The PPE18 of *Mycobacterium tuberculosis* Interacts with TLR2 and Activates IL-10 Induction in Macrophage

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The complete decoding of the *Mycobacterium tuberculosis* genome revealed the presence of two unique PE and PPE family of genes which accounts ~10% of the total coding capacity of the genome. The PE family has a conserved N-terminal domain with proline-glutamic acid (PE) motif and the PPE family is characterized by the presence of conserved N-terminal domain containing proline-glutamic-glutamic acid (PPE) motif with considerable variation in the C-terminal region (1). Because these genes are highly expanded in the pathogenic strains of mycobacteria, it is possible that they may play important roles in the pathophysiological functions in these organisms (2). The PE (3, 4) and PPE (5, 6) families have been thought to play important roles in generating antigenic variation and immune evasion. Some of them are known to induce strong B cell response (7), however, their exact pathophysiological roles are yet to be understood.

Emerging evidence suggests that one of the major immune evasion strategies used by *M. tuberculosis* is its ability to evoke a deliberate Th2 response which in turn down-regulates the antimycobacterial Th1 response (8). Differential expressions of cytokines such as IL-12 p40 and IL-10 by macrophages are known to dictate the development of Th1 and Th2 cell responses and thereby determine the course of infection to a great extent. For example, induction of higher levels of IL-10 can result in decreased production of IL-12 p40 and/or IFN-γ that fabricates a strong Th2 response (9). Being an intracellular infection, in tuberculosis, from clinical point of view, it is important to devise approaches by which mycobacteria favoring Th2 component can be switched off (8). Therefore, it is critical to understand the mechanisms by which the bacilli modulate the host immune response toward a favorable Th2 phenotype that is critical for its survival. Overproduction of IL-10 is shown to inhibit host’s protective response and subsequently increase susceptibility to mycobacterial infection (10, 11). Mycobacteria have been shown to specifically target macrophages for infection and use strategies to trigger IL-10 induction in macrophages (12) during the early stages of infection, which possibly helps the bacilli to establish a favorable Th2 environment (13). Although the exploitation of IL-10 by *M. tuberculosis* to favor its survival is well understood (10, 14), the effectors or the mechanisms involved in triggering IL-10 production are not well understood.

One of the PPE proteins, PPE18 (also known as Rv1196 or mtb39a) was found to elicit T cell proliferation; nonsecretory and PPE18-immunized mice had lower bacterial load upon challenge with *M. tuberculosis* (15). PPE18 appears to be located in a cluster with ESAT-6-like proteins (2) and is one of the highly expressed genes in *M. tuberculosis* (15). PPE18 appears to be located in a cluster with ESAT-6-like proteins (2) and is one of the highly expressed genes in *M. tuberculosis* (15).
ROLE OF PPE18 IN ACTIVATING IL-10

Introduction

PPE18, a member of the family of PPE proteins, is known to bind to the TLR2 receptor and activate macrophages to produce IL-10. In this study, we aimed to investigate the role of PPE18 in activating IL-10 production in murine macrophages.

Materials and Methods

Expression and purification of recombinant PPE18 (rPPE18) protein

The open reading frame encoding PPE18 protein of M. tuberculosis was amplified by PCR from H37Rv genomic DNA by using the forward primer 5’ TTGATGCCATGGTGTTTCTCGCGGTAGACA 3’ and the reverse primer 5’ GCCATCTCCAGCAGGGCCGCTACCA 3’. The PCR product was cloned into the intermediate vector pGEM-T-Easy (Promega) and confirmed by sequencing. The full-length gene was then subcloned into the bacterial expression vector pRSET-A (Invitrogen) in frame with a N-terminal histidine tag. Polyclonal tagged recombinant protein was purified using TALON resin according to manufacturer’s recommendation for purification of protein under native condition. SDS-PAGE analysis of the purified protein revealed it to be an essentially homogenous preparation of PPE18 protein of 39-kDa protein (supplementary Fig. S1A). The purity of the recombinant PPE18 protein was further confirmed by gel filtration (BioRad) using prepacked Superose 12 column (HR 10/30, Amersham Pharmacia Biotech). Approximately 250 μg/ml of the protein preparation was injected and the profile was recorded (supplementary Fig. S1B). The protein sample was dialyzed against several changes of PBS. The protein concentration was estimated using bichoninic acid method of protein estimation (Pierce). To remove endotoxin contamination, the rPPE18 protein was incubated with 10% v/v polymeric B-agarose (Sigma-Aldrich) for 1 h at 4°C as described earlier (20). The preparation had a very low endotoxin content (<0.05 EU/ml) as measured by the E-toxase (Limulus amebocyte lysate) kit (Sigma-Aldrich).

Generation of rPPE18-specific polyclonal Ab

The Ab to the PPE18 protein was generated in C57BL/6 mice maintained in the animal house facility of National Institute of Nutrition (NIN), Hyderabad. The experiments were conducted following the institutional rules and the reagents were obtained from National Centre for Cell Science (NCCS), Pune, India. The suspension cell line was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% v/v heat inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen) at 37°C in a 5% CO2 humidified incubator. Human monocytes THP-1 cells were differentiated to macrophages by incubation with 20 ng/ml PMA (Sigma-Aldrich) for 24 h followed by a rest for overnight in medium containing 1% FBS. Human embryonic kidney cell line (HEK293) was obtained from NCCS, Pune, India and was maintained in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Isolation of monocyte-derived macrophages (MDM)

MDM were isolated as described earlier (21). In brief, PBMC were isolated from heparinized blood obtained from the antecubital vein of the healthy volunteers (all were previously vaccinated with Bacillus Calmette-Guérin) by density gradient centrifugation on Ficoll-Hypaque (Sigma-Aldrich) and centrifuged at 400 × g for 25 min. Cells were harvested from the interface, washed twice with RPMI 1640. Adherent monolayers were obtained by incubating PBMC in 24-well tissue culture plates for 24 h at 37°C. Non-adherent cells were removed using a transfer pipette and used for purification of T cells. The adherent cells were washed and adjusted to density of 3 × 10⁶/ml. Approximately 3 × 10⁶ monocytes per well were incubated for 7 days at 37°C under 5% CO2 in fresh RPMI 1640 medium supplemented with 15% FBS. The bioethics committee of CDFD approved the present study.

Macrophage activation assay

PMA-differentiated THP-1 macrophages were treated in triplicate with increasing concentrations of rPPE18 in the absence or presence of bacterial LPS (Sigma-Aldrich). The rPPE18 protein was also preincubated (30 min) with pepstatin F(ab’), antihuman PPE18 Ab or NMS and added into the THP-1 cultures. In some experiments, THP-1 macrophages were pretreated with increasing concentrations of anti-human TLR2 mAb, TL2.1 (mouse IgG2a (IgG2a) (Imgenex) for 30 min at 37°C and subsequently exposed to 3 μg/ml PPE18 protein in the absence or presence of bacterial LPS (Sigma-Aldrich). In control groups, isotype-matched Ab of IgG2a type (BD Pharmingen) was added at the same concentrations. After 48 h, the culture supernatants were collected for estimating IL-10 and IL-12 p40 levels. In some experiments, IL-10 induction by rPPE18 (3 μg/ml) was estimated in undifferentiated THP-1 cells. Also, macrophages were incubated with inhibitors for p38 MAPK (SB203580), ERK 1/2 (PD98059), and JNK (JNK inhibitor I(L)-form) for 1 h before adding rPPE18 (3 μg/ml) and IL-10 induction in the supernatants was estimated after 48 h by EIA. The SB203580 or PD98059 or JNK inhibitor I (L)-form was added to the cell culture at a final DMSO concentration of 0.1% and thus DMSO was used as a solvent control. SB203580, PD98059, and JNK inhibitor I (L)-form and the JNK 1(-) negative control peptide were obtained from (Calbiochem-Novabiochem).

T cell activation assay

Autologous T cells were purified from nonadherent fraction of theuffy coat that was used to purify MDM, using nylon wool column as described earlier (22). In brief, the nonadherent cells were incubated in prewashed nylon wool at 37°C in 5% CO2. After 60 min, the nylon wool column was washed twice with RPMI 1640. Adherent monolayers were obtained by incubation with 20 ng/ml PMA for 24 h followed by a rest for overnight in medium containing 1% FBS. Human monocytes THP-1 were differentiated to macrophages by incubation with 20 ng/ml PMA (Sigma-Aldrich) for 24 h followed by a rest for overnight in medium containing 1% FBS. Human embryonic kidney cell line (HEK293) was obtained from NCCS, Pune, India and was maintained in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Total RNA isolation and RT-PCR for IL-10 and IL-12 p40

For RT-PCR, PMA-differentiated THP-1 macrophages were incubated either with titrating concentrations of native or with a fixed concentration of

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3 μg/ml rPPE18 protein subjected to autoclaving (A) or treated with proteinase K (PK). In some experiments, bacterial LPS (3 μg/ml) was added as stimulator. Total RNA was extracted from these groups using TRIzol according to manufacturer’s protocol (Invitrogen). In brief, 2 μg of total RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) as per the manufacturer’s instruction. The PCR was performed at an annealing temperature of 57°C with gene specific (IL-10) PCR pair primer provided in the gene specific relative RT-PCR kit (Ambion), which generates a product of 297 bp. For specific detection of IL-12 p40 gene, PCR was performed using the following primers derived from previously published sequence data (24). IL-12 p40 forward primer used was 5’-GGACCAAGGACTTGGGTCCTT-3’ whereas IL-12 p40 reverse primer used was 5’-CCTCGTTTGTGCCTCCTGA-3’. The house keeping gene β-actin was used as an internal control with the forward primer 5’-CATGATTGCTGCTGCTGCTGA-3’ and reverse primer 5’-TTAATGTCACGGAGATTCTCC-3’. The amplification conditions for IL-12 p40 and β-actin were as follows: denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 2 min. After 30 cycles, the amplified products for IL-12 p40 (373 bp) and β-actin (510 bp) were resolved by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

Transient transfection assay
The full-length human TLR2 or TLR4 cDNA cloned in pcDNA3.1 plasmid vector as well as a deletion mutant lacking the cytoplasmic tail of TLR2 were kind gifts from Dr. Manikutala Kundu (Bose Institute, Kolkata, India). The FLAG-tagged wild-type (WT) TLR2 as well as TLR2 ectodomain deletion mutants Mut1 (leucine rich repeat (LRR) 1–5), Mut2 (LRR 5–10), Mut3 (LRR 10–15), and Mut4 (LRR 15–20) were all gifts from Dr. Carsten J. Kirkishing (Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany). The dominant negative mutant of p38 (DNp38) was a gift from Dr. Jiahua Han (The Scripps Research Institute, La Jolla, CA). All the plasmid constructs were transfected in THP-1 or HEK293 cells using the cationic lipid suspension lipofectamine 2000 (Invitrogen). Expression vector (pcDNA3.1) without any insert was used as negative control. After 24 h of transfection, cells were used either for measuring the surface expression of TLR2 or TLR4 by flow cytometry using anti-TLR2/TLR4 mAb or incubated with biotinylated rPPE18 for checking rPPE18 binding by flow cytometry or cultured in the presence of rPPE18 for measuring IL-10 induction by EIA. The pSV-β-galactosidase (Promega) vector was used for transfection efficiency control. The β-galactosidase expression was measured by β-galactosidase EIA (Roche Diagnostics) as per the manufacturer’s protocol.

Flow cytometry
For TLR2 and TLR4 staining, cells were treated (30 min at 4°C) with anti-human TLR2 mAb (Imgenex) or anti-human TLR4 mAb HTA125 (mouse IgG2a) (Imgenex) or isotype control IgG2a and then incubated with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) at 4°C for a further 30 min. Then, cells were washed at room temperature with increasing concentrations of rPPE18 conjugated to biotin (PPE18-biotin) for 10–15 min. For competition assay for binding of rPPE18, cells were preincubated with 100 and 10-fold excess of PamCSK4 (Calbiochem) for 15 min on ice followed by incubation with rPPE18-biotin. These cells were then incubated with streptavidin-FITC (1/1000, Sigma-Aldrich), washed and resuspended in PBS. The fluorescence was measured by flow cytometry in FACs Vantage flow cytometer (BD Biosciences) using CellQuest (BD Biosciences) data analysis software.

Immunoprecipitation assay
Cells transfected with WT-TLR2 or pcDNA3.1 were washed twice with PBS and lysed with 500 μl of lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 10% v/v glycerol, 150 mM NaCl, 20 mM NaF, and protease inhibitor mixture from Roche Diagnostics). After centrifugation, the supernatant from the cell lysate was incubated with TALON immobilized on metal affinity chromatography (M. tuberculosis) for 2 h at 4°C overnight on a rotator. The beads were then washed, boiled in 4× Laemmli buffer for 5 min. The proteins were separated on 10% SDS-PAGE and then transferred to nitrocellulose membrane (Amersham Biosciences). The membranes were incubated with anti-TLR2 mAb followed by incubation with anti-mouse IgG-HRP conjugate (Sigma-Aldrich). In some experiments anti-TLR1 or anti-TLR4 or anti-TLR6 Ab used for IP (Sigma-Aldrich) to check the PPE18 interaction with TLRs. Immunoreactive bands were detected by chemiluminescence using manufacturer’s protocol (Amersham Biosciences) to detect His-tagged PPE18 bound to TLR2.

Protein–protein docking studies
The PPE18 sequence (from residue 1–173) showed around 34% sequence identity to Chain B of a PE/PPE Protein Complex from M. tuberculosis (2G38) when subjected to blastp. No significant alignment was obtained for the rest of the protein. Because our initial studies indicated that TLR2–receptor complex of PPE18 fits within the N terminus, we did homology modeling for N terminus domain of PPE18 till the residue P173. The PPE18 sequence was aligned with a PE/PPE protein complex of M. tuberculosis (2G38) using online program (http://www.ebi.ac.uk/Tools/chalst/). This alignment was used as an input for homology modeling. The homology modeling was done using the MODELLER software (http://salilab.org/modeler/) (25, 26). With this rPPE18 version, the model, with best G-score of PROCHECK (27) and with best VERIFY-3D profile (http://nihserver.mbi.ucla.edu/Verify_3D/) (28), was subjected to energy minimization. The GROMOS96 43A1 force field (27) was used for the minimization with SPC (Simple Point Charge) water (29) and steepest descent algorithm. The energy-minimized structure was used for the docking on human TLR2 structure. The crystal structure of TLR1–TLR2 heterodimer induced by binding of a tri-acylated lipopeptide (2x82) (30) was used as static structure for docking whereas the PPE18 protein is kept as mobile. The energy-minimized structure (2x82 Chain A) corresponding to TLR2 was then used for docking. The docking program was used 3D-Dock (31) (www.bnnm.icnet.uk/docking/download.html). The package is a suite of programs where fidoCK performs the docking using shape complementarity and electrostatics to rank the docked complexes. Another program, epscore, then re-ranks the complexes using residue pair potentials. Hence, first 20 top scoring PPE18-TLR2 complexes were generated.

Measurement of phospho-p38, JNK, and ERK 1/2 by flow cytometry
The expression of phosphorylated p38, JNK, and ERK 1/2 in macrophages was determined by flow cytometry using protocol described elsewhere (32). In brief, cells (1 × 10^6) were treated with rPPE18 for the times indicated and then fixed with 1.5% formaldehyde (Sigma-Aldrich). After washing, permeabilization was conducted using freshly prepared 90% ice-cold methanol for 30 min on ice. The cells were washed three times in staining buffer (0.1% BSA in PBS), and incubated with Ab to phospho-p38 or phospho-JNK or phospho-ERK 1/2 (Cell Signaling Technology) for 30 min at 37°C. Cells were then washed in staining buffer and incubated with anti-mouse or anti-rabbit IgG-FITC (Sigma-Aldrich). Flow cytometry analysis of the FITC-stained cells was conducted on Becton Dickinson FACSVantage equipment.

Western blotting of p38 MAPK
The p38 MAPK was detected by Western blotting as described earlier (33). In brief, cells were lysed using Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue), separated on a 10% SDS-PAGE. Following electrophoretic transfer, the nitrocellulose membranes were incubated with Ab to either phosphorylated or total p38 MAPK (Cell Signaling Technology), followed by incubation with secondary conjugate (anti-mouse IgG-HRP from Sigma-Aldrich). Bound enzyme was detected by chemiluminescence following the manufacturer’s protocol (Amersham Biosciences).

Microorganisms, medium, and transformation
The M. tuberculosis PPE18 null mutant (Mutant ID 1440, MT1234) and the corresponding wild-type (CDC1551) strains were obtained from Colorado State University (as a part of National Institutes of Health NIAID Contract No. HHSN266200400009C entitled, “Tuberculosis Vaccine Testing and Research Materials”). The M. smegmatis mc(2)155 (American Type Culture Collection) and the M. tuberculosis strains were grown in Middlebrook 7H9 broth (HiMedia) supplemented with 0.2% glycerol, 0.5% Tween 80, and 10% Middlebrook albumin-dextrose-catalase in a BSL-3 facility at ICGEB, New Delhi.

The full length PPE18 gene was amplified from M. tuberculosis H37Rv genomic DNA and cloned into pMV261 (34) and the recombinant plasmid pMV-PPE18 was transformed into M. smegmatis mc(2)155 by electroporation (35). Transformants were selected on Middlebrook 7H11 agar plates (HiMedia) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment in the presence of 50 μg/ml kanamycin.

Infection of macrophages
PMA-differentiated THP-1 macrophages were infected with various mycobacterial strains at two different multiplicity of infections (MOIs) of 5 and 10. After 3 h, cells were treated with gentamicin (Life Technologies)
The recombinant PPE18 protein stimulates IL-10 induction in macrophages. A, PMA-differentiated THP-1 macrophages (3 × 10⁴/well/200 μl) were incubated with various concentrations of rPPE18 or rPPE18 (3 μg/ml) treated with proteinase K (PK) or subjected to autoclaving (A) or preincubated with F(ab)₂ of anti-PPE18 Ab or normal mouse serum (NMS). After 48 h, supernatants were collected and assayed for IL-10 levels (mean ± SD) by EIA. B, IL-10 induction by rPPE18 (3 μg/ml) was assessed in MDM. C, Semiquantitative RT-PCR analysis of IL-10 mRNA in total RNA extracted from PMA-differentiated THP-1 macrophages incubated with either various concentrations of native rPPE18 or rPPE18 (3 μg/ml) treated with proteinase K or subjected to autoclaving. The mRNA level was normalized to β-actin mRNA. Results are mean ± SD of three different experiments.

Results

The recombinant PPE18 protein activates IL-10 induction in macrophages

We overexpressed the M. tuberculosis PPE18 protein with a N-terminal 6× histidine tag in Escherichia coli and purified the soluble protein to homogeneity of >95% (supplemental Fig. S1, A and B). The preparation had very low endotoxin content (<0.05 U/ml) and was used for all in vitro experiments. The PMA-differentiated THP-1 macrophages were treated with various concentrations of the recombinant PPE18 (rPPE18) and after 48 h, the culture supernatants were collected to measure various cytokines (IL-10 or IL-12 p40) by EIA. The rPPE18 protein was found to specifically increase IL-10 production in a dose-dependent manner (Fig. 1A; bars 2, 3, 4, and 5) without eliciting detectable levels of other cytokines like TNF-α, IL-12, p40, or NO (data not shown). The effect was specific to rPPE18 as proteinase K digestion or heat denaturation of the protein abrogated IL-10 production (Fig. 1A, compare bars 6 and 7 with bar 4; p < 0.0001 in both the cases). Further, preincubation of rPPE18 protein (3 μg/ml) with F(ab)₂ fragments of the anti-PPE18 Ab (polyclonal Ab developed in C57BL/6 mice; supplemental Fig. S2) but not of NMS abrogated its ability to induce IL-10 indicating that the observed effects were specific to rPPE18 protein (Fig. 1A, compare bar 8 with bar 9; p < 0.001). Similar results were obtained when human MDM were stimulated with rPPE18 (Fig. 1B). In addition, when THP-1 macrophages were stimulated with rPPE18, levels of IL-10 transcripts were also increased in a dose-dependent manner (Fig. 1C).

The PPE18 targets the TLR2 pathway to activate IL-10 induction in macrophages

Because PPE18 is present in the M. tuberculosis lysate but not in the culture filtrate proteins (15), we speculated that this protein may be exposed in the cell surface and probably interacts with some of the macrophage surface receptor(s) and trigger IL-10 induction. Therefore, to test our hypothesis, we incubated PMA-differentiated THP-1 macrophages with increasing concentrations of biotin labeled rPPE18 (PPE18-biotin) and measured the surface-bound rPPE18 using flow cytometry. The bound PPE18 was detected with streptavidin-FITC conjugate. As observed in the histogram, there was a dose-dependent increase in PPE18 binding (Fig. 2A). Preincubation of rPPE18 protein (3 μg/ml) with F(ab)₂ of anti-PPE18 Ab eliminated binding of rPPE18 to surface of macrophages whereas F(ab)₂ of NMS failed to do so (Fig. 2A). The maximum amount of binding was observed up to 10–15 min. These data indicate that PPE18 interacts with some receptors on the surface of the macrophages.

Because we observed significantly enhanced binding of rPPE18 to PMA-differentiated THP-1 as compared with undifferentiated cells (Fig. 2B), we speculated that the molecule(s) involved in binding of PPE18 is likely one of those expressed at higher levels in PMA-differentiated THP-1 macrophages. Interestingly, there was also a marked up-regulation of IL-10 induction by rPPE18 in PMA-differentiated THP-1 in contrast to undifferentiated THP-1 (Fig. 2C, compare bar 4 with bar 3; p < 0.001), concurrent to its enhanced binding to PMA-differentiated cells (Fig. 2B). PMA is known to increase various receptors, especially TLR2, TLR4, and CD14 (36) in THP-1 cells as also observed by us (data not shown). Therefore, it may be possible that some of these receptors might be crucial for rPPE18-mediated induction of IL-10. To identify the receptor, we performed an inhibition assay by preincubating the PMA-differentiated THP-1 macrophages with mAb to TLR2 or TLR4 or CD14 and found that anti-TLR2 mAb but not isotype-matched control Ab inhibited IL-10 production by rPPE18 in a...
dose-dependent manner (Fig. 2D) and 20 μg/ml anti-TLR2 mAb prevented rPPE18-mediated IL-10 production by >90% (Fig. 2D, compare bar 5 with bar 2; p < 0.001). In contrast, preincubation with both anti-TLR4 and anti-CD14 mAb did not show any inhibitory effect (Fig. 2D). These observations suggested that TLR2 was the possible interacting partner of PPE18 in the observed IL-10 activation. Again, anti-TLR2 mAb was found to inhibit rPPE18 binding in a concentration-dependent manner (supplemental Fig. S3) indicating that PPE18 protein specifically interacts with TLR2 receptors. As expected, both anti-TLR4 mAb and anti-CD14 mAb did not affect rPPE18 binding activity (data not shown).

To further substantiate that PPE18 interacts with TLR2, we transfected HEK293 cells that naturally lack TLRs to express specific TLRs. The HEK293 cells were transfected transiently with either the backbone vector pcDNA3.1 alone (which served as a negative control) or the WT-TLR2 or the WT-TLR4 and binding of rPPE18 was assessed. The cells transfected with TLR2 and TLR4 constructs showed expression of these proteins (Fig. 3A, i and ii). As expected, PPE18 binding could be detected in HEK293 cells overexpressing TLR2 (Fig. 3A, iii), but not the cells overexpressing TLR4 (Fig. 3A, iv). The vector control group did not show any PPE18 binding (Fig. 3A) indicating that PPE18 specifically interacts with TLR2. To further ascertain whether PPE18 physically interacts with TLR2 molecule, we conducted pull-down assay using whole cell extracts from HEK293 cells transfected with either pcDNA3.1 or WT-TLR2 construct. These cell extracts were incubated with rPPE18 immobilized on TALON beads. Western blots probed with anti-TLR2 mAb showed that immobilized rPPE18 could pull down TLR2 (Fig. 3B). No band was visible in the vector control group or the group containing only TALON beads (Fig. 3B). These observations suggest that PPE18 interacts specifically and predominantly with the TLR2. Specific interaction of PPE18 with TLR2 was further confirmed by pull-down assay using whole cell extracts from PMA-differentiated THP-1 macrophages which endogenously express TLR2 and other TLRs (37). An interaction of TLR2 with PPE18 was detected, but no interaction could be observed with other TLRs tested i.e., TLR1, TLR4, and TLR6 (Fig. 3C). The above observations suggest that PPE18 interacts predominantly with the TLR2.

In the earlier section we found that rPPE18 induced IL-10 production in MDM (Fig. 1B). To examine binding of PPE18 to primary human macrophages, we incubated human MDM with biotin-labeled rPPE18 in the presence of isotype-matched control Ab or anti-TLR2 blocking mAb (20 μg/ml). In line with our results...
obtained from THP-1 cell line, PPE18 was found to bind specifically to TLR2 receptors on human MDM also as blocking with anti-TLR2 mAb abrogated binding of rPPE18 to the macrophage surface (Fig. 3D). These results indicate specific interaction of PPE18 to TLR2 and IL-10 activation was not restricted to a transformed cell line like THP-1 but could be observed in primary human macrophages.

**PPE18 specifically interacts with the LRR 11–15 region of TLR2**

We observed that the PPE18 construct lacking the N-terminal domain comprising of amino acid 1–180 (PPE18ΔN) did not bind to TLR2, but the truncated PPE18 with deletions in the C-terminal region comprising of amino acid 180–391 (PPE18ΔC) retained their ability to bind to TLR2 (Fig. 4A). Thus, probably the C-terminal region comprising amino acid 180–391 of PPE18 is not important for TLR2 binding, and the TLR2-interacting domain is localized within the N-terminal PPE domain (amino acid 1–180). An insight into the way TLR2 and PPE18 (N-terminal region) interact with each other was obtained by performing computational docking studies. We used 3D-dock (http://www.bmm.icnet.uk/docking) a molecular docking program that uses shape complementarity to assess the interaction of protein molecules, to predict possible sites for interaction between the TLR2 and the PPE18. Interestingly, the top ranking docking scores for PPE18 was found to be located at the TLR2 convex region that stretches the border of the central and C-terminal domain overlapping with the LRR 11–15 of TLR2 (Fig. 4B). Pam3CSK4, a synthetic TLR2 ligand is known to interact in a similar way with TLR2 through a different region (30, 38). A competition assay using Pam3CSK4 revealed no
significant inhibition in binding of PPE18 on THP-1 (supplemental Fig. S4) confirming that PPE18 and Pam 3CSK4 probably interact with TLR2 at different regions. Nonetheless, the region involved in PPE18 binding to TLR2 ectodomain overlaps with both the ligand binding and dimerization domain as proposed earlier (30). Furthermore, Meng et al. (39) reported based on deletion mutagenesis experiments that TLR2 contains multiple binding domains for ligands which may contribute to characterization of its promiscuous molecular pattern recognition (39). To further confirm that PPE18 binds to the TLR2 LRR domain 11/H1101115, we transfected HEK293 cells with TLR2 LRR/LRR-like motif deletion constructs and assessed for their ability to bind PPE18 by flow cytometry. It was found that a deletion of LRR 10–15 named as Mut3, abolished the binding of PPE18 while other deletion mutants like Mut1, Mut2, and Mut4 encompassing LRR 1–5, LRR 5–10, and LRR 15–20 respectively showed similar binding comparable to the WT TLR2 construct (Fig. 4C). All the TLR-2 (WT and the mutants) proteins were found to be efficiently expressed in these transfected cells as determined by immunoblotting using anti-FLAG Ab as described by Meng et al. (39) (supplemental Fig. S5).

**IL-10 induction by PPE18 is dependent on signaling through TLR2**

To examine whether the TLR2-initiated signaling is critical for the generation of IL-10 in response to rPPE18, we transfected undifferentiated THP-1 macrophages with WT-TLR2 or dominant negative TLR2 (DN-TLR2, lacking the downstream signaling activity due to amino acid deletion in the C terminus) (40). After 24 h post-transfection, cells were incubated with biotinylated rPPE18 for 10–15 min and examined for the binding of rPPE18. A parallel experiment was set up where these cells were cultured for 48 h with rPPE18 for measuring IL-10 induction. Flow cytometric analysis revealed that the THP-1 cells transfected with DN-TLR2 expressed cell surface TLR2 to the same extent as that of WT-TLR2 construct (Fig. 5Ai). Further, binding of rPPE18 was equivalent and correlated well with the fold changes observed in TLR2
followed by incubation with anti-mouse FITC or incubated with 3 μg/ml biotinylated rPPE18 (10–15 min) and then with streptavidin-FITC. Flow cytometric analysis was conducted to detect the expression of TLR2 (i) and amount of rPPE18 bound to THP-1 cells expressing wild-type or mutant TLR2 (ii). B, THP-1 cells transfected with WT-TLR2 or DN-TLR2 construct were treated with 3 μg/ml rPPE18. The control group was transfected with pcDNA3.1 vector alone. After 48 h, culture supernatants were harvested to estimate IL-10 levels by EIA. Results are mean ± SD of three different experiments.

**FIGURE 5.** IL-10 induction by PPE18 is dependent on signaling competent TLR2. A, The undifferentiated THP-1 cells were transfected transiently with either WT-TLR2 or mutant TLR2 (DN-TLR2) or with backbone vector (pcDNA3.1). After 24 h, cells were either stained with anti-TLR2- or isotype matched Ab followed by incubation with anti-mouse FITC or incubated with 3 μg/ml biotinylated rPPE18 (10–15 min) and then with streptavidin-FITC. Flow cytometric analysis was conducted to detect the expression of TLR2 (i) and amount of rPPE18 bound to THP-1 cells expressing wild-type or mutant TLR2 (ii). B, THP-1 cells transfected with WT-TLR2 or DN-TLR2 construct were treated with 3 μg/ml rPPE18. The control group was transfected with pcDNA3.1 vector alone. After 48 h, culture supernatants were harvested to estimate IL-10 levels by EIA. Results are mean ± SD of three different experiments.

expression in both DN-TLR2- and WT-TLR2-transfected cells (Fig. 5Aii). However, despite the equivalent binding of rPPE18, only the cells transfected with WT-TLR2 showed increased induction of IL-10 in response to rPPE18 as compared with the control group transfected with pcDNA3.1 alone (Fig. 5B, compare bar 4 with bar 2; p < 0.001) and treatment of the DN-TLR2-transfected THP-1 cells with rPPE18 induced IL-10 (Fig. 5B, bar 6) similar to the basal level as observed in the control group (Fig. 5B, bar 2) suggesting that IL-10 induction by rPPE18 is dependent on the signaling through TLR2.

**PPE18-mediated induction of IL-10 in macrophages is dependent on TLR2-mediated activation of p38 MAPK**

Several studies pointed out that an intricate balance of p38 MAPK and ERK 1/2 in macrophages determines the levels of proinflammatory and anti-inflammatory cytokines (41–44). Although ERK 1/2 activation is mainly required for TNF-α production by macrophages (43, 44), IL-10 secretion is generally dependent on p38 MAPK activation to a dominant extent (42, 45). Interestingly, we observed that rPPE18 induced a rapid (as early as 10 min) and sustained phosphorylation of p38 MAPK relative to untreated THP-1 macrophages while both the JNK and ERK 1/2 phosphorylation levels remained unaffected at the same time points (Fig. 6A). Bacterial LPS was used as positive control for induction of phosphorylation of p38 MAPK, ERK 1/2, and JNK (46). These data indicate that PPE18 specifically triggers phosphorylation of p38 MAPK in PMA-differentiated THP-1 macrophages (Fig. 6A). The rPPE18 induced p38 MAPK phosphorylation also in undifferentiated THP-1 cells (supplemental Fig. S6). The degree of p38 MAPK activation by PPE18-mediated induction of IL-10. These results indicate that p38 MAPK activation is essential for PPE18-mediated IL-10 induction. To further confirm the role of p38 MAPK, we next used dominant-negative mutant of p38 (DNp38) (33). The PMA-differentiated THP-1 macrophages were cotransfected with either backbone vector (pcDNA3.1) or DNp38 construct along with β-galactosidase vector (pSV-β-galactosidase) and then both the groups were treated with rPPE18 (3 μg/ml) to measure IL-10 production. It was observed that IL-10 induction was significantly reduced in macrophages transfected with the DNp38 compared with the macrophages transfected with the pcDNA3.1 (Fig. 6E, upper panel, compare bar 4 with bar 2; p < 0.001). β-galactosidase protein expression was found to be equivalent in all the groups indicating equal transfection efficiency (Fig. 6E, lower panel). These results further demonstrate a specific role of p38 MAPK in rPPE18-mediated induction of IL-10.

**PPE18 inhibits LPS-induced IL-12 p40 production in macrophages**

Because IL-10 is known to be a potent inhibitor of IL-12 p40 (9, 50), we examined whether IL-12 p40 induction in activated macrophages was suppressed by PPE18. Accordingly, PMA-differentiated THP-1 macrophages were stimulated with 3 μg/ml LPS in the absence or presence of various concentrations of rPPE18 protein. The recombinant protein was found to suppress LPS-induced IL-12 p40 gene expression (Fig. 7A) and IL-12 p40 protein production (Fig. 7B) in dose-dependent manner. The PPE18 inhibited IL-12 p40 levels also in MDM in response to both LPS (data not shown) and PPD (supplemental Fig. S8). Blocking the TLR2 receptors using anti-TLR2 mAb restored the PPE18 effect on IL-12 p40 inhibition (data not shown) indicating a role of TLR2 signaling in the PPE18-mediated inhibition of IL-12 p40.

**PPE18 skews the anti-PPD T cell response biased toward the Th2 type**

The T cell proliferation and effector commitment depends to a great extent on the cytokines induced in the activated macrophages...
It has been observed that T cell response to PPD is predominantly skewed to Th2 type in tuberculosis patients (8, 13, 51). As rPPE18 protein can strongly activate IL-10 production from macrophages (and subsequently inhibit IL-12 p40 cytokine), it is likely that PPE18 is one of the effectors that may be involved in the development of Th2-type response bias. Therefore, we examined whether PPE18 could modulate anti-PPD T cell response toward a Th2 type. The MDM from healthy volunteers were incubated for 2 h with 3 μg/ml rPPE18 or recombinant GST protein (as negative control). Cells were washed to remove any excess rPPE18 or GST and used as APCs. The purified T cells were cultured with autologous macrophages pretreated with rPPE18 or GST and PPD (10 μg/ml) as recall Ag. After 96 h, the culture supernatants were tested for the levels of IL-5 and IFN-γ cytokines. We used IFN-γ secreted by T cells as marker for Th1 response while IL-5 was used as an indicator for Th2-type T cell response.

**FIGURE 6.** The p38 MAPK downstream of TLR2 is important for IL-10 induction by rPPE18. A, The PMA-differentiated THP-1 macrophages (1–2 × 10^6) were either left untreated or incubated with rPPE18 (3 μg/ml) for various time points as indicated and then fixed with 1.5% formaldehyde. After permeabilization, the macrophages were incubated with Ab to either phospho-p38 or phospho-JNK or phospho-ERK 1/2 followed by incubation with anti-mouse IgG-FITC or anti-rabbit IgG-FITC and cell-bound fluorescence was analyzed with FACS Vantage flow cytometer. LPS (3 μg/ml for 15 min) was used as positive control for phospho-p38, phospho-JNK, and phospho-ERK 1/2. B, Flow cytometric analyses of the expression of phosphorylated p38 at 10 min time point with different concentrations of rPPE18 (1–10 μg/ml) is shown. C, PMA-differentiated THP-1 macrophages were preincubated with increasing concentrations of anti-TLR2 Ab or isotype control Ab for 30 min and were subsequently incubated with rPPE18 (3 μg/ml). After 10 min, cells were lysed and aliquots of total cell lysates were separated by SDS-PAGE and immunoblotted. Blots were incubated overnight with Ab to either phosphorylated or total p38 MAPK followed by incubation with anti-mouse IgG-HRP and were visualized by chemiluminescence. D, The PMA-differentiated THP-1 macrophages were incubated with 3 μg/ml rPPE18 in the absence or presence of SB203580 or PD98059 or JNK inhibitor (L)-form or JNK 1(-) negative control peptide and after 48 h IL-10 levels were estimated by EIA. E, The PMA-differentiated THP-1 macrophages were also transiently transfected with either the empty vector (pcDNA3.1) or p38 dominant negative (DNp38) expression vector along with β-galactosidase construct (pSV-β-Galactosidase). Twenty-four hours after transfection, cells were incubated with rPPE18 (3 μg/ml) for 48 h; supernatants were harvested and analyzed for IL-10 levels by EIA (upper panel). The β-galactosidase expression was measured following β-galactosidase EIA (lower panel). Data shown are mean ± SD of three different experiments.
The PPE18 protein has similar effect when presented as part of the whole mycobacterium.

The data from the previous section using recombinant protein suggest that PPE18 induces IL-10 production by interacting with TLR2 on macrophages. However, the in vitro effect of recombinant PPE18 protein may differ from its pathophysiological effect during infection on account of differences in local concentration, orientation, and exposure of different parts of the molecule or of copresentation with competing TLR2 agonists and other signaling ligands of *M. tuberculosis*. To investigate the pathophysiological function of this protein in the context of the whole bacillus, we compared the kinetics of IL-10 and IL-12 p40 cytokines elicited in macrophages infected with either WT (CDC1551) or PPE18 null mutant at different MOIs. Macrophages infected with the PPE18 null mutant had decreased levels of IL-10 as compared with the macrophages infected with the WT strain at MOIs of 5 and 10 (Fig. 9Ai; *p < 0.001*). Concurrently, there was an increased production of IL-12 p40 in macrophages infected with the PPE18 null mutant as compared with the WT strain at later time points of infection (Fig. 9Ai; *p < 0.001* at 48 h at MOIs of 5 and 10). These results are consistent with our earlier results obtained using recombinant PPE18 protein suggesting that the PPE18 could be one of the important molecules in triggering of IL-10 induction by the *M. tuberculosis*.

To further corroborate our findings, we overexpressed PPE18 in *M. smegmatis* using pMV261 under the control of hsp60 promoter, because in the *M. smegmatis* genome, the PPE proteins are conspicuously absent barring only two PPE proteins (2). In the more recently annotated database, only four putative PPE proteins (MSMEG_0064, MSMEG_0619, MSMEG_2737, and MSMEG_5350) could be identified (http://cmr.jcvi.org/cgi-bin/CMR/shared/AnnotationSearch.cgi?sub_org = *Mycobacterium*+smegmatis+MC2!&sub_org_val = gms!&search_string = &evidence_search_type &accession_search_type &org_search &org_val = smegmatis!&crumbs = search&search_string = ppe&annotation_search_string = ppe&annotation_search_type = &annotation_match_type = &classification_search_string = &evidence_search_string = &evidence_search_type = &org_search = &org = gms!&feat_type = ). In addition, when the PPE18 protein sequence was BLASTed against the *M. smegmatis* peptide database (http://blast.jcvi.org/cmr-blast/), no significantly similar protein matching the PPE18 was returned. The recombinant PPE18 protein was found to be associated with the insoluble cell wall rich “pellet” fraction prepared as described by Delogu et al. (52) (supplemental...
Fig. S9A). Further, flow cytometry analyses using anti-PPE18 Ab indicate that PPE18 is probably surface exposed (supplemental Fig. S9B).

We used these PPE18 overexpressing strains (M. smegmatis-pMV-PPE18) to infect PMA-differentiated THP-1 macrophages at different MOIs. The levels of IL-10 and IL-12 p40 were measured by EIA in the postinfection culture supernatants. It was found that THP-1 macrophages produced significantly higher amount of IL-10 at 12 h onwards when infected with M. smegmatis-pMV-PPE18 compared with those infected with the control bacilli harboring vector alone (M. smegmatis-pMV), at both MOIs of 5 and 10 (Fig. 9Bi; p < 0.001). In contrast, macrophages infected with M. smegmatis-pMV-PPE18 had significantly lower levels of IL-12 p40 at 12 h onwards compared with those infected with the control M. smegmatis-pMV (Fig. 9Bii; p < 0.001 at both MOIs of 5 and 10). These observations indicate that PPE18, when presented in the context of whole mycobacteria, induces IL-10 production in vitro.

Discussion

Our data suggest that one of the PPE proteins of M. tuberculosis, PPE18 triggers an anti-inflammatory response by activating IL-10 and concurrently decreases the levels of proinflammatory indicators like IL-12 p40 in activated macrophages. The recombinant PPE18 was found to directly interact with the TLR2 on macrophages to induce IL-10 and our data are also suggestive of a critical role of p38 MAPK for IL-10 activation. Interestingly, the anti-inflammatory effect of PPE18 is in contrast to the actions of other TLR2 ligands from mycobacterium which induce a proinflammatory response such as production of TNF-α (53, 54). Therefore, it appears that downstream TLR2 signaling cascades could be either pro- or anti-inflammatory depending on the specific ligand TLR2 interacts with. However, the mechanisms that dictate whether a TLR2-triggered cascade will swing toward proinflammatory or anti-inflammatory response is not well understood. One of the determining factors could be whether TLR2 ligands of mycobacteria trigger a downstream p38 MAPK or ERK 1/2 activation, as activation of ERK 1/2 mostly leads to induction of TNF-α, whereas activation of p38 MAPK is found to be essential for IL-10 induction (41).

It seems that the LRR motifs in the TLR ectodomain play an important role in recognition of various TLR ligands (30, 39, 55, 56). In this study, we found that the site of interaction between the PPE18 and the TLR2 ectodomain encompasses a region that lies between LRR 11–15 on the convex face, a region that does not overlap with the binding site for a synthetic lipopetide Pam3CSK4 (30, 38). A deletion in the LRR 11–15 domain abolished the binding of PPE18 to TLR2 indicate that PPE18 specifically interacts with TLR2 through this region. Interestingly, we found that PPE18 activates p38 MAPK whereas Pam3CSK4 is known to generally induce ERK 1/2 activation (57). Therefore, it is tempting to speculate that different TLR2 ligands interact with different ligand-specific sites on TLR2 ectodomain that may trigger specific conformational changes in TLR2 leading to different downstream signaling.

Interestingly, structural genomic data predict that the cooperonic PE/PPE pairs are likely to be functional as dimers where α1 and α2 helices of PE domain interact with the α2 and α3 helices of the PPE domain (58). Because PPE18 and PE13 belong to the same operon, they are likely to form a functional dimer, however, its pathophysiological functions as a heterodimer are yet to be understood. Interestingly, the TLR2-binding N-terminal PPE domain of PPE18 overlaps with the putative site of interaction with the PE13. More recently, PPE18 is also predicted to form dimer with another PE protein PE31 who are not operon pairs (59). Although, direct physical interactions of PPE18 with PE13 and PE31 are yet to be
demonstrated, the available data suggest the ability of PPE18 to form different heterodimeric complexes that may be required to execute different pathophysiological functions of this protein. Therefore, we speculate that, the differential interactions of PPE18 with PE13, PE31 and TLR2 may constitute an important point of regulation that may determine the pathophysiological functions of the PPE18 during the host pathogen interactions. It will be interesting to further investigate the different pathophysiological functions, if any, executed by the PPE18 as an interacting partner with PPE13 or PPE31 and their spatial regulation during infection.

The observed effects of the recombinant PPE18 in vitro may be different from its actual pathophysiological effects during infection owing to differences in local concentration, orientation, and exposure of different parts of the molecule or competition with other TLR2 ligands of M. tuberculosis. Therefore, we investigated the effect of this protein in the context of the whole bacillus. We compared the kinetics of IL-10 and IL-12 p40 cytokines elicited in macrophages infected with either the WT (CDC1551) or a PPE18 null mutant strain of M. tuberculosis. It was found that there was a significant reduction in the IL-10 production in macrophages infected with PPE18 null mutant strain as compared with those infected with the WT strain. In contrast, the levels of IL-12 p40 induced by the macrophages infected with the WT strain were lower compared with those infected with the PPE18 null mutant. This effect was found to be particularly pronounced at 48 h post-infection. To further elaborate the role of this protein, we also carried complementation experiment where we overexpressed the PPE18 gene in a heterologous mycobacteria, M. smegmatis which lack this gene, under the control of a strong and constitutive hsp60 promoter. Cell fractionation studies and flow cytometry analyses indicate that the PPE18 is present in the cell wall component of M. smegmatis and at least part of it is exposed on its surface. Therefore, PPE18 is likely to be available for interaction with the host components. Similar strategy has been used by other groups to determine cellular localization and/or functions of other PE/PPE proteins using M. smegmatis overexpression model (52, 60). When these M. smegmatis transformants, overexpressing the PPE18, were used to infect the macrophages, it was found that the IL-10 levels were significantly higher than those infected with the M. smegmatis transformants harboring the vector alone. These observations are in line with our earlier in vitro observations in macrophages treated with recombinant PPE18 that induced IL-10 production.

One of the characteristics of M. tuberculosis is that it can establish a state of chronic infection, during which the Th1/Th2 balance is critical determinant for its persistence (8, 61). There are evidences that pathogen-specific information are sensed through different TLRs (53, 62) and can subsequently dictate the Th1/Th2 balance through differential production of IL-12 and IL-10. Indeed there is substantial divergence in the ability of TLR2 and TLR4 signaling pathways to influence the nature (pro- vs anti-inflammatory) and magnitude of inflammatory response. TLR4 signaling pathway is typically a potent mediator of IL-12, whereas TLR2 agonists may have varied cytokine response depending on nature of ligands. For example, it has been shown that TLR2 agonist like mycobacterial ESAT-6 can inhibit IL-12 production (63) or PE_PGRS33 activate production of TNF-α (54). Our findings that PPE18, upon binding to TLR2, could induce IL-10 production further extend this notion. These observations emphasize the potential importance of TLR2 receptor in modulating progression of tuberculosis disease. Because, transgenic mice over expressing IL-10 are found to be more susceptible to M. tuberculosis infection (11), and IL-10 knockout mice are more resistant to infection (12), it is likely that IL-10 plays a definitive role in favoring survival of the bacilli. IL-10 is known to inhibit Th1 response by inhibiting IL-12 (50) and IFN-γ production (9, 64) and thus favor a Th2-biased environment. In the present study, we found that interaction of PPE18 with TLR2 induces a biased Th2 response against PPD through production of IL-10. We speculate that PPE18 could be a component of the mycobacterial interactome that may be required for a pronounced Th2-type response. Our data are also in accordance with the emerging paradigm that TLR2 signaling favors development of Th2-type phenotype (65).

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Disclosures

The authors have no financial conflict of interest.

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