC-SIGN making it likely that these proteins are not redundant in their functions, particularly with regard to M.tb carbohydrate recognition. In this context, the association profile for the PIM families and LM differed markedly between these two C-type lectins. In the case of the MR, association was highest for the higher-order PIMs. For DC-SIGN, mannan-inhibitable association was independent of the number and location of the mannose moieties in PIMs and LM. Therefore, DC-SIGN appears to recognize mannos-oligosaccharides of any length. The multiple linear CRDs located in the extracellular domain of the MR (46) appear to favor the recognition of the mono- and dimannoside cap-like structures present in higher-order PIMs. In contrast, DC-SIGN has a single CRD and forms tetramers on the cell surface, where the DC-SIGN tetramer clusters its CRDs to enable high avidity binding to mannos-containing glycoconjugates (46). Together, these results are consistent with the idea that the spatial orientation of MR CRDs limits its sugar recognition repertoire, whereas the clustered CRD conformation of tetrameric DC-SIGN enables a broader range of mannos glycoconjugate recognition.

Our data indicate that the nature of the fatty acids in PIMs play a role in their recognition by the MR. In our bead model, the degree of acylation of the PIMf markedly affected their ability to associate with the MR (Ac1PIMf >> Ac5PIMf) pointing toward the importance of the fatty acids in the conformation and accessibility of the terminal mannosyl cap-like structure. This was demonstrated using both MDMs and COS-1-MR cells. There are contradictory data in the literature regarding the importance of the acyl groups of PIMs in generating proinflammatory cytokines depending on the model used. Some studies showed that when presented free in solution, mixtures of PIMs, PIM2, and PIM6 families and purified species of PIMf and PIMf induce TNF-α via a TLR-2-dependent pathway irrespective of their acyl forms (19, 22, 47). In contrast, a recent study using the liposome model showed that the presence of a fourth fatty acid in the C3 position of the myoinositol of Ac5PIM2 diminished the production of proinflammatory cytokines (48). Based on our findings, it is possible that this observation was related to lower binding of Ac5PIM2 to its receptor(s). Our data show lower association of the tetra-acylated PIM species with the MR. Thus, the presence of this fourth fatty acid, especially in Ac5PIM6, may induce a spatial conformational change which diminishes its efficient interaction with the CRDs of the MR. Although it is possible that our findings could be related to the bead model used, perhaps with altered coating of Ac5PIM6, this phenomenon was not observed for association of PIMs with DC-SIGN making this possibility unlikely. Thus, the presence of the fourth fatty acid attached to the myo-inositol may be critical for C-type lectin recognition and this would be particularly pronounced for the PIMs, which are small glycolipids relative to the larger lipoglycans such as ManLAM or LM. This concept is supported by a recent study with ManLAM and the human pulmonary C-type lectin surfactant protein A where the authors suggested that the lipid moiety of ManLAM is responsible for the macromolecular organization of this lipoglycan that enables C-type lectin interaction (49).

In M.tb, the fourth fatty acid of PIMs is most frequently palmitic acid, tuberculostearic (TBST) acid or oleic acid (17, 22). Although the importance of the nature of this fourth fatty acid is not clear for C-type lectin recognition by the macrophage, previous studies performed in dendritic cells (48) attribute proinflammatory responses to the TBST. Three dimensional computer modeling of Ac1PIMf vs Ac5PIMf (Fig. 9), when both are bound to a flat surface, showed that Ac5PIMf adopts a spatial conformation with a different plane of axis of the nonreducing terminus when compared with Ac1PIMf (Fig. 9). We speculate that this change in axis may explain the marked difference in association with the MR between these two species from the same family (i.e., the dimannoside termini in Ac1PIMf are in a more favorable conformation to bind to the MR). In addition, when we evaluated how the nature of the fatty acid in Ac5PIMf affects its spatial conformation, there was a slight affect on the axis when the fourth fatty acid was substituted with palmitic or oleic acids relative to TBST. These findings lend support to the importance of the type and nature of fatty acids in dictating the spatial conformation of the carbohydrates in the PIMs.

![Diagram](image.png)

**FIGURE 9.** Carbohydrate linkage and fatty acid composition of M.tb PIMs determine their recognition by human macrophage C-type lectins. Shown is a three-dimensional prediction (0° and 90° rotation around the phosphate, see directional arrow) of Ac1PIMf vs Ac5PIM6 depicting the role of the acyl groups in determining the spatial conformation of the PIM terminal mannosyl cap. The change in spatial conformation observed in the cap may explain the marked difference in binding to the CRDs of the MR between Ac1PIMf and Ac5PIMf. Ac1PIMf beads associate to a much greater extent with the macrophage MR than Ac5PIMf beads. Ac5PIMf has three fatty acids (two in the glycerol and the third in the C6 position of the mannos attached to the C2 position of its myo-inositol). The three fatty acids of Ac1PIMf bound to the bead allow for a spatial conformation of the cap that is optimal for recognition by the MR (equivalent to ManLAM). The addition of a fourth fatty acid located in the C3 position of the myo-inositol alters the conformation of the cap of Ac5PIMf such that the association of this lipoglycan with the MR is markedly reduced.
Evidence is emerging for the importance of mycobacterial PIMs in host cell recognition and response (23, 50). These studies showed that human macrophages preincubated with PIM$_{A2}$ and PIM$_{A3}$ inhibited phagocytosis of mycobacteria. PIM$_{A3}$-coated beads were found to associate with complement receptor 3 and not the MR (23). These results complement our observations, where the PIM$_{A2}$ associated poorly with the MR and is heavily expressed in the cell wall of M. smegmatis when compared with M. tb. However, our results showed that the low level of PIM$_{A2}$ association with macrophages was mannan inhibitable raising the possibility for involvement of a C-type lectin. In this regard, we found that purified species of the PIM$_{A2}$ (Ac$_{1}$PIM$_{A2}$ and Ac$_{2}$PIM$_{A2}$) associate well with DC-SIGN. Because MDMs express negligible DC-SIGN, it remains unclear whether this is an important PRR for the PIM$_{A2}$ on macrophages.

A study using murine bone marrow-derived dendritic cells has shown that PIM$_{A1}$ liposomes activate a marked proinflammatory cytokine response, a finding that the authors speculated was due to an interaction with TLRs (48). Our findings provide evidence that the PIM$_{A2}$ is recognized differently from higher-order PIMs by macrophages and potentially by dendritic cells. Whereas the PIM$_{A2}$ associates with complement receptor 3 (23), DC-SIGN (our study), and potentially TLRs, we demonstrate that higher-order PIMs preferentially associate with the MR and DC-SIGN. M. tb engagement of the MR and DC-SIGN has been shown to inhibit the proinflammatory response of phagocytes (14, 51). We make the important observation that M. tb expresses low quantities of the PIM$_{A2}$ on its surface and, instead, expresses abundant higher-order PIMs. This result along with previous studies with ManLAM indicate that virulent M. tb has evolved to more heavily decorate its abundant cell wall lipoglycoconjugates with $\alpha(1\rightarrow2)$ mannosylated termini. These termini resemble those present in high mannose N-linked oligosaccharides of newly produced glycoproteins in eukaryotic cells (52). Such heavily $\alpha(1\rightarrow2)$ mannosylated N-glycoproteins released into circulation are cleared by macrophages via the MR by pinocytosis to maintain homeostasis (53). We speculate that increased $\alpha(1\rightarrow2)$ mannosylation on the surface of M. tb allows for preferential engagement of the MR on macrophages in a form of exploitation to enhance its survival.

Consistent with this notion, we have recently determined that MR-mediated phagocytosis of ManLAM-coated microspheres and M. tb leads to limited P-L fusion in human macrophages (12). In this study, we show that in addition to ManLAM, higher-order PIMs that bind to the MR (i.e., Ac$_{1}$PIM$_{A}$ and Ac$_{2}$PIM$_{A}$ but not Ac$_{3}$PIM$_{A}$) demonstrate a significant increase in P-L fusion upon MR blockade further emphasizing the importance of the MR pathway in directing vesicle trafficking for M. tb within the macrophages. Our P-L fusion results with Ac$_{2}$PIM$_{A}$ (as a representative of the lower-order PIMs) support previous studies where it was shown that a mixture of PIM$_{A2}$ and PIM$_{A3}$ did not inhibit P-L fusion (44). The fact that components of the PIM$_{A2}$ did not bind to the MR adds further evidence for the specificity of the MR pathway in limiting P-L fusion.

Recently, we have shown that a strain of M. smegmatis over-expressing a phosphomannomutase (manB) involved in the biosynthesis of GDP-mannose (the mannoside donor for PIM biosynthesis (4)), expressed large amounts of higher-order PIMs on its surface (mainly Ac$_{1}$PIM$_{A}$, Ac$_{2}$PIM$_{A}$, and Ac$_{3}$PIM$_{A}$) (42). As a consequence of overexpression of higher-order PIMs, this strain demonstrated a 13-fold increase in binding to the human macrophage MR when compared with control strains (42). This fact, along with our current findings, further support the idea that higher-order PIMs expressed on the surface of M. tb will bind to the MR and participate together with ManLAM in restricting P-L fusion and consequently aiding the survival of the bacillus in human macrophages.

In summary, we purified the different PIM families and species found in M. tb and have characterized their association with two important host C-type lectins, the MR and DC-SIGN, in tuberculosis pathogenesis. Whereas higher-order PIMs and ManLAM, both heavily expressed on the M. tb surface, preferentially engage the MR and direct P-L fusion inhibition, these lipoglycoconjugates along with lower-order PIMs (expressed in abundance in M. smegmatis) and LM engage DC-SIGN. Association of higher-order PIMs with the MR is dependent on the number of fatty acids in the lipid anchor. The generation of highly purified M. tb cell envelope lipoglycoconjugates will allow us to do more detailed studies of the relationship between the MR and DC-SIGN and their signaling and trafficking pathways. Our results suggest that the balance between the relative amounts of ManLAM plus higher-order PIMs (preferentially recognized by the MR) and PIM$_{A2}$ plus LM (recognized by DC-SIGN and other receptors) exposed on the surface of different M. tb strains will influence the immune response to M. tb infection.

Acknowledgments

We thank Dr. Joanne Turner for careful reading of this manuscript and the help provided by the Mass Spectrometry and Proteomics Facility in the Campus Chemical Instrument Center at Ohio State University.

Disclosures

The authors have no financial conflict of interest.

References


