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Mycobacterial Phosphatidylinositol Mannosides Negatively Regulate Host Toll-like Receptor 4, MyD88-dependent Proinflammatory Cytokines, and TRIF-dependent Co-stimulatory Molecule Expression^{*[5]}

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Mycobacterium tuberculosis modulates host immune responses through proteins and complex glycolipids. Here, we report that the glycosylphosphatidylinositol anchor phosphatidyl-*myo*-inositol hexamannosides PIM₆ or PIM₂ exert potent anti-inflammatory activities. PIM strongly inhibited the Toll-like receptor (TLR4) and myeloid differentiation protein 88 (MyD88)-mediated release of NO, cytokines, and chemokines, including tumor necrosis factor (TNF), interleukin 12 (IL-12) p40, IL-6, keratinocyte-derived chemokine, and also IL-10 by lipopolysaccharide (LPS)-activated macrophages. This effect was independent of the presence of TLR2. PIM also reduced the LPS-induced MyD88-independent, TIR domain-containing adaptor protein inducing interferon β (TRIF)-mediated expression of co-stimulatory receptors. PIM inhibited LPS/TLR4-induced NF κ B translocation. Synthetic PIM₁ and a PIM₂ mimetic recapitulated these *in vitro* activities and inhibited endotoxin-induced airway inflammation, TNF and keratinocyte-derived chemokine secretion, and neutrophil recruitment *in vivo*. Mannosyl, two acyl chains, and phosphatidyl residues are essential for PIM anti-inflammatory activity, whereas the inosityl moiety is dispensable. Therefore, PIM exert potent antiinflammatory effects both *in vitro* and *in vivo* that may contribute to the strategy developed by mycobacteria for repressing the host innate immunity, and synthetic PIM analogs represent powerful anti-inflammatory leads.

Multifold interactions between *Mycobacterium tuberculosis* and host phagocytes determine immune responses to *M. tuberculosis* and tuberculosis pathogenesis (for review, see Refs. 1 and 2). Alveolar macrophages, the primary host cells for *M. tuberculosis*, and dendritic cells that carry mycobacterial antigens from the infection site to the draining lymph nodes to establish a T cell-

mediated immune response contribute to modulate the innate immune response by secreting cytokines after recognition of microbial motives. Among them, TNF² is an essential mediator for granuloma formation and containment of *M. tuberculosis* infection. Similarly, IL-12, interferon γ , but also IL-1, IL-18, IL-23, and nitric oxide are required for host defense (1–6). Phagocytes are also a source of immuno-modulatory cytokines, such as IL-10 and transforming growth factor- β , which dampen the immune response and inflammation. Mycobacteria-derived molecules down-modulating the immune system have been described, including the protein ESAT-6, mannose-capped lipoarabinomannan (ManLAM), and lipomannans (LM) (7–12). Here, we report that phosphatidyl-*myo*-inositol mannosides (PIM), the glycosylphosphatidylinositol (GPI) anchor structure of LAM and LM, exert strong anti-inflammatory activities.

Mycobacterial cell wall LAM, LM, and PIM are recognized by macrophages and dendritic cells through various pattern recognition receptors, including Toll-like receptors (TLRs) (13–16) and C-type lectins such as mannose receptor (MR/CD206) and dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN/CD209), central to *M. tuberculosis* binding and internalization by human dendritic cells (17–20). DC-SIGN and mannose receptor were proposed to mediate ManLAM inhibition of LPS-induced IL-12 production in dendritic cells, an activity ascribed to the mannosylated cap (8, 9). We showed recently that mycobacterial LM have a dual potential for pro-inflammatory and anti-inflammatory effects (11), tri- and tetra-acylated LM fractions exerting stimulatory effects through TLR2, TLR4, and MyD88 (21), whereas diacylated LM inhibit LPS-induced cytokine response independently of TLR2, SIGN-R1, and mannose receptor (12).

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Schemes 1–3 and Figs. 1–4.

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² The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; BCG, bacillus Calmette Guérin; DC-SIGN, dendritic cell-specific intracellular adhesion molecule-3 grabbing nonintegrin; KC, keratinocyte-derived chemokine; MR, mannose receptor; LAM, lipoarabinomannan; LM, lipomannans; Man, mannosyl unit; ManLAM, mannose-capped LAM; MyD88, myeloid differentiation protein 88; PI, phosphatidyl-*myo*-inositol; PIM, PI mannosides; TLR, Toll-like receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; GPI, glycosylphosphatidylinositol; TRIF, TIR domain-containing adaptor protein inducing interferon β ; KO, knock out.

PIM Inhibit TLR4-mediated Host Innate Responses

PIM are biosynthetic precursors of LM and LAM (22–25). Dimannoside (PIM₂) and hexamannoside (PIM₆) PIM are the two most abundant classes of PIM found in *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG (see Fig. 1). PIM purification and molecular chemical characterization revealed four major acyl forms, mono- to tetra-acylated (lyso-PIM for one acyl, PIM for two acyl, Ac₁PIM for three acyl, and Ac₂PIM for four acyl, respectively; see Fig. 1) for both PIM₂ and PIM₆ (26–29). Higher order PIM with mannose cap-like structures were found to preferentially associate with human MR and to contribute to phagosome-lysosome fusion (20). The degree of acylation influenced higher order PIM association with the MR, whereas PIM₂ was recognized by DC-SIGN independently of its acylation degree. The complete synthesis of the different PIM has recently been reported (30–33).

Here, we analyzed isolated acyl forms of PIM and identified PIM₂ and PIM₆ but also synthetic PIM₁ and a mimetic of PIM₂ as strong inhibitors of endotoxin-induced proinflammatory responses *in vitro* and *in vivo*. Using macrophages from genetically modified mice, we characterized PIM inhibitory effects on MyD88, TRIF, and NFκB signaling pathways. Hence, not only complex mycobacterial lipoglycans like ManLAM and LM but also small molecular weight PIM analogues are potent inhibitors of host inflammatory responses.

EXPERIMENTAL PROCEDURES

Purification of LM Acyl Forms—The PIM-containing lipidic extract was obtained through purification of the *M. bovis* BCG phenolic glycolipids (34) as summarized in Gilleron *et al.* (26). *M. bovis* BCG PIM₂ and PIM₆ mono-, di-, tri-, and tetra-acylated forms were further fractionated using hydrophobic interaction chromatography as described by Gilleron *et al.* (21). The purity of the different acyl forms was assessed by ³¹P NMR and matrix-assisted laser desorption/ionization mass spectrometry.

Synthetic PIM—PIM₁ containing a C16 and a C18 chain in the glycerolipid unit was prepared following largely published procedures (30, 32, 35, 36) with some modifications (for details, see the [supplemental information](#)). The reference compound phosphatidylinositol (PI) was prepared by an analogous method. PIM₂ mimetic was prepared by bisglycosylation of commercial 2-*O*-benzyl glycerol using tetra-*O*-methoxyacetyl- α -D-mannopyranosyl trichloro-acetimidate in the presence of trimethylsilyl triflate, debenzoylation of the glycerol-2-*O* position, phosphorylation with the same phosphoramidite as used in the synthesis of PIM₁, and deprotection by treatment with *tert*-butylamine (for the selective cleavage of the methoxyacetyl group) and final hydrogenolysis (for details, see the [supplemental information](#)).

Mice—6–12-Week-old mice deficient for TLR2 (37), MyD88 (38), TRIF (39), CD1 (40), ST2 (41), mannose receptor (42), or SIGNR1 (43) and wild-type control C57Bl/6 (B6) mice were bred at the Transgenose Institute animal breeding facility (Orleans, France).

Primary Macrophage Cultures—Murine bone marrow cells were isolated from femurs and cultivated (10⁶/ml) for 7 days in Dulbecco's minimal essential medium supplemented with 2 mM L-glutamine, 20% horse serum, and 30% L929 cell-conditioned medium as a source of macrophage colony-stimulating

factor. After a further 3 days in fresh medium, the cell preparation contained a homogenous population of macrophages (97–98% CD11b+F4/80+). The bone marrow-derived macrophages (10⁵ cells/well) in Dulbecco's minimal essential medium supplemented with 2 mM L-glutamine, 2 × 10⁻⁵ M β -mercaptoethanol, and 0.1% fetal calf serum were stimulated with 100 ng/ml LPS (*Escherichia coli*, serotype O111:B4; Sigma), 0.5 μ g/ml synthetic bacterial lipopeptide Pam₃CSK₄ ((*S*)-2, 3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser-Lys₄-OH) tri-hydrochloride, EMC Microcollections, Tuebingen, Germany), 30 ng/ml MALP2 (*S*-(2,3-bisacyloxypropyl)-cysteine-GNNDENISFKEK (Alexis Biochemicals, Lausanne, Switzerland), 0.125 μ M CpG ODN1826 (tccatgacgttcctgacgtt), or 3 μ g/ml of poly(I-C) (poly(I)-poly(C) double strand, Amersham Biosciences). The PIM preparations or DMSO controls are added at the indicated concentrations 30 min before the stimuli. Lyophilized PIM preparations were solubilized in DMSO and added to the cultures at a non-cytotoxic 1% DMSO final concentration (3–7 μ g/ml unless otherwise stated). The macrophages were activated with interferon- γ (500 units/ml) to study IL-12 release, and the supernatants were harvested after 24 h for further analysis. Alternatively, cells were collected at the indicated times for PCR gene expression analysis. Primary lung, bronchoalveolar or peritoneal resident macrophages, and spleen adherent cells were also activated with LPS in the presence of PIM. The absence of cytotoxicity of the stimuli was controlled using MTT incorporation.

Nuclear Translocation of NFκB—Bone marrow-derived macrophages stimulated with LPS plus PIM as above on microscopic slides for 1–4 h, washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with Triton 100 × 0.5% were incubated with goat anti-murine NF-κBp65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, washed, and incubated with swine anti-goat IgG FITC Ab (Sigma). Cells presenting NFκB nuclear translocation were scored by confocal microscopy (Leica SP2).

Flow Cytometric Analysis (FACS)—Cells were saturated with mouse serum before staining for 20 min with fluorescence-conjugated antibodies CD11b-PerCP (clone M1/70), PE-F4/80 (clone BM8), fluorescein isothiocyanate anti-CD86 (clone GL1), PE-CD40 (clone 3/23), and isotype-matched control antibodies (all from Pharmingen except BM8, which was from eBioscience), and analyzed using a FACSCalibur flow cytometer and CellQuest Software (BD Biosciences).

Cytokine Enzyme-linked Immunosorbent Assay—Supernatants were harvested and assayed for cytokine content using commercially available enzyme-linked immunosorbent assay reagents for TNF, KC, IL-6, IL-10, and IL-12 p40 (DuoSet R&D Systems, Abingdon, UK).

Nitrite Measurements—Nitrite concentrations in cell supernatants were determined using the Griess reaction (3% phosphoric acid, 1% *p*-aminobenzene sulfonamide, 1% *N*-1-naphthyl ethylenediamide) as previously described (44).

Real-time Reverse Transcription-PCR—Expression of the indicated cytokine and chemokine genes was measured by real-time reverse transcription-PCR of bone marrow-derived macrophages 2 and 6 h after LPS stimulation (0.1 μ g/ml) in the presence of synthetic PI, PIM₁, PIM₂ mimetic, deacylated

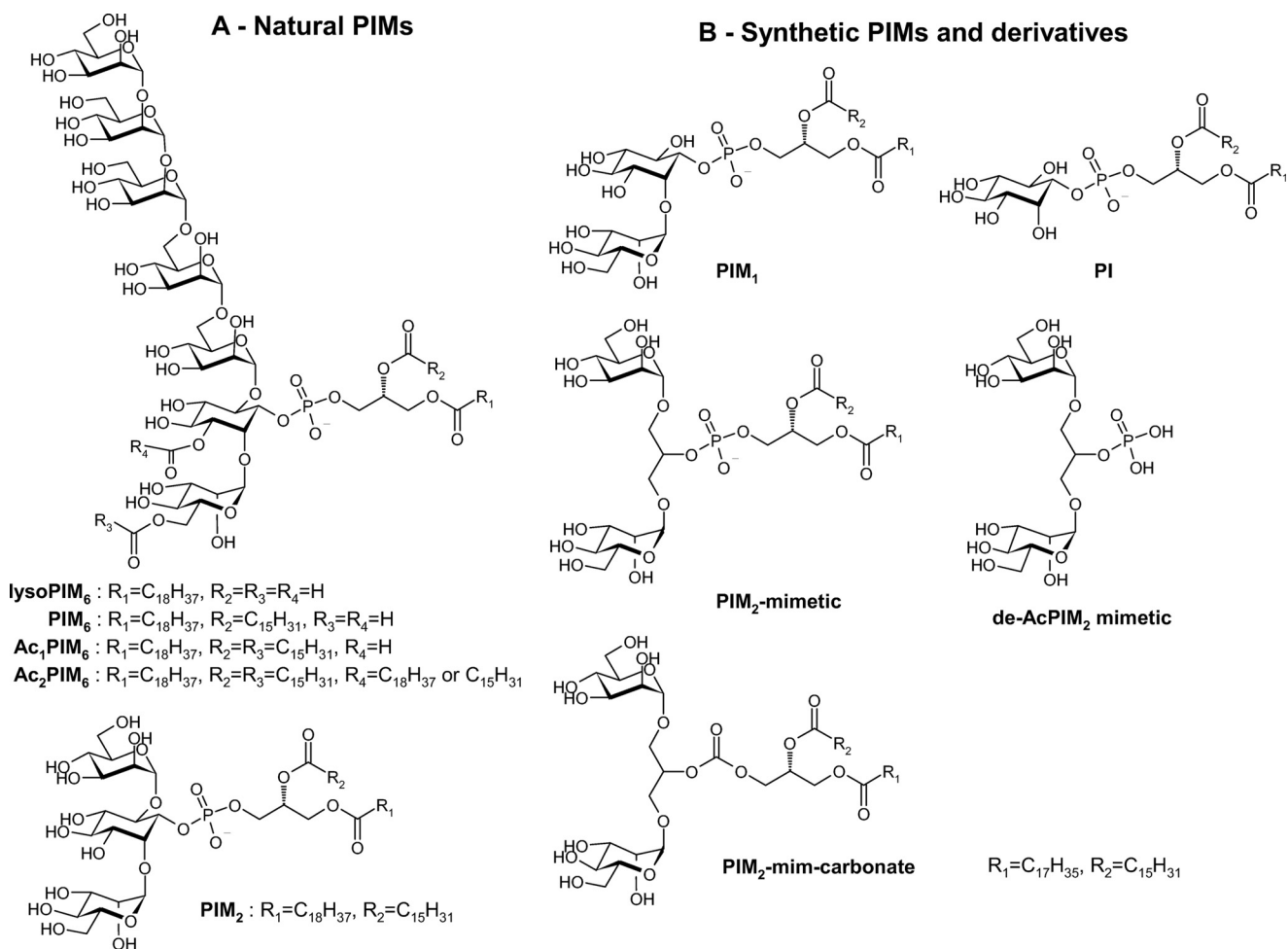


FIGURE 1. Natural PIM and synthetic PIM₁ and PIM₂ mimetics used in the study. Shown is a schematic representation of natural lyso-PIM₆, PIM₆, Ac₁PIM₆, Ac₂PIM₆, and PIM₂ (A) and synthetic PIM₁ (B) showing the C16 and C18 acyl groups on glycerol chain positions *sn*-2 and *sn*-1, the precursor PI, a synthetic mimetic of PIM₂ (PIM₂ mimetic) bearing C16 and C18 acyl chains, the de-acetylated control molecule precursor of the PIM₂ mimetic (de-AcPIM₂ mimetic), and a PIM₂ mimetic with replacement of the phosphodiester moiety by a carbonate.

Ac₂PIM₂ mimetic (all at 10 μg/ml). Expression of *Hprt1*, glyceraldehyde-3-phosphate dehydrogenase, 18 S, and β₂-microglobulin housekeeping genes was used for normalization.

Airway Inflammation—LPS (1 μg) from *E. coli* (serotype O55:B5; Sigma) in saline containing PIM₁ or PIM₂ mimetic (50 μg) in DMSO (1.25% final) or saline plus DMSO alone was applied by nasal instillation in a volume of 40 μl under light ketamine-xylazine anesthesia. Airways resistance was evaluated by whole-body plethysmography (EMKA Technologies, Paris, France) over a period of 3 h (45). Enhanced respiratory pause as a measure of airway dysfunction (for details see Ref. 46) was registered and analyzed using Datanalyst Software (EMKA Technologies). At 24 h, myeloperoxidase activity was evaluated in lung, and bronchoalveolar lavage fluid was collected as described (46) for cytokine analysis and cell differential counts on 200 cells with Diff-Quik staining (Merz & Dade AG, Dudingen, Switzerland).

Statistical Analysis—Statistical significance was determined with Graph Pad Prism software (Version 4.0, San Diego, CA) by one way non-parametric analysis of variance followed by the Tukey post test. *p* values of <0.05 were considered statistically significant.

RESULTS

Inhibition of LPS-induced Macrophage Stimulation by PIM₆-purified Acyl Forms—Unfractionated PIM stimulates TNF production by a monocytic cell line (47), and we showed previously that purified PIM₆ are also slightly proinflammatory (27). However, because LM pro- and anti-inflammatory activities were separated according to the degree of acylation of the LM molecules, we asked whether certain PIM fractions could also inhibit macrophage activation. An enriched fraction of PIM₆ was prepared from *M. bovis* BCG (26) and acyl forms bearing one to four fatty acids (see Fig. 1) were further purified as described (27). The inhibitory effect of lyso-PIM₆, PIM₆, Ac₁PIM₆, and Ac₂PIM₆ fractions on LPS-induced TNF secretion was first assessed using RAW 264.7 monocytic cell line and confirmed on primary bone marrow-derived macrophages (Fig. 2). The most effective inhibitors of TNF release were PIM₆ and Ac₁PIM₆, whereas Ac₂PIM₆ was slightly less effective, and essentially no inhibition was seen with lyso-PIM₆ (Fig. 2A). Similar results were obtained for NO release (data not shown). All PIM fractions were titrated and tested at a concentration at which only lyso-

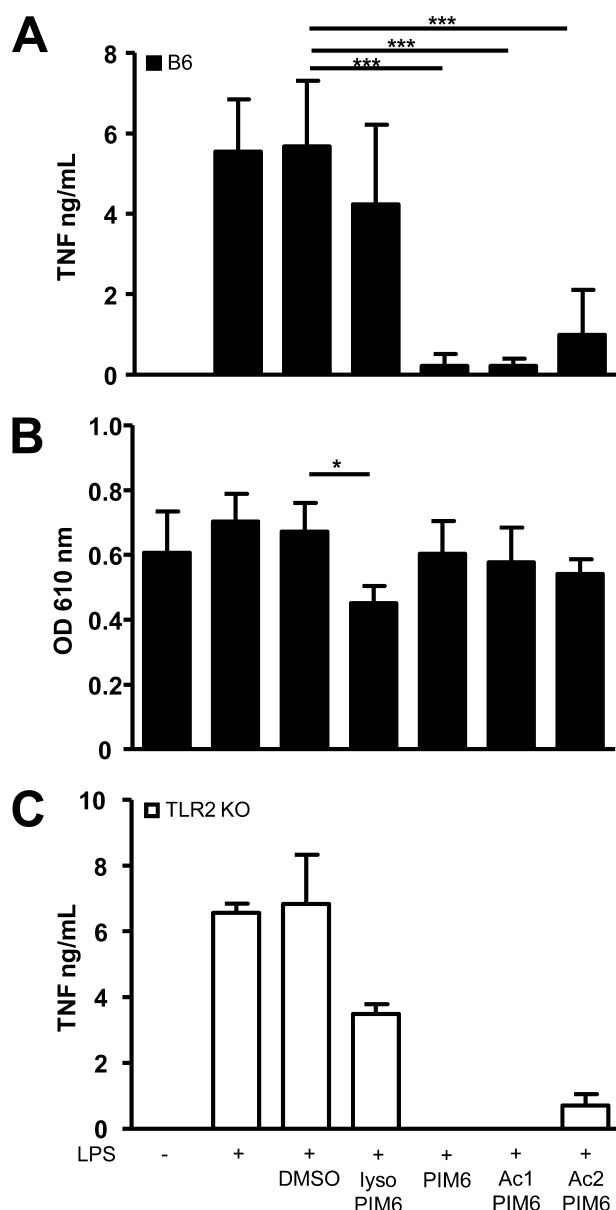


FIGURE 2. PIM₆-purified fractions inhibit TNF release by LPS-stimulated macrophages, independently of TLR2. Bone marrow-derived macrophages from wild-type (A and B) or TLR2-deficient (C) mice were incubated for 24 h with LPS in the presence of purified PIM₆-acylated forms (lyso-PIM₆, PIM₆, Ac₁-PIM₆, and Ac₂-PIM₆; all at 6.7 μg/ml) or DMSO vehicle alone. TNF (A and C) was measured in the supernatants, and potential cytotoxicity (B) was verified on the cells by MTT bioassay (absorbance, 610 nm). Results are the mean ± S.D. from n = 6 mice per group from three independent experiments; *, p < 0.05; ***, p < 0.01. ko, knock out.

PIM₆ exhibited a slight cytotoxicity (Fig. 2B). Thus, PIM-purified fractions inhibited TNF and NO secretion by primary macrophages depending on their level of acylation.

The Inhibition of LPS-induced TNF by PIM Is TLR2-independent—The unfractionated PIM preparation was shown to be a TLR2 agonist based on a reporter assay with cell lines transfected with the *tlr2* gene (47), and we showed previously that the slight macrophage activation in response to PIM₂ and PIM₆ isolated fractions was dependent on the TLR2 pathway (27). We then examined whether TLR2 recognition was involved in the PIM anti-inflammatory activity using bone marrow-derived macrophages prepared from mice deficient for TLR2. The inhibitory

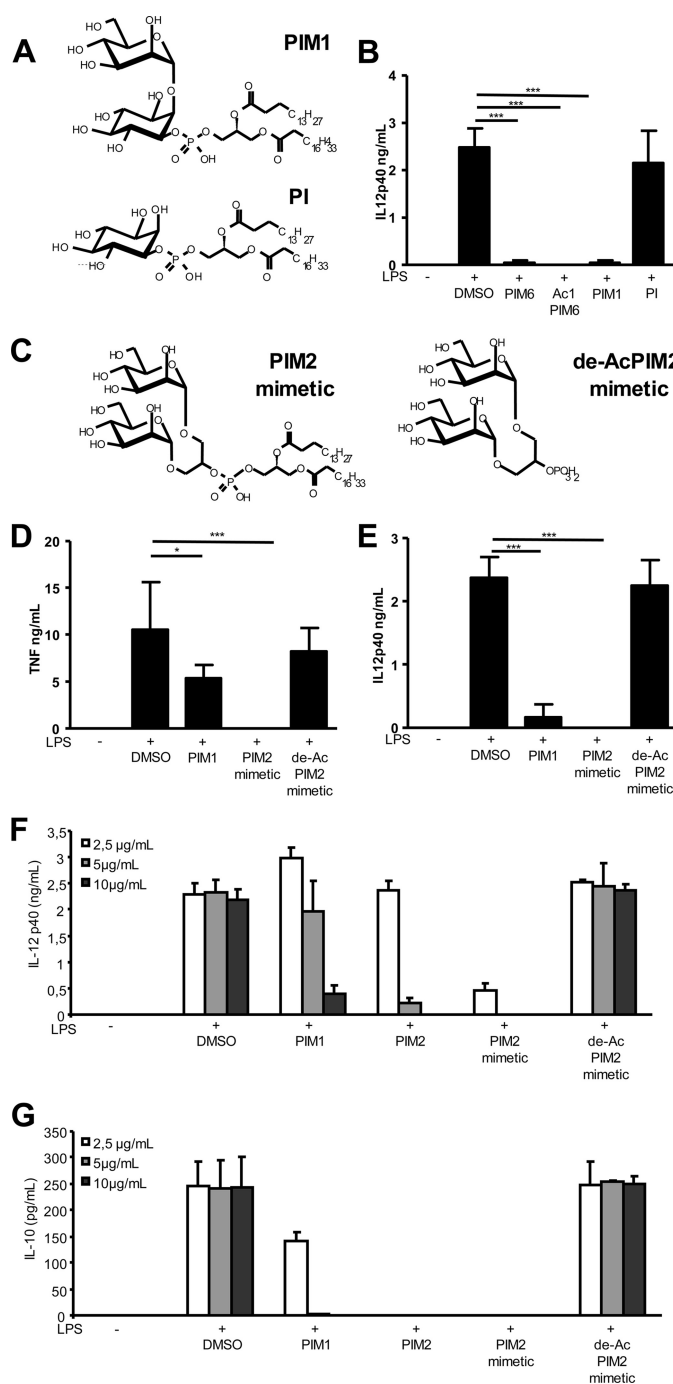


FIGURE 3. Anti-inflammatory activity of synthetic PIM₁ and PIM₂ mimetic. A, schematic representation of synthetic PIM₁ showing the C16 and C18 acyl groups on glycerol chain positions *sn*-2 and *sn*-1 and the precursor PI. B, comparison of synthetic PIM₁ to natural PIM₆ or Ac₁-PIM₆-purified fractions or to non-acylated PI (all at 10 μg/ml) for inhibiting LPS-induced release of IL-12 p40 by wild-type bone marrow-derived macrophages. C, schematic representation of a synthetic mimetic of PIM₂ (PIM₂ mimetic) and the de-acylated control molecule (de-AcPIM₂ mimetic). Also shown are a comparison of synthetic PIM₁, synthetic PIM₂ mimetic, and non-acylated de-AcPIM₂ mimetic for inhibiting LPS-induced release of TNF (D) and IL-12 p40 (E). Titration of natural PIM₂ and synthetic PIM₂ mimetic inhibitory activities as compared with PIM₁ and non-acylated de-AcPIM₂ mimetic for the inhibition of IL-12 p40 (F) and IL-10 (G) is shown. Results are the mean ± S.D. from n = 4–8 mice per group from 2–4 independent experiments; *, p < 0.05; ***, p < 0.01.

effect of PIM fractions on LPS-induced TNF was independent of their recognition by TLR2, as cells deficient for TLR2 were efficiently inhibited by the PIM₆ acyl fractions (Fig. 2C). The inhibi-

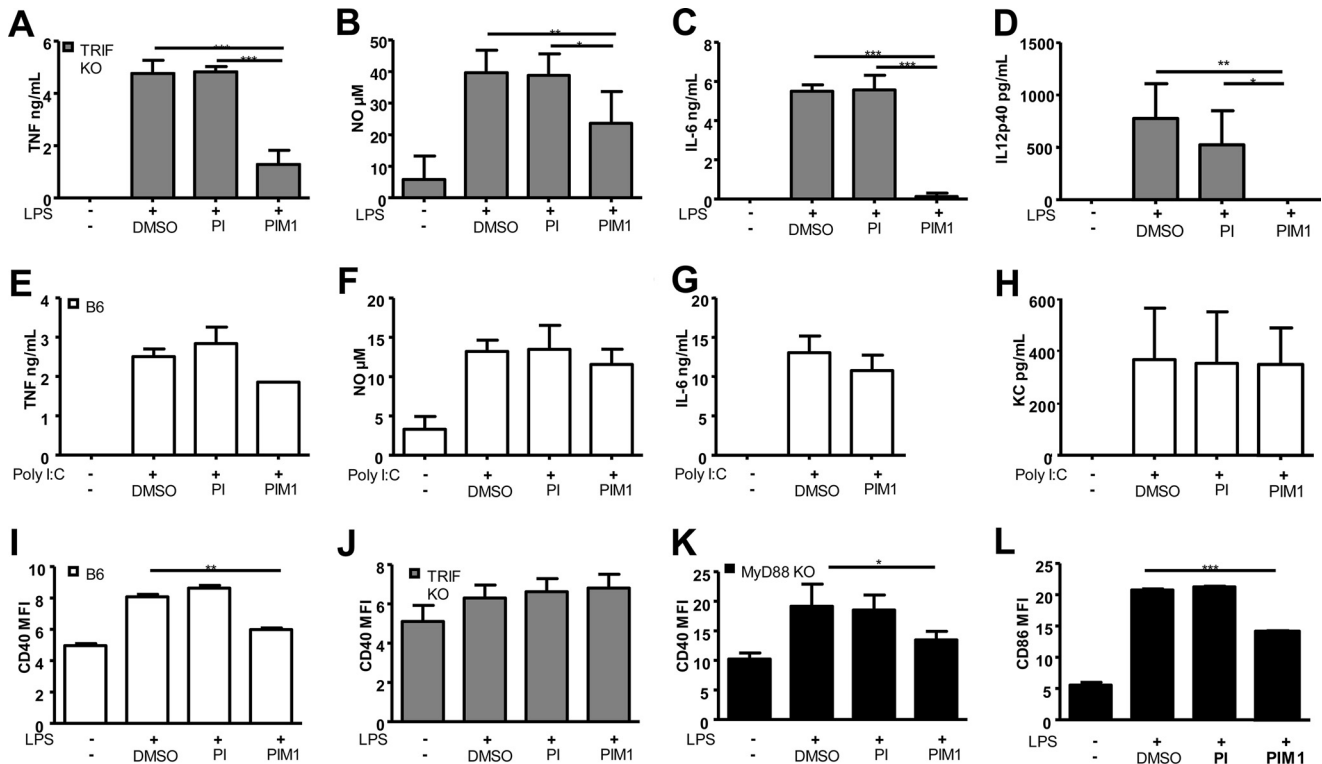


FIGURE 4. Inhibition of MyD88 versus TRIF-dependent signals by synthetic PIM₁. Wild-type, TRIF KO, or MyD88 KO bone marrow-derived macrophages were activated for 24 h with TLR4 agonist LPS or TLR3 agonist poly(I:C) in the presence of synthetic PIM₁ (10 μg/ml), PI (10 μg/ml), or DMSO vehicle alone as indicated. PIM₁ inhibited TNF (A), NO (B), IL-6 (C), and IL-12 p40 (D) by LPS-activated TRIF KO macrophages. Macrophage stimulation by poly(I:C), a TLR3 ligand using TRIF-dependent signaling, was not inhibited by PIM₁ in terms of TNF (E), NO (F), IL-6 (G), or KC (H) release. LPS induction of CD40 was inhibited by PIM₁ in wild-type macrophages (I). There was little CD40 induction in TRIF KO macrophages (J) as expected, and PIM₁ inhibited CD40 and CD86 expression in MyD88 KO macrophages (K and L). Results are the mean ± S.D. from *n* = 4 mice per genotype and are from two experiments representative of three to five independent experiments; *, *p* < 0.05; ***, *p* < 0.01. *MFI*, mean fluorescence intensity.

tion of LPS-induced TNF by PIM₆, Ac₁PIM₆, or Ac₂PIM₆ was even accentuated in the absence of TLR2, which can be explained by their slight TLR2 agonist activity, whereas lyso-PIM₆ remained poorly inhibitory. Thus, TLR2 is not required for mediating the inhibition of LPS-induced cytokine release by PIM fractions.

Specificity of PIM Fractions Inhibitory Effects for TLR4-mediated Stimulation—We next asked whether the inhibitory effects of the PIM fractions were specific for the TLR4 activation pathway. Specific TLR agonists, namely TLR4 agonist LPS, TLR2/TLR1 agonist Pam₃CSK₄ (bacterial lipopeptide), TLR2/TLR6 agonist Malp2, and TLR9 agonist CpG, were used to activate macrophages in the absence or in presence of PIM fractions. PIM₆, Ac₁PIM₆, and Ac₂PIM₆ inhibited the production of NO and TNF (supplemental Fig. 1A and not shown) after stimulation by LPS but not after stimulation by bacterial lipopeptide, Malp2 or CpG. The inhibitory effect of PIM fractions on IL-12 p40 expression (supplemental Fig. 1B) after LPS stimulation was stronger and was also partially seen after Malp2 stimulation. Therefore, the inhibitory effects of the PIM fractions are preferentially targeted to the TLR4 signaling pathway, although the specificity does not seem absolute for IL-12 p40 release.

Separation of Anti- Versus Pro-inflammatory Activity in Synthetic PIM₁—To confirm the inhibitory activity seen in mycobacterial-purified PIM fractions and to fully separate the anti-inflammatory activity from the slight TLR2-dependent proinflammatory activity seen with the natural PIM, we synthe-

sized a monomannosylated PI (PIM₁) bearing C16 and C18 chains (Fig. 3A). Synthetic PIM₁ inhibited the LPS-induced secretion of IL-12 p40 (Fig. 3B), TNF, and NO (not shown) as efficiently as the natural fractions containing PIM₆ or Ac₁PIM₆, whereas synthetic PI did not. Moreover, PIM₁ was neither stimulatory for cytokine or NO production nor cytotoxic at the concentrations used (not shown). Thus, through synthesis we could definitely ascertain that the anti-inflammatory activities seen in the natural purified fractions was associated with the PIM structure, separate the PIM anti-inflammatory from the pro-inflammatory activity and demonstrate that a monomannoside PIM structure was sufficient for the anti-inflammatory activity.

Synthetic PIM₂ Mimetic Recapitulates PIM Anti-inflammatory Activities—To avoid the long synthesis of natural PIM₂, we prepared a structural analogue that conserves the main features of PIM₂, namely the C16 and C18 acyl chains, two α-mannosyl residues, the diacylglycerol unit, and the phosphodiester linkage, all three groups being carried by a simple three-carbon glycerol scaffold instead of the complex *myo*-inositol (PIM₂ mimetic; Fig. 3C). A related molecule was reported earlier to induce some interferon γ release by splenocytes *in vitro* (48). We assessed the inhibitory activity of the PIM₂ mimetic on macrophages activated with LPS, in comparison to synthetic PIM₁, and to a precursor of the PIM₂ mimetic, dimannoside phosphate, devoid of acyl residues (de-AcPIM₂ mimetic). PIM₂ mimetic strongly inhibited the production of TNF and IL-12

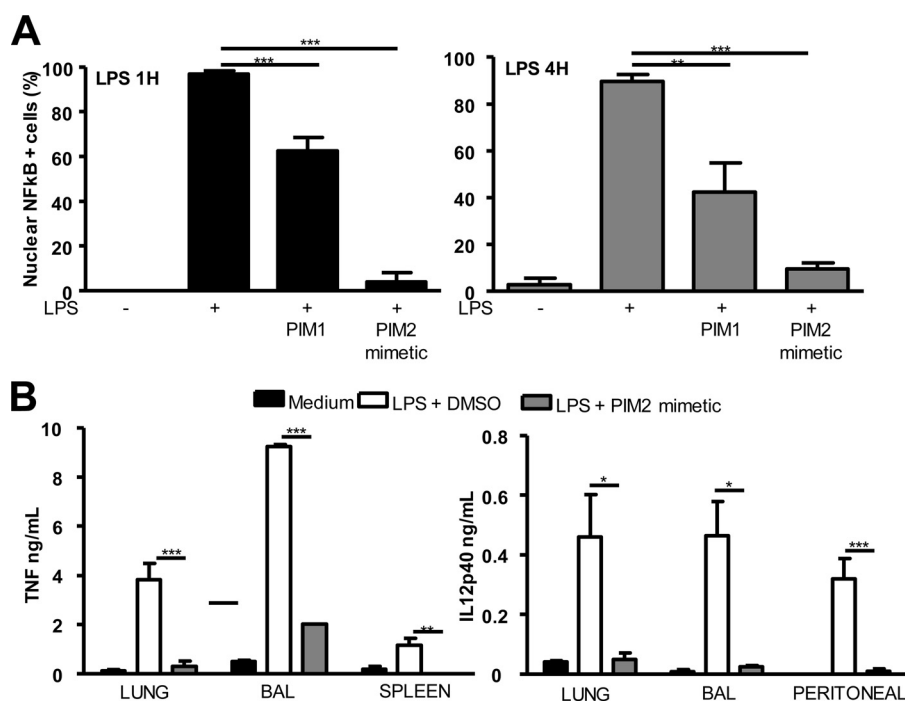


FIGURE 5. Synthetic PIM analogues inhibit NFκB nuclear translocation and act on different resident macrophage populations. A, NFκB translocation in LPS-stimulated macrophages; quantification of the cells exhibiting only NFκB nuclear staining 1 and 4 h after LPS stimulation in the absence or in the presence of PIM₁ or PIM₂ mimetic (*n* = 3 from two experiments). B, lung, bronchoalveolar (BAL), or peritoneal resident macrophages or spleen adherent cells were either untreated (medium) or activated with LPS in the presence of PIM₂ mimetic (10 μg/ml) or DMSO vehicle alone and TNF or IL-12 p40 concentrations in the supernatants were measured at 24 h. Results are the mean ± S.D. from *n* = 6 mice per group from two independent experiments; *, *p* < 0.05; ***, *p* < 0.01.

p40, whereas the nonacylated precursor molecule was inactive (Fig. 3, D and E). Moreover, the compounds were neither stimulatory nor cytotoxic at concentrations up to 10 μg/ml.

PIM₂ mimetic seemed more active than synthetic PIM₁ (Fig. 3, D and E, and data not shown). To assess whether this was because of the presence of a second mannose moiety, PIM₂ mimetic was further compared with the naturally purified PIM₂. Both PIM₂ mimetic and natural PIM₂ strongly inhibited the production of IL-12 p40 (Fig. 3F) and TNF (data not shown) but also IL-10 (Fig. 3G) and NO (not shown) by LPS-activated macrophages, and they were more potent than PIM₁, suggesting that indeed a second mannose moiety may increase the inhibitory effect. Replacement of the phosphodiester moiety by a carbonate (see Fig. 1) abrogated PIM₂ mimetic inhibitory activity (supplemental Fig. 2), indicating that although the inositol moiety is dispensable for the anti-inflammatory activity, the phosphodiester moiety is essential. Therefore, such PIM₂ mimetics are much more readily accessible by total synthesis and recapitulate the anti-inflammatory activities seen in natural or synthetic PIM.

PIM Inhibition of MyD88 Versus TRIF-dependent Signals—Because LPS/TLR4 can signal through two different pathways using, respectively, the adaptors MyD88/TIRAP or TRIF/TRAM, we next asked whether the inhibitory effects of the PIM fractions specifically target one of these pathways. PIM inhibitory activity was, thus, tested in MyD88- versus TRIF-deficient macrophages. Proinflammatory cytokine release upon LPS stimulation is strongly dependent on the MyD88 pathway (49) but largely independent of TRIF (Fig. 4, A–D). TNF, IL-12 p40,

IL-6, and NO release by TRIF-deficient macrophages was strongly inhibited by synthetic PIM₁ (Fig. 4, A–D), whereas PI was inactive, similar to what is seen in wild-type macrophages (data not shown and Fig. 3B). Conversely, TNF, IL-6, KC, or NO release after activation by TLR3 agonist poly(I·C), which stimulates a TRIF-dependent, MyD88-independent pathway, was poorly inhibited by PIM₁ (Fig. 4, E–H). Both sets of results suggested that PIM inhibition of cytokine or NO release in response to LPS activation was independent of the TRIF pathway.

We next asked whether the expression of the co-stimulatory molecules CD40 and CD86, which should be largely TRIF-dependent and MyD88-independent, was affected by PIM (Fig. 4, I–L). LPS-induced expression of co-stimulatory molecules such as CD40 was slightly reduced in the presence of synthetic PIM₁ but not of PI in wild-type macrophages (Fig. 4I). As expected, LPS-induced CD40 and

CD86 expression was essentially absent in TRIF-deficient macrophages (Fig. 4J and not shown). The expression of LPS-induced CD40 and CD86 was also strongly reduced in the presence of PIM₁, but not of PI, in MyD88-deficient macrophages (Fig. 4, K and L). Therefore, PIM₁ efficiently inhibited both signals emanating from LPS-TLR4 interaction, namely the MyD88-mediated TRIF-independent cytokine and NO release and the MyD88-independent, TRIF-mediated expression of co-stimulatory molecules CD40 and CD86.

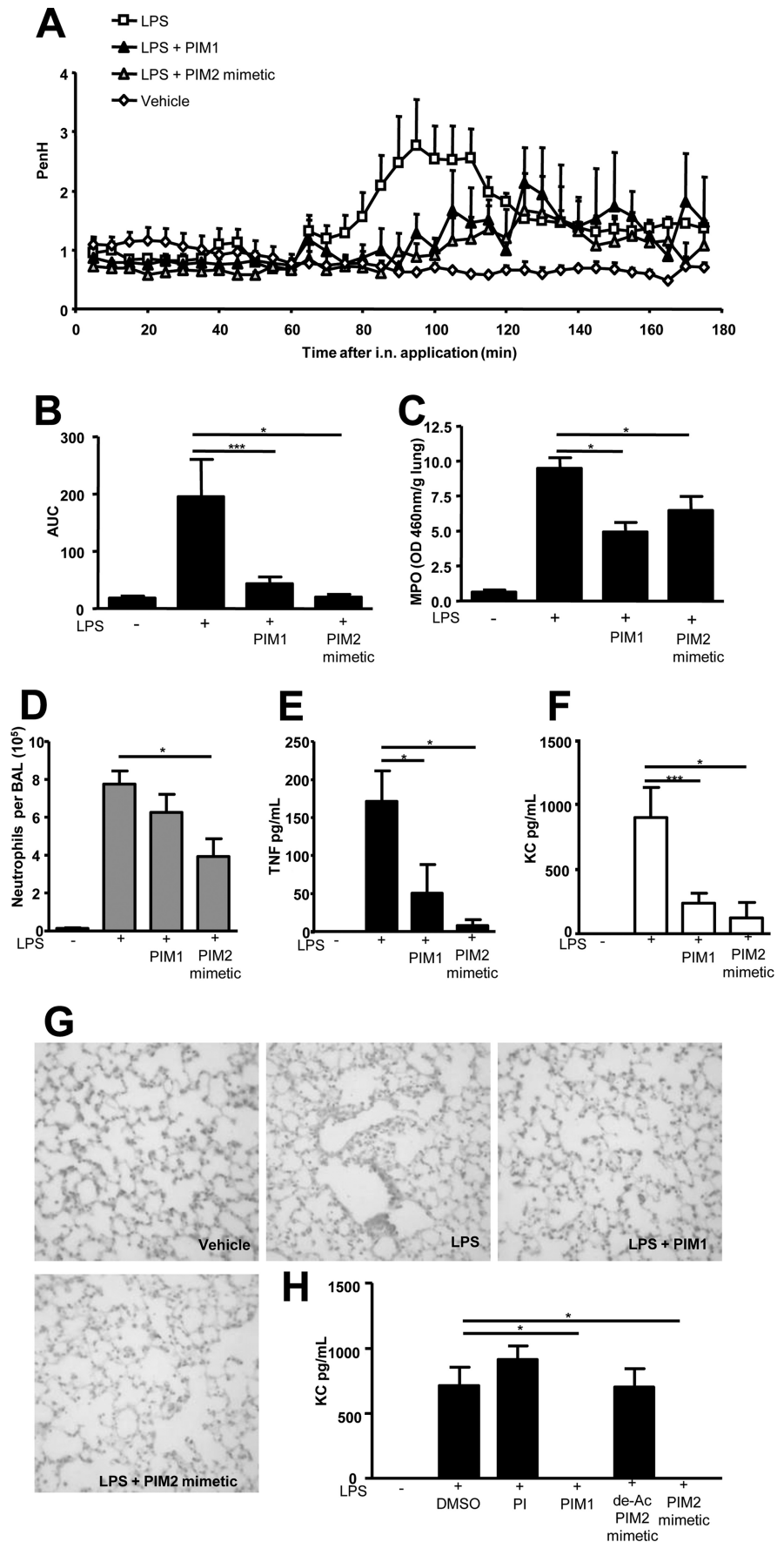
Inhibition of NFκB Translocation by PIM—TLR4-dependent expression of *IL12b*, *IL6*, and *Tnf* genes is dependent upon NF-κB, and we next asked whether PIM activity affects NFκB translocation. NFκB staining is cytoplasmic in unstimulated macrophages and clearly nuclear 1 h after LPS stimulation. In the presence of PIM₁ or PIM₂ mimetic, cells exhibiting only NFκB nuclear staining after LPS stimulation were decreased by 25 and 90%, respectively (Fig. 5A). Similar results were obtained 4 h after LPS stimulation, indicating that NFκB translocation was not merely delayed in the presence of PIM (not shown). Furthermore, we verified that PIM₂ mimetic inhibited the release of TNF and IL-12 p40 in primary macrophage populations, including lung and alveolar macrophages (Fig. 5B). Therefore, PIM₁ and PIM₂ mimetic reduced LPS-induced NFκB translocation, and the cytokine inhibitory effect was seen on different primary macrophage populations.

In Vivo Inhibition of Endotoxin Induced Airway Inflammation by Synthetic PIM—TNF is essential for acute LPS induced respiratory dysfunction as shown in TNF-deficient mice (46, 50). We then asked whether the inhibition of cyto-

kine release by synthetic PIM₁ or PIM₂ mimetic was sufficient for reducing the airway response to endotoxin. Mice exposed to intranasal application of LPS developed an acute increase of enhanced respiratory pause, which was markedly decreased by co-administration of PIM₁ or PIM₂ mimetic (Fig. 6, A and B). The recruitment of inflammatory cells, mostly neutrophils, in the alveolar space seen after LPS treatment was decreased by co-administration of PIM₁ or PIM₂ mimetic (Fig. 6D) and so were neutrophils in the lung, assessed by lung tissue myeloperoxidase activity (Fig. 6C). In the bronchoalveolar fluid LPS-induced secretion of TNF, and KC, the neutrophil attracting chemokine, was reduced by PIM₁ and PIM₂ mimetic (Fig. 6, E and F). Microscopically, lung tissue sections showed strong inflammation and neutrophil infiltration after local LPS, which was partially reduced in the presence of PIM₁ or PIM₂ mimetic (Fig. 6G). Indeed, both compounds abrogated KC secretion by LPS-induced macrophages *in vitro* (Fig. 6H). Furthermore, PIM₁ and PIM₂ mimetic potently inhibited the expression of a series of pro-inflammatory chemokines and cytokines, including CXCL1 (KC), CXCL2 (MIP-2 α), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), IL-1 α , IL-1 β , IL-12/IL-23 p40, IL-12 p35, IL-18, IL-33, TNF, granulocyte-macrophage colony-stimulating factor, oncostatin M, and interferon β but also of IL-10 as early as 2–6 h after LPS stimulation *in vitro* (Fig. 7). Therefore, the synthetic PIM₁ or PIM₂ mimetic effectively inhibited the airway inflammation in response to local LPS exposure *in vivo*, an activity likely reflecting their potent inhibition of a series of proinflammatory chemokines and cytokines.

DISCUSSION

Primary infection by *M. tuberculosis* concerns one-third of the glo-



PIM Inhibit TLR4-mediated Host Innate Responses

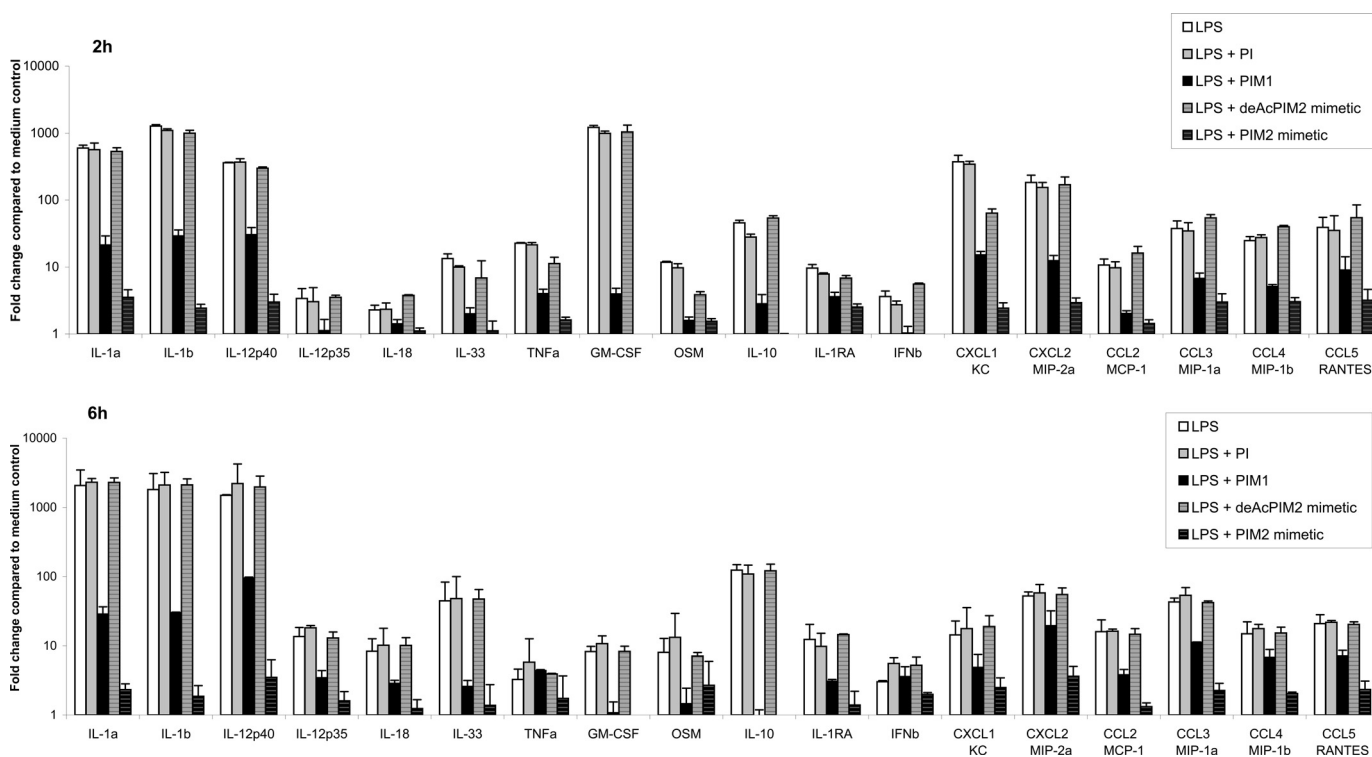


FIGURE 7. Inhibition of chemokine and cytokine gene expression by PIM analogues in LPS-stimulated macrophages. Real-time reverse transcription-PCR quantification of the expression of the indicated cytokine and chemokine genes 2 h (top) and 6 h (bottom) after LPS stimulation (0.1 $\mu\text{g}/\text{ml}$) of bone marrow-derived macrophages in the presence of synthetic PI, PIM₁, PIM₂ mimetic, and de-acetylated Ac₂PIM₂ mimetic (all at 10 $\mu\text{g}/\text{ml}$) or DMSO vehicle alone. The data for the different genes are normalized versus the expression of *Hprt1*, glyceraldehyde-3-phosphate dehydrogenase, 18 S, β_2 -microglobulin house-keeping genes and presented as ratio of stimulated cells over unstimulated controls. Results are expressed as the mean \pm S.D. of $n = 2$ mice and are from one experiment representative of two independent experiments. *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *IFNb*, interferon β . *OSM*, oncostatin M.

bal population, but it remains clinically silent in 9 of 10 infected individuals. The bacterium is still present and viable, ready to flare-up when the immune surveillance fails. To keep control of the immune system, there must be a fine balance between the mycobacteria and the host defense. Several mycobacterial products, including the protein ESAT-6, or the cell wall complex glycolipids LAM or LM down-modulate the host immune responses (7–12). Here we show that the small molecular weight PIM, previously shown to be weak TLR2 agonists, in fact also contain molecular moieties strongly inhibiting the host immune response.

In terms of structure/function relationships, at least one mannosyl and two fatty acids are required on the PIM molecule to inhibit the LPS-induced inflammatory response. Indeed, the most effective inhibitors of cytokine and NO release were PIM₂, PIM₆, and Ac₁PIM₆, whereas Ac₂PIM₆ was slightly less effective, and lyso-PIM₆ was inactive. We reported previously that Ac₂LM exhibited an inhibitory effect, whereas Ac₁LM was inactive, clearly indicating that two fatty acids are required on the LM molecule to inhibit the LPS-induced TNF production, whereas three or four acyl chains are associated with LM pro-

inflammatory activity (11, 12, 21). At least di-acylation of *M. bovis* BCG ManLAM was also necessary to inhibit LPS-induced IL-12 production by human dendritic cells (8). To further confirm that the PIM structure bearing two fatty acids was required and sufficient for anti-inflammatory activity, we synthesized PIM₁. Indeed, we could show that a synthetic preparation of PIM₁ was a potent inhibitor of LPS-induced release of cytokines and NO *in vitro*, whereas PI, which lacks the mannosyl moiety, was not inhibitory. Furthermore, using synthetic PIM derivatives, we could show that the inositol moiety is dispensable for the anti-inflammatory activity, whereas the phosphodiester is essential.

ManLAM anti-inflammatory activity has been largely ascribed to the induction of the anti-inflammatory cytokine IL-10 (9, 10) through C-type lectins such as the mannose receptor and DC-SIGN. In contrast, PIM activity was not mediated by IL-10 overexpression, as PIM strongly inhibited not only pro-inflammatory cytokines like TNF, IL-12/IL-23 p40, and IL-6 but also IL-10 release. In fact, the inhibition of early transcriptional responses was suggestive of a direct effect as the expression of a large panel of LPS-induced cytokines and che-

FIGURE 6. *In vivo* inhibition of endotoxin-induced airway inflammation by synthetic PIM₁ and PIM₂ mimetic. Wild-type C57BL/6 mice were challenged intranasally (*i.n.*) with 1 μg of LPS in the absence or in the presence of synthetic PIM₁ or PIM₂ mimetic or vehicle (1.25% DMSO in saline). A representative experiment showing enhanced respiratory pause (*Penh*) recorded for 180 min using whole body plethysmography is shown (A). The bar graph in B represents the calculated area under the curve (AUC) from two independent experiments (shown in B–F). Neutrophil myeloperoxidase (MPO) activity in the lung was evaluated 24 h after challenge (C). The bronchoalveolar fluid (BAL) was analyzed for neutrophil counts (D) and concentration of TNF (E) and KC (F). PIM₁ and PIM₂ mimetic prevented the recruitment of neutrophils in the lung, as assessed by histological analysis. Representative hematoxylin and eosin staining of lung sections are shown (G, magnification, $\times 400$). The inhibition of KC release by LPS-stimulated bone marrow-derived macrophages after incubation with PIM analogues (used as in Fig. 3) is shown in H. The values represent the mean \pm S.D. of $n = 6$ mice per group from three independent experiments; *, $p < 0.05$.

mokines messages was drastically inhibited by PIM as early as 2 h after macrophage activation. Furthermore, the absence of C-type lectins mannose receptor and SIGN-R1 did not affect PIM inhibition of murine macrophage response (supplemental Fig. 3 and data not shown). Because PIM₆ have a high affinity for human CD1b molecules (51) and PIM₂ form complexes to mouse CD1d (52), we verified using CD1d-deficient macrophages that binding to CD1d was not required for PIM inhibition of proinflammatory cytokines (supplemental Fig. 3).

PIM inhibited TLR4 activation triggered not only by LPS but also by other TLR4 agonists such as *M. tuberculosis* H37Rv LM (12) or a synthetic lipid-A analog OM-197-MP-AC (53) (supplemental Fig. 4). Within the pathways triggered by TLR4, PIM inhibited clearly the MyD88-dependent, TRIF-independent secretion of pro-inflammatory cytokines but also the MyD88-independent, TRIF-dependent expression of co-stimulatory molecules. Central to cytokine transcription in response to TLR trigger is NF κ B activation and translocation. Here, we showed a clear inhibitory effect of PIM on NF κ B translocation induced by LPS/TLR4 activation.

PIM are GPI anchors of mycobacterial LAM and LM. LAM inserts into the plasma membrane of lymphomonocytic cells through their GPI anchors (54). PIM₆ competitively inhibited LAM insertion, and the glycan moiety was important as PI was not as effective. LAM preferentially incorporated into specialized plasma membrane domains enriched in endogenous, host GPI-anchored molecules (54). On the other hand, protozoa GPI-anchor molecules have been shown to contribute to the regulation of host immune response by parasites such as *Trypanosoma* or *Plasmodium*. Although some GPI anchors have been reported to stimulate host inflammatory responses, GPI-anchored mucin from *Trypanosoma cruzi* membrane abrogated monocyte TNF and IL-12 expression (55), and treatment with GPI moiety of *T. cruzi* variant surface glycoproteins reduced macrophage TNF, IL-6, and IL-12 release while increasing IL-10 (56). Here we propose that GPI-anchor PIM are a potent additional weapon for mycobacteria to dampen and control the host immune responses. The GPI-anchor PIM activities are distinct from those of mycobacterial proteins such as ESAT-6, mediated through TLR2 and Akt, and of Man-LAM, mediated through IL-10 overexpression and attributed to the mannosylated cap, absent in PIM structures. PIM activities are also distinct from those of *T. cruzi* GPI anchors as they are not mediated through IL-10 expression, and PIM do not trigger alternative macrophage activation (data not shown). Models of MD-2/TLR4 heterotetramer complex indicate multiple stabilizing contacts between TLR4 and MD-2 in the presence of a full agonist such as lipid A (57). PIM might interfere with the formation or the interactions of activated LPS·MD·2·TLR4 complex. However, we were able to reverse PIM inhibition of LPS-induced IL-12 p40 using inhibitors of kinases known to down-regulate IL-12 expression, such as phosphatidylinositol 3-kinase (data not shown), suggesting that the downstream signaling rather than the primary LPS·MD·2·TLR4 complex is affected by PIM.

We further asked whether PIM could contribute to the modulation of the innate immune response *in vivo*. In particular, because PIM traffic out of the mycobacterial phagosome and

are released to the medium and bystander uninfected cells (58), it was important to determine whether PIM could dampen the innate immune responses in the lung, a target organ for mycobacterial infections. Indeed, we show that local administration of synthetic PIM₁ or PIM₂ mimetic inhibit endotoxin induced lung inflammation in terms of cytokine and chemokine secretion, inflammatory cell recruitment in the airways, and airway dysfunction. Readily available PIM₂ structural analogs such as PIM₂ mimetic that recapitulate or even improve the activity seen in natural or synthetic PIM are of considerable value as potential pharmacologically active leads.

In conclusion, we report the anti-inflammatory activities of PIM. Modulation of PIM release may represent an additional means of regulating the host innate immunity for mycobacteria. Indeed, GPI-anchor PIM could contribute to dampen the activation of infected macrophages and neighboring cells in the tuberculous granuloma and contribute to control the immune response in latent infection. PIM also represent a non-peptidic, small molecular weight, pathogen-derived immunomodulatory molecules with potential as immunotherapeutics.

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