Mycobacterial PIMs Inhibit Host Inflammatory Responses through CD14-Dependent and CD14-Independent Mechanisms

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Abstract

Mycobacteria develop strategies to evade the host immune system. Among them, mycobacterial LAM or PIMs inhibit the expression of pro-inflammatory cytokines by activated macrophages. Here, using synthetic PIM analogues, we analyzed the mode of action of PIM anti-inflammatory effects. Synthetic PIM1 isomer and PIM2 mimetic potently inhibit TNF and IL-12 p40 expression induced by TLR2 or TLR4 pathways, but not by TLR9, in murine macrophages. We show inhibition of LPS binding to TLR4/MD2/CD14 expressing HEK cells by PIM1 and PIM2 analogues. More specifically, the binding of LPS to CD14 was inhibited by PIM1 and PIM2 analogues. CD14 was dispensable for PIM1 and PIM2 analogues functional inhibition of TLR2 agonists induced TNF, as shown in CD14-deficient macrophages. The use of rough-LPS, that stimulates TLR4 pathway independently of CD14, allowed to discriminate between CD14-dependent and CD14-independent anti-inflammatory effects of PIMs on LPS-induced macrophage responses. PIM1 and PIM2 analogues inhibited LPS-induced TNF release by a CD14-dependent pathway, while IL-12 p40 inhibition was CD14-independent, suggesting that PIMs have multifold inhibitory effects on the TLR4 signalling pathway.

Introduction

Mycobacterium tuberculosis induces the formation of granuloma, a “super cellular” structure involving cells both from the innate and the adaptive immune responses, that may play a dual role, for infection containment on the one side, and immune evasion and persistence of viable mycobacteria on the other side. M. tuberculosis are recognized by multiple pattern recognition receptors expressed on alveolar macrophages, their primary host cells, which in turn modulate the immune responses by secreting cytokines and chemokines. TNF, an essential mediator for granuloma formation, is essential for controlling M. tuberculosis infection [1,2], together with IL-12, IFNγ or IL-1 [3–9]. Macrophages also express cytokines that dampen the immune response such as IL-10. Mycobacteria produce a series of molecules modulating the immune system, including the protein ESAT-6, lipomannans (LM), mannose-capped lipoarabinomannan (ManLAM) and their precursors mono- to tetra-acylated phosphatidyl-myo-inositol mannosides (PIM; lyso-PIM for one acyl, PIM for two acyl, Ac₃PIM for three acyl and Ac₄PIM for four acyl, respectively) [10–19].

Several pattern recognition receptors have been implicated in the recognition of mycobacterial LAM, LM and PIMs by macrophages and dendritic cells, such as Toll-like receptors (TLRs) and C-type lectin mannose receptor (CD206) and dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN/CD209) [15–17,20–28]. Tri and teta-acylated LM fractions are pro-inflammatory through TLR2, TLR4 and myeloid differentiation protein 88 (MyD88), and purified fractions of dimannoside PIM₂ and hexamannoside PIM₆, the two most abundant classes of PIMs found in M. tuberculosis H37Rv and M. bovis BCG (bacillus Calmette Guérin), may be proinflammatory through TLR2 [29,30]. Higher-order PIMs with mannose cap-like structures seem to associate with human mannose receptor and to contribute to phagosome-lysosome fusion depending of their degree of acylation, while PIM₂ are recognized by DC-SIGN independently of their acylation degree [31].

Among the anti-inflammatory activities, ManLAM inhibition of LPS-induced IL-12 production in dendritic cells was attributed to DC-SIGN [15]. We showed recently that di-acylated LM, but also purified fractions of PIM₂ and PIM₆ and synthetic PIM₁ and PIM₂ analogues inhibit LPS/TLR4-induced cytokine response independently of TLR2, SIGN-R1 and mannose receptor [18,19]. Suppression of ovalbumin-induced allergic airway eosinophilia, a model dependent on LPS response [32], by natural or synthetic PIMs, and by a PIM₂ analogue was reported [33–35]. Thus, not only complex mycobacterial lipoglycans like ManLAM and LM, but also small molecular weight PIMs are potent inhibitors of host inflammatory responses.
LAM were also shown to insert into mononuclear cell plasma membranes [36] and to modify the signalling machineries of rafts/microdomains [37]. LAM GPI anchor PIMs competitively inhibited LAM insertion into plasma membranes, likely into specialized domains enriched in endogenous GPI-anchored molecules [36]. Although TLR4 is a major receptor for the cellular response to LPS, cells need to express co-receptors such as the GPI-anchored CD14 or MD2 to mount a full response to LPS. MD2 is indeed necessary for the processing and membrane expression of TLR4 as well as for LPS signalling [38–40] while the GPI-anchored CD14 is required for the LPS binding to MD2/TLR4 and subsequent signalling [41,42].

Here, using synthetic PIM1 and PIM2 analogues, we analyzed the mode of action of PIM anti-inflammatory effects. We investigated LPS binding on TLR4/MD2/CD14 expressing cells and found that PIMs inhibit this step and more specifically the LPS binding to CD14. By using a shorter form of LPS, rough-LPS, that stimulates TLR4 pathway independently of CD14 [41], we investigated LPS binding on TLR4/MD2/CD14 expressing cells. Our data show that PIM1 and PIM2 analogues inhibit the LPS-induced TNF production by a CD14-dependent pathway while the IL-12 p40 inhibition is CD14-independent, suggesting that PIMs have multifold inhibitory effects on TLR4 signalling pathway.

Materials and Methods

Ethics statement

The study of immune responses to mycobacteria infections was approved by the Regional ethics committee for animal experiments (CL2008-011).

Mice

Six to 12 week old mice deficient for TLR2 [43], TLR4 [44], CD14 (obtained from Freeman, M.W [45]), MD2 [39] and wild-type C57Bl/6 mice were bred at the Transgenose Institute animal facility (UPS4 TAAM, Orleans, France).

Synthetic PIMs

PIM1 containing a C16 and a C18 chain in the glycerolipid unit (2-O-α-D-mannopyranosyl-1-O-phosphatidyl-D-myo-inositol), together with the PIM1 isomer (1-O-α-D-mannopyranosyl-2-O-phosphatidyl-D-myo-inositol), PIM2 mimetic [1,3-bis(α-D-mannopyranosyl)-2-O-phosphatidyl glycerol], and the reference compound phosphatidyl inositol (PI, 1-O-phosphatidyl-D-myo-inositol), were prepared as described [19].

LPS binding to cells

Human embryonic kidney (HEK) 293 cells were obtained from the Centre for Applied Microbiology and Research (Porton Down, Salisbury, Wiltshire, UK) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mm L-glutamine and maintained at 37°C in a humidified atmosphere of 5% CO₂. HEK293 cells transfected with TLR4/MD2/CD14 (HEK-MTC) were obtained from Invivogen (San Diego, CA) and maintained in the same medium as above supplemented with Hygrogold (Invivogen) and blastocidin (Invivogen). HEK-MTC cells (1×10⁶ cells in 50 mL in DMEM 10% FCS) were incubated with 10 μg/mL of PIM or vehicle for 30 min at 37°C under gentle agitation prior incubation with biotinylated smooth LPS (S-LPS; Escherichia coli, serotype O111:B4, InvivoGen) at a final concentration of 2.5 μg/mL prepared in DMEM 10% FCS for 15–20 min. Cells were washed with ice cold PBS and stained with streptavidin-ITC on ice. After fixation with 3% paraformaldehyde, binding of S-LPS-biotin to cells was measured on a BD FACS CaliburTM. S-LPS-binding on bone marrow derived macrophages (see below) was also investigated by using DMEM supplemented with 0.1% FCS and a final concentration of 5 μg/mL of S-LPS-biotin prepared in DMEM 0.1% FCS and S-LPS-binding was measured with a BD FACS CantoTM II.

LPS binding to soluble CD14

Soluble recombinant mouse CD14 was coated overnight at 4°C (5 μg/mL on Nunc 96-well plates; R&D systems, Abingdon, UK) and non specific binding saturated with 2% BSA in PBS for 1 hr at 37°C. The plates were washed three times in PBS before incubation with synthetic PIMs (10 μg/mL; 1 hr at 37°C) before addition of biotinylated S-LPS for 2 hrs at 37°C (100 ng/mL, InvivoGen) in PBS containing 1% of fetal calf serum. Alternatively, 0.1% serum from wild-type or LBP-deficient mice [46] was used, as indicated. Unbound S-LPS-biotin was removed with four PBS washes, and bound S-LPS-biotin was detected with horseradish peroxidase avidin D conjugate (1/2000, Vector laboratories) diluted in 1% BSA in PBS. After addition of the ABTS substrate (2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid at 0.3 g/L in 0.1 M anhydrous citric acid containing 0.3% H₂O₂), absorbance at 405 nm was measured with a microplate reader (Bio-Tek Instrument, INC). Competition with increasing concentrations of ultrapure S-LPS (E. coli, serotype O111:B4, InvivoGen, San Diego, CA) was performed to assess binding specificity.

Cell culture

Murine bone marrow cells were isolated from femurs and cultivated (10⁶/mL) for 7 days in DMEM supplemented with 2 mM L-glutamine, 20% horse serum and 50% L929 cell-conditioned medium as source of M-CSF. After further three days in fresh medium, the cell preparation contained a homogenous population of macrophages (97–98% CD11b⁺F4/80⁺). The bone marrow derived macrophages (BMDM; 10⁶ cells/well) in DMEM supplemented with 2 mM L-glutamine and 0.1% FCS were stimulated with 100 ng/mL of S-LPS (E. coli, serotype O111:B4, CD14-dependent, ultrapure S-LPS from InvivoGen or Sigma, St Louis, MO), 0.5 μg/mL of synthetic bacterial lipopeptide Pam,C5K4 (S-[2,3-bis-(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys₉-Oh), tri hydrochloride, EMC Microcollections, Tuebingen, Germany), 30 ng/mL Malp₂ (S-[2,3-bis Acyloxypropyl]-cysteine-GNNDNSISFKEK, Alexius Biochemicals, Lausanne, Switzerland), 0.125 μM of Cpg ODN1826 (ttcatgcgtctgctcttggt, Invivogen), 3 μM Taxol (Alexis). A CD14-independent, rough-LPS (Re-LPS; E. coli, serotype 515, Alexis) was also used, as indicated. Lyophilised PIM preparations were solubilised in DMSO and added to the cultures at the indicated concentration 30 min prior to the stimuli in a solution containing a maximum non-cytotoxic, 1% DMSO final concentration. Vehicle controls at the relevant DMSO concentration are included in each experiment. PIMs were incubated in presence or absence of 5 μg/mL recombinant mouse CD14 Fc chimera (endotoxin <1.0EU/μg protein; R&D system). The macrophages were activated with IFN-γ (300 U/mL) to study IL-12 release, and the supernatants harvested after 24 hours for further analysis. Absence of cytotoxicity of the stimuli was controlled using MTT incorporation. To control PIMs anti-inflammatory activity on human embryonic kidney (HEK) cells, HEK-MTC cells (6×10⁴ cells/well) were stimulated with S-LPS in the presence of PIM.
analogues as above and human IL-8 concentration was measured in the supernatant after overnight incubation.

**Cytokine ELISA**

Supernatants were harvested and assayed for cytokine content using commercially available ELISA reagents for murine TNF, murine IL-12 p40, and human IL-8 (Duoset R&D Systems).

**Statistical analysis**

Statistical significance was determined with Graph Pad Prism software (version 4.0, San Diego, CA) by one or two way parametric ANOVA test followed by Bonferroni post-test. P values <0.05 were considered statistically significant.

**Results**

**Interference of PIMs with LPS binding to cells**

We showed previously that synthetic PIM1 and PIM2 mimetic (Figure S1) inhibit TNF and IL-12 p40 release by macrophages stimulated with low dose LPS ([19] and Figure 1A, B) at micromolar concentrations (Figure 1C, D). We thus asked whether PIMs could interfere with LPS-binding to cells (Figure 1E-J). By using HEK cells transfected with TLR4, MD2 and CD14, we showed that binding of biotinylated smooth LPS (S-LPS; E. Coli serotype O111:B4) was partially inhibited by PIM1 (Figure 1F), a PIM4 isomer (isoPIM1) (Figure 1G) and a PIM2 mimetic (Figure 1I) but not by phosphatidyl inositol (PI; Figure 1E) or by a deacylated (Figure 2G). Therefore, the inhibitory effects of synthetic PIM1 inhibition of S-LPS response could be seen in the absence of TLR2 effect is independent of the TLR4 pathway. Conversely, the release by S-LPS-stimulated HEK cells (Figure 1L). PIM 1, of TLR4/MD2/CD14 (Figure 1J). The inhibition of S-LPS binding of S-LPS-biotin was detected on HEK cells in the absence specific cellular binding of biotinylated S-LPS. However, no as PIMs, indicating a non-saturable, and maybe partially non-unlabelled S-LPS competed only partially the binding of (Figure S1) inhibit TNF and IL-12 p40 release by macrophages analogue inhibition of S-LPS-induced TNF (Figure S4A). Soluble CD14 binding to sCD14, in an LBP-independent way, but CD14 may occurred in mouse serum from wild-type or LBP-deficient mice, while essentially absent in CD14-deficient BMDM macrophages, while it was only slightly reduced in TLR4-deficient macrophages and similar in MD2-deficient and wild-type macrophages (Figure S5).

**Synthetic PIM analogues potently inhibit TLR4 and TLR2 induced pathways**

We showed previously that the inhibitory effects of the natural PIM4 fractions were preferentially targeted to the TLR4 signalling pathway, although the specificity was not absolute for IL-12 p40 release [19]. Using more potent synthetic PIM1 and PIM2 analogues, we readdressed TLR specificity. Specific TLR4 agonist S-LPS, TLR2/TLR1 agonist Pam3CSK4, TLR2/TLR6 agonist Malp2, and TLR9 agonist CpG, were used to activate candidate. Indeed, natural PIM4 from M. kansasii was shown to interact with CD14 [50] and S-LPS binding was shown to depend on the presence of CD14 [51]. We first confirmed that S-LPS binding depended on the presence of CD14 as S-LPS binding was shown to depend primarily on the presence of CD14 [52]. S-LPS-biotin binding to sCD14 coated on a solid phase was strongly inhibited by PIM1, isoPIM1, and PIM2 mimetic but not by PI and deAcPIM2 mimetic controls (Figure 1H). Excess of unlabelled S-LPS competed only partially the binding of biotinylated S-LPS (data not shown), although to the same extent as PIMs, indicating a non-saturable, and maybe partially non-specific cellular binding of biotinylated S-LPS. However, no binding of S-LPS-biotin was detected on HEK cells in the absence of TLR4/MD2/CD14 (Figure 1J). The inhibition of S-LPS binding (Figure 1K) was accompanied with an inhibition of IL-6 release by S-LPS-stimulated HEK cells (Figure 1L). PIM1, isoPIM1, and PIM2 mimetic also affected S-LPS-binding to primary macrophages (Figure S2). Thus, there was a partial decrease of S-LPS-biotin binding to TLR4/MD2/CD14 expressing HEK cells as well as primary macrophages in the presence of PIMs.

**Not only amphiphilic, acylated TLR agonists, but also Taxol is inhibited by PIM**

The TLR2 and TLR4 ligands tested above were acylated amphiphilic molecules. Since LAM were shown to form micelles [47] and PIMs may also do so, we next wanted to exclude that PIMs act by scavenging the different acylated TLR4-agonist S-LPS, or TLR2-agonists Pam3CSK4 and Malp2. We thus asked whether PIMs could also inhibit macrophage activation triggered by Taxol, a TLR4 agonist of a different molecular class [48,49]. The contribution of potentially contaminating endotoxins in this stimulation was excluded by pre-incubating Taxol with polymyxin B at a concentration sufficient to neutralise 100 ng/mL of LPS (data not shown). As shown in Figure 3A, Taxol is not acylated, it requires the presence of TLR4 to stimulate TNF release by bone marrow-derived macrophages (Figure 3B), and Taxol stimulation is potently inhibited by isoPIM1 and PIM2 mimetic but not by PI and deAcPIM2 mimetic controls (Figure 3C). Thus, a TLR4 ligand unlikely to form micelles is also susceptible to PIM inhibition.

**Interference of PIM analogues with smooth LPS binding to CD14**

Since synthetic PIM analogues could target both TLR2 and TLR4 pathways we hypothesized that they may interact with a co-receptor common to TLR2 and TLR4. CD14 was a likely candidate. Indeed, natural PIM4 from M. kansasii was shown to interact with CD14 [50] and S-LPS binding was shown to depend on the presence of CD14 [51]. We first confirmed that S-LPS binding depended on the presence of CD14 as S-LPS binding was essentially absent in CD14-deficient BMDM macrophages, while it was only slightly reduced in TLR4-deficient macrophages and similar in MD2-deficient and wild-type macrophages (Figure S5).

We then tested directly the ability of PIMs to interfere with S-LPS binding to soluble CD14 (sCD14) in presence of serum. Indeed, LPS-binding protein (LBP) present in serum increases LPS binding to sCD14 (not shown; [52]). S-LPS-biotin binding to sCD14 coated on a solid phase was strongly inhibited by PIM1, isoPIM1, and PIM2 mimetic but not by PI and deAcPIM2 mimetic controls (Figure 4). To avoid the contribution of LBP in this interaction, we compared S-LPS-binding to sCD14 in fetal calf serum (Figure 4A), or in serum from wild-type (Figure 4B) or LBP-deficient mice (Figure 4C). Inhibition of S-LPS-biotin binding to sCD14 by PIM1, isoPIM1, and PIM2 mimetic but not by PI and deAcPIM2 mimetic controls occurred in mouse serum from wild-type or LBP-deficient mice, thus in the presence or in the absence of LBP. The inhibition of S-LPS binding to sCD14 by PIM1 was slightly weaker than the inhibition by isoPIM1 or PIM2 mimetic, similar to the effect seen on whole cell S-LPS-binding. Binding of biotinylated S-LPS was effectively competed by unlabelled S-LPS in this system (Figure 4D).

To address the functional relevance of this interaction, we asked whether soluble CD14 might “scavenge” some PIM molecules and reduce PIM inhibition on S-LPS-induced TNF response. Addition of sCD14 had essentially no effect on isoPIM1 or PIM2 mimetic inhibition of S-LPS-induced TNF (Figure S4A). Soluble CD14 was used at a concentration effective for restoring some S-LPS functional effect in CD14-deficient macrophages (Figure S4B). Therefore, anti-inflammatory PIMs can prevent S-LPS binding to sCD14, in an LBP-independent way, but CD14 may not be directly involved in PIM inhibitory effects.

**PIM inhibition of TLR2-induced cytokine responses is independent of CD14**

CD14 is able to recognize different ligands beside LPS and it has been involved in TLR2-signaling induction in response to...
CD14 Involvement in PIM Inhibition of Inflammation

A and B: Graphs showing the effect of S-LPS on TNF and IL-12p40 levels. Vehicle, deAcPIM2 mimetic, and PIM2 mimetic treatments are compared.

C and D: Graphs showing the percentage inhibition of TNF and IL-12p40 by PIM2 mimetic.

E, F, G, H, I, J: Flow cytometry histograms comparing different treatments including Vehicle, PI, PIM1, isoPIM1, deAcPIM2 mimetic, and PIM2 mimetic.

K and L: Graphs showing % LPS binding and IL-8 levels with S-LPS treatments including Vehicle, PI, PIM1, isoPIM1, deAcPIM2 mimetic, and PIM2 mimetic.
CD14 involvement in PIM inhibition of inflammation

Figure 1. Synthetic PIM analogues inhibit S-LPS-induced responses and binding to HEK cells expressing TLR4, MD2 and CD14. Bone marrow derived macrophages (A, B) were stimulated with increasing concentrations of S-LPS in presence of 10 μg/mL of PIM1 mimetic or deAcPIM2 mimetic, or vehicle control, and TNF (A) and IL-12 p40 (B) were measured in supernatant after overnight incubation. Results are mean ±/− SEM from n = 6 mice from three independent experiments. PIM analogues were titrated (C, D) in the presence of 0.1 μg/mL S-LPS and a 10 μg/mL dose was chosen as this concentration was sufficient for the active PIMs to strongly inhibit LPS-induced TNF (C) and IL-12 p40 (D) release without cytotoxicity. (E–J) HEK cells stably transfected with TLR4, MD2 and CD14 were incubated with PI (E), PIM1 (F), isoPIM1 (G), deAcPIM2 mimetic (H) or PIM2 mimetic (I) (10 μg/mL; dotted line) prior to incubation with biotinylated S-LPS (2.5 μg/mL) and streptavidin FITC (black line) or only streptavidin FITC (grey histogram). Non transfected HEK cells were incubated with biotinylated S-LPS as a control (J). Results are from one experiment representative of three independent experiments. (K) Percentage of S-LPS-binding to HEK-MTC cells in presence of vehicle, PI, isoPIM1, deAcPIM2 mimetic or PIM2 mimetic. Results are the mean ±/− SD from three independent experiments. (l) Human IL-8 was measured in the supernatant after overnight S-LPS stimulation of HEK-MTC cells. Results are mean ±/− SD from triplicates, from one experiment representative of three independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001 versus vehicle; †, p<0.05; ††, p<0.01; †††, p<0.001 indicate significant differences between PIM1 or isoPIM1 versus PI as control; ††, p<0.01; †††, p<0.001 indicate significant differences between PIM2 mimetic and deAcPIM2 mimetic as control.

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To further address the role of CD14 in the PIM inhibition of S-LPS-induced response, we next investigated PIM anti-inflammatory effect on the CD14-independent stimulation by high S-LPS concentrations. Interestingly, TNF release stimulated by 1–3 μg/mL of S-LPS, in a CD14-independent way (see Figure 6), was not inhibited by PIM2 mimetic (Figure 8A), while CD14 independent release of IL-12 p40 induced by S-LPS at 1–3 μg/mL concentrations was strongly inhibited by PIM2 mimetic (Figure 8B). When titrated in parallel by increasing the ratio of S-LPS over PIM concentrations, TNF release was clearly less inhibited than IL-12 p40 release.

Thus, the fact that both CD14-independent Re-LPS-induced IL-12 p40 release and CD14-independent, high dose S-LPS-induced IL-12 p40 were inhibited by isoPIM1 or PIM2 mimetic indicated that PIMs affect IL-12 p40 release independently of CD14. Conversely, the fact that both CD14-independent Re-LPS-induced TNF release and CD14-independent, high dose S-LPS-induced TNF were not inhibited by isoPIM1 and PIM2 mimetic, while CD14-dependent S-LPS induced TNF was inhibited, suggested that PIM inhibition of TNF release targeted a CD14-dependent pathway.

Discussion

Bacterial pathogens have developed numerous strategies to undermine host innate responses and promote infection [56,57]. PRRs such as TLR2 or TLR4 are crucial to detect different PAMPs and to coordinate signals that allow host cells to induce a range of defence mechanisms, including oxidative stress, autophagy and cell death. However, PRRs are also targets for microorganisms to subvert both immune recognition and intracellular signalling. Here we show that PIM1 and PIM2 analogues interfere with the pathways activated by both TLR2 and TLR4. M. tuberculosis and M. bovis were also shown to trigger TLR2 and TLR4 pathways and produce TLR2 but also TLR4 agonists such as M. bovis tetra-acylated LM or M. tuberculosis LM [19]. Mycobacteria thus produce on the one hand PAMPs that are recognized by the host, and on the other hand molecules that can interfere with the host innate immune responses, with a possible balance between those. Indeed, PIMs inhibit macrophage activation by M. tuberculosis LM [19].

We reported previously that some natural and synthetic PIMs inhibit the expression of NO, a potent mycobacterial mediator, and of pro-inflammatory cytokines essential for host response to mycobacteria such as TNF, IL-12p40 and IL-1 in vitro and in vivo in response to LPS [19]. In line with this, natural or synthetic PIMs [33,34] or a synthetic PIM2 analogue [35] suppress ovalbumin-induced allergic airway eosinophilia, a model in which LPS contaminant has been shown to play a crucial role [32]. Here, in order to further understand the role that PIMs may play in immune evasion, we thus addressed molecular mechanisms.
involved in the inhibition by PIM analogues of LPS pro-inflammatory responses.

PIMs were described as mycobacterial adhesins mediating binding to mammalian cells, but this effect was mostly attributed to high order, polar PIM5 or PIM6 [58]. PIMs interact with several cell surface receptors, including not only TLR2 but also CD1d [59,60], and C-type lectins mannose receptor or DC-SIGN [31]. However, we showed previously that synthetic PIM1 or PIM2 mimetic analogues are not TLR2 agonists as they do not trigger inflammatory responses at micromolar concentrations, and that

**Figure 2. Synthetic PIM analogues inhibit TLR4 and TLR2 pathways but not TLR9.** Macrophages from C57Bl/6 (A–D), TLR4 KO (E, F) or TLR2 KO mice (G) were activated with TLR2 agonist Malp2 (A, E) or Pam3CSK4 (Pam3; B, F), TLR4 agonist S-LPS (C, G) or TLR9 agonist CpG (D) in presence of synthetic PI, isoPIM1, deAcPIM2 mimetic, PIM2 mimetic (all at 10 µg/mL), or vehicle. TNF production was measured in the supernatant after overnight incubation. Results are from n = 4–6 mice from two to three independent experiments (A–B, D–F) or n = 2 mice from one experiment representative of two independent experiments (C, G). ND: not detected. *, p<0.05; **, p<0.01; ***, p<0.001 indicate significant differences between isoPIM1 versus PI as control; {, p<0.05; {, p<0.01 indicate significant differences between PIM2 mimetic and deAcPIM2 mimetic as control.

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CD1d, mannose receptor and SIGN-R1 are dispensable for PIM inhibition of LPS-induced pro-inflammatory response in murine macrophages [19]. We thus asked whether ‘anti-inflammatory’ PIM analogues could compete with LPS for binding on target cells. Using flow cytometry to quantify LPS-binding to HEK cells expressing synthetic PI, isoPIM1, deAcPIM2 mimetic, PIM2 mimetic (all at 10 μg/mL), or vehicle (C) and TNF concentrations were measured in the supernatant after overnight incubation. Results are mean ± SD from n = 2 mice from one experiment representative of 2 to 3 independent experiments. ND: not detected.

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**Figure 3. Synthetic PIM analogues inhibit TLR4-dependent, Taxol induced TNF release.** The antituomral compound Taxol (A) was used at increasing concentrations to stimulate macrophages from C57Bl/6 and TLR4 KO mice, showing the TLR4 specificity (B). BMDM from C57Bl/6 mice were stimulated with Taxol (3 μM) in the presence of synthetic PI, isoPIM1, deAcPIM2 mimetic, PIM2 mimetic (all at 10 μg/mL), or vehicle (C) and TNF concentrations were measured in the supernatant after overnight incubation. Results are mean ± SD from n = 2 mice from one experiment representative of 2 to 3 independent experiments. ND: not detected.

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**Figure 4. Synthetic PIM analogues inhibit S-LPS-binding to soluble CD14.** The effects of PIMs on the binding of biotinylated S-LPS to sCD14 was investigated in presence of 1% FCS (A, D), or 0.1% serum from wild-type mice (B) or from LBP-deficient mice (C). Solid phase adsorbed sCD14 was incubated (1 hr at 37 °C) in the presence of synthetic PI, PI, isoPIM1, deAcPIM2 mimetic and PIM2 mimetic (all at 10 μg/mL) or vehicle, before addition of biotinylated S-LPS (0.1 μg/mL; 2 hrs at 37 °C). Binding specificity was determined by incubation with increasing concentrations of non biotinylated S-LPS 1 hr prior to biotinylated S-LPS (D). Results are expressed as percentage of biotinylated S-LPS-binding to sCD14 as compared to incubation with vehicle and are mean ± SD from three independent experiments. **,** p < 0.001 versus vehicle; **,** p < 0.001 versus isoPIM1 as control; **,** p < 0.001 indicate significant differences between isoPIM1 versus PI as control; **,** p < 0.001 indicate significant differences between PIM2 mimetic and deAcPIM2 mimetic as control.

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**Figure 3. Synthetic PIM analogues inhibit TLR4-dependent, Taxol induced TNF release.** The antituomral compound Taxol (A) was used at increasing concentrations to stimulate macrophages from C57Bl/6 and TLR4 KO mice, showing the TLR4 specificity (B). BMDM from C57Bl/6 mice were stimulated with Taxol (3 μM) in the presence of synthetic PI, isoPIM1, deAcPIM2 mimetic, PIM2 mimetic (all at 10 μg/mL), or vehicle (C) and TNF concentrations were measured in the supernatant after overnight incubation. Results are mean ± SD from n = 2 mice from one experiment representative of 2 to 3 independent experiments. ND: not detected.

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**Figure 4. Synthetic PIM analogues inhibit S-LPS-binding to soluble CD14.** The effects of PIMs on the binding of biotinylated S-LPS to sCD14 was investigated in presence of 1% FCS (A, D), or 0.1% serum from wild-type mice (B) or from LBP-deficient mice (C). Solid phase adsorbed sCD14 was incubated (1 hr at 37 °C) in the presence of synthetic PI, PI, isoPIM1, deAcPIM2 mimetic and PIM2 mimetic (all at 10 μg/mL) or vehicle, before addition of biotinylated S-LPS (0.1 μg/mL; 2 hrs at 37 °C). Binding specificity was determined by incubation with increasing concentrations of non biotinylated S-LPS 1 hr prior to biotinylated S-LPS (D). Results are expressed as percentage of biotinylated S-LPS-binding to sCD14 as compared to incubation with vehicle and are mean ± SD from three independent experiments. **,** p < 0.001 versus vehicle; **,** p < 0.001 versus isoPIM1 as control; **,** p < 0.001 indicate significant differences between isoPIM1 versus PI as control; **,** p < 0.001 indicate significant differences between PIM2 mimetic and deAcPIM2 mimetic as control.

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CD1d, mannose receptor and SIGN-R1 are dispensable for PIM inhibition of LPS-induced pro-inflammatory response in murine macrophages [19]. We thus asked whether ‘anti-inflammatory’ PIM analogues could compete with LPS for binding on target cells. Using flow cytometry to quantify LPS-binding to HEK cells expressing LPS receptor and co-receptors TLR4, MD2 and CD14 or to primary macrophages, we show that anti-inflammatory PIM1, isoPIM1 and PIM2 mimetic partially inhibited the binding of biotinylated S-LPS to cells while inactive controls PI and non-acylated, deAcPIM2 mimetic did not. The extent of competition achieved with active PIM1 and PIM2 analogues was similar to that observed with an excess of unlabelled S-LPS, although incomplete, which might indicate some non-saturable and potentially non-specific cellular binding of biotinylated S-LPS. Increased internalization of TLR4 was unlikely responsible for the decreased S-LPS-binding by PIMs. Indeed, PIMs prevented the down-regulation of TLR4 mRNA expression seen 2 h after S-LPS-stimulation (data not shown). Furthermore, macrophage pre-treatment with cytochalasin D did not affect PIMs inhibitory activities (data not shown).

Natural PIMs inhibited preferentially the TLR4 pathway [19], suggesting a specific interaction of the PIMs with TLR4 or TLR4 pathway. However, using more active, synthetic PIM analogues we demonstrated PIM inhibitory effects on macrophage responses to either TLR2 or TLR4 agonists. The inhibition of TLR2/TLR1 agonist PamC3SK4 and TLR2/TLR6 agonist Malp2 induced responses occurred even in the absence of TLR4 and, conversely, the inhibition of TLR4 agonist S-LPS response occurred in the
Figure 5. Inhibition of TLR2 signaling by PIM analogues is independent of CD14. Macrophages from C57Bl/6 mice (A, C) or CD14 KO mice (B, D) were incubated with synthetic PI, isoPIM1, deAcPIM2 mimetic, PIM2 mimetic (10 μg/mL) or control vehicle prior to stimulation with Malp2 (30 ng/mL; A, B) or Pam3CSK4 (Pam3; 0.5 μg/mL; C, D). TNF release was measured in supernatants after overnight incubation. Results are mean ± SD from n = 4 mice from two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus vehicle. ##, p < 0.01; hhh, p < 0.001 indicate significant differences between isoPIM1 versus PI as control; †, p < 0.05; †††, p < 0.001, indicate significant differences between PIM2 mimetic versus deAcPIM2 mimetic as control.

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Figure 6. CD14-dependency of TNF and IL-12 p40 release induced by S-LPS versus Re-LPS. Macrophages from C57Bl/6 or CD14 KO mice were stimulated with increasing concentrations of S-LPS (A, B) or Re-LPS (C, D). TNF (A, C) and IL-12 p40 (B, D) concentrations were measured in the supernatants after overnight incubation. Results are mean ± SEM from n = 4 mice from two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 indicate significant differences between C57Bl/6 and CD14 KO.

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Figure 7. Differential inhibition of S-LPS versus Re-LPS induced TNF and IL-12 p40 release by PIMs. Concentrations of TNF (A–C) and IL-12 p40 (D–F) in supernatants of wild type (A, B, D, E) or CD14-deficient (C, F) macrophages stimulated overnight with 100 ng/mL of S-LPS (A, D) or Re-LPS (B, C, E, F) in the presence of synthetic PI, isoPIM1, deAcPIM2 mimetic, PIM2 mimetic (10 μg/mL), or vehicle. Results are mean ± SD from n = 4 mice from two independent experiments representative of three independent experiments. ND: not detected. **, p < 0.01; ***, p < 0.001 versus vehicle. 0.01; ***, p < 0.001 indicate significant differences between isoPIM1 versus PI as control; ††, p < 0.01; †††, p < 0.001 indicate significant differences between PIMs, mimetic versus deAcPIM2 mimetic as control.

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absence of TLR2. These results indicated that cytokine responses to both TLR2 and 4 pathways can be inhibited by active PIMs and suggested that PIMs were unlikely to act through an exclusive interaction with TLR4. We hypothesised that PIMs may target a co-receptor common to both TLR2 and TLR4. Since PIMs are GPI-anchor, amphiphilic structures with acylated moieties, they might interfere with the organization of supramolecular coreceptors/receptors multimeric complexes involved in both TLR2 and TLR4 pathways. Indeed, LAM GPI anchor PIM6 competitively inhibit the insertion of LAM into mononuclear cell plasma membranes, likely into specialized domains enriched in endogamous GPI-anchored molecules [36]. LAM were shown to modify the signalling machineries of rafts/microdomains [37]. We investigated CD14, one of the GPI-anchored proteins present in hematopoietic cell microdomains, as a potential target candidate for PIMs effect on the TLR2 and TLR4 pathways. Indeed, CD14 is necessary for S-LPS binding to cells and subsequent signalling [51] and CD14 was also implicated as a first step in Pam3CSK4 recognition, inducing physical proximity with TLR2/TLR1 and formation of the TLR2 signalling complex [61]. Natural PIM2 from M. kansasii was shown to interact with CD14 [50], and CD14 was implicated in mycobacterial LM and H37Ra LAM pro-inflammatory activities [54,62]. Here, we documented the inhibition of S-LPS binding to soluble CD14 by the anti-inflammatory PIM1, isoPIM1 and PIM2 mimetic, but not by PI or a deacylated PIM2 analogue. Thus, PIM derivatives interfered with S-LPS binding to cells, and S-LPS-interaction with CD14 was a likely target for this inhibition. However, PIM inhibition of S-LPS-interaction with sCD14 was independent of the presence of LBP. Further, PIM inhibition of S-LPS-induced TNF release was not restored by addition of soluble CD14 to cells, indicating that PIMs might not directly compete with S-LPS for binding to CD14, but might rather affect an earlier step independent of LBP. Indeed, several receptors found in serum are involved in LPS disaggregation like HMGB1 [63], and might be considered.

We then addressed the functional implication of CD14 in PIM anti-inflammatory effects by using macrophages deficient for CD14. A partial CD14-dependency was reported for Malp2, but not for Pam3CSK4 induced TNF response [42], while in our hands Malp2-induced TNF release was CD14 independent. The CD14 independent activation of TLR2 agonists Malp2 and Pam3CSK4 was reduced by isoPIM1 and PIM2 mimetic, indicating that active PIMs inhibit TLR2 signalling pathways by a mechanism independent of CD14. We next asked whether PIM interference with LPS-CD14 was a necessary component of the functional inhibition of LPS-induced pro-inflammatory responses by PIMs, at different levels. Active PIM analogues inhibited CD14-independent Re-LPS-induced IL-12 p40 as well as CD14-independent IL-12 p40 stimulation induced by high S-LPS concentrations. However, while CD14-dependent TNF release was potently inhibited by PIM1 and PIM2 analogues, neither CD14-independent Re-LPS induced TNF release, nor CD14-independent, high dose S-LPS-induced TNF were affected by PIMs. Thus, CD14-independent IL-12 p40 release was inhibited by PIM1 and PIM2 derivatives, while the CD14-independent TNF release was not. These data suggest that PIMs affected IL-12 p40 release independently of CD14 while PIMs targeted a CD14-dependent pathway for inhibition of TNF release.

We propose that PIMs may exert their inhibitory activity through different ways, by inhibiting S-LPS binding to CD14, and by interfering at another level. Indeed, CD14 participates in LPS-induced TNF production in RAW cells and peritoneal macrophages while a CD14-independent pathway is used in Kupffer cells [64]. Further, although CD14 is essential for cell binding and activity of low dose smooth LPS, CD14 is dispensable at high doses of S-LPS and for binding and cell activation by rough LPS [41,42], confirming that TLR4 ligands can induce TNF and IL-12 production by different mechanisms which might not be equally affected by PIMs. IL-12 p40 release after S-LPS stimulation requires CD14 in macrophages, but other receptors such as CD11b and CD18 (Mac-1) have been involved in the optimal expression of IL-12 p40 and IL-12 p35 genes in response to LPS or Taxol [65]. The regulation of IL-12 p40 expression is complex [66]. One major regulator of IL-12 p10 production is the anti-
inflammatory cytokine IL-10. We showed previously that PIM inhibitory activity was not dependent on an increase in IL-10 expression as this cytokine is also inhibited by PIMs [19]. Combined activation of TLRs and other pattern recognition receptors or co-receptors may result in agonistic or antagonistic interactions and, in particular, the regulation of IL-12 expression in response to TLR trigger is the net result of complex activation and down-regulations implicating different kinases such as PI3K or AKT (reviewed in [66,67]). The potential interference of PIMs with other mechanisms or signalling pathways involved in the expression of IL-12 will require further investigations.

In conclusion, as summarized schematically in Figure S6, we show that PIMs inhibit macrophage activation in response to TLR2 or TLR4 pathways at different levels. PIMs block LPS binding to CD14, which may explain PIM inhibition of CD14-dependent LPS functional responses through TLR4. However, not all TLR responses need CD14, and this is particularly so for TLR4 response to rough LPS or to high dose smooth LPS, but also for some TLR2 responses. In these cases, PIM inhibitory effect has to be explained at another level, likely downstream of TLRs.

Supporting Information

Figure S1 Structure of synthetic PIM1, isoPIM1 and PIM2 mimetics. Schematic representation of synthetic PIM1 showing the C16 and C18 acyl groups on glycerol chain positions sn-2 and sn-1, an isomer of PIM1 (isoPIM1) carrying the phosphatidylycholine group at position O-2 and the mannosyl residue at O-1 of D-myo-inositol, the precursor PI, a synthetic mimetic of PIM2 (PIM2 mimetic) bearing C16 and C18 acyl chains, and the de-acylated precursor of the PIM2 mimetic (deAcPIM2 mimetic) as control molecule.

Figure S2 Synthetic PIM analogues inhibit S-LPS-binding to macrophages. Bone marrow derived macrophages from C57Bl/6 mice were incubated with 10 µg/mL of deAcPIM2 mimetic and PIM2 mimetic or vehicle control. After overnight incubation, TNF (A) and IL-12 p40 (B) concentrations were measured in supernatants. Results are mean +/- SEM from n = 6 mice from three independent experiments. ***, p<0.001 versus vehicle. †, p<0.05 indicate significant differences between deAcPIM2 mimetic and PIM2 mimetic.

Figure S3 CD14 is an important co-receptor for S-LPS-binding to macrophages. Bone marrow derived macrophages from C57Bl/6 (A), TLR4 KO (B), MD2 KO (C) or CD14 KO (D) mice were incubated with biotinylated S-LPS and streptavidine FITC (black line). DeAcPIM2 mimetic did not displace S-LPS and was superimposed with S-LPS plus vehicle (A). In controls, macrophages were stained only with streptavidin FITC (grey histogram). Results shown are from cells derived from one mouse representative of cells from four different mice. (C) Percentage of S-LPS binding to macrophages in presence of PIMs or vehicle. Mean +/- SD from n = 4 mice from 2 independent experiments. ***, p<0.001 versus vehicle. †††, p<0.001 indicate significant differences between deAcPIM2 mimetic and PIM2 mimetic.
from one mouse to macrophages representative of four mice. (E) Percentage of S-LPS-binding to macrophages compared to C57Bl/6 binding level. Mean +/- SD from n = 4 mice from two to four independent experiments. **, p<0.01, *** p<0.001 versus C57Bl/6. (TIF)

Figure S4 Addition of sCD14 does not affect PIM inhibition of S-LPS-induced TNF. (A) Macrophages from C57Bl/6 mice were incubated with murine soluble CD14 (sCD14; 5 µg/mL) and PIMs (10 µg/mL) as indicated prior to stimulation with S-LPS (100 ng/mL). (B) Wild type or CD14 KO macrophages were stimulated with S-LPS in the absence or in the presence of murine soluble C14 (sCD14; 5 µg/mL). TNF concentration was measured in the supernatants after overnight incubation. Mean +/- SD from n = 4 mice from two experiments representative of three independent experiments. ***, p<0.001 versus vehicle. 0.001 indicate significant differences between isoPIM1 versus PI as control, †††, p<0.001 indicate significant differences between deAcPIM2 mimetic and PI mimetic. (TIF)

Figure S5 Differential inhibition of induced TNF and IL-12 p40 release by PIMs at low doses of Re-LPS. Concentrations of TNF (A) and IL-12 p40 (B) in supernatants of IL-12 p40 release by PIMs at low doses of Re-LPS. However, not all TLR responses need CD14, as indicated for TLR4 response to rough LPS or to high micromolar doses of smooth LPS, but also for TLR2/TLR1 response to Pam3CysK4 and TLR2/TLR6 response to Malp2. In these cases, PIM inhibitory effect may be downstream of TLRs. In addition, IL-12p40 expression requires other surface molecules to be complete, such as CD11b and CD18, and this may in part explain the different sensitivity of TNF and IL-12p40 to the inhibition by PIMs. (TIF)

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Author Contributions

Conceived and designed the experiments: NC ORM LON FE VFJQ. Performed the experiments: NC SR MLB SF JKDK EFK. Analyzed the data: NC SR JKDK EFK LON FE VFJQ. Contributed reagents/materials/analysis tools: SF ORM LON. Wrote the paper: NC FE VFJQ.

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