Original Paper

***Mycobacterium Tuberculosis* PPE Family Protein Rv1808 Manipulates Cytokines Profile via Co-Activation of MAPK and NF- κ B Signaling Pathways**

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Key Words

Mycobacterium tuberculosis • Rv1808 • Macrophages • Immunomodulatory Cytokines • MAPK • NF- κ B • Immune evasion

Abstract

Background/Aims: *Mycobacterium tuberculosis* is an extremely successful intracellular pathogen armed with multiple tactics to subvert host immunity. PPE (Pro-Pro-Glu) family exclusively distributed in mycobacteria might be responsible for the virulence and pathogenicity of *M.tuberculosis*. The up-regulation of Rv1808(PPE32) in many conditions prompted us to define its role in host innate immune response. **Methods:** The Rv1808 encoding gene was expressed in nonpathogenic fast growing *Mycobacterium smegmatis*, mycobacteria-*Escherichia coli* shuttle plasmid pNITmyc served as control. RT-PCR and ELISA were used to detect the transcription and translation of host cytokines in culture supernatant from macrophage incubated with purified Rv1808 protein. Pharmacological inhibitors were applied to confirm the specificity of the effector interfering of host signaling. **Results:** Recombinant Ms_Rv1808 survived better than Ms_pNITmyc within macrophage, accompanied by slightly higher host cell death. Rv1808 protein is associated with the cell wall and exposed on the cell surface. Physical binding of Rv1808 to TLR2 resulted in increase in the secretion of anti-inflammatory cytokine interleukin-10 (IL-10) and pro-inflammatory cytokines tumor necrosis factor (TNF- α) and interleukin-6 (IL-6) possibly via co-activation of NF- κ B and MAPK (p38MAPK, JNK and ERK) signalling. **Conclusion:** Cell wall associated Rv1808 protein manipulated the host cytokines via MAPK and NF- κ B signaling pathways.

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Introduction

Tuberculosis (TB) remains one of the most prevalent and deadly infectious diseases worldwide. There are estimated 8.7 million new cases of TB and 1.4 million deaths from TB in latest statistics (http://www.who.int/mediacentre/events/annual/world_tb_day/en/index.html). Multiple factors have synergistically exacerbated the dire scenario, such as the lengthy duration of treatment with a combination of three to four antibiotics, the poor patients compliance, the shortage of novel drugs, the emergency of multi-drug resistant (MDR) and extensive drug resistant (XDR), even total drug resistant (TDR) *M. tuberculosis* strains, increasing incidence of HIV co-infection, and the unreliable drug supply [1, 2]. In-depth understanding of the biology of *M. tuberculosis*, particularly their antigenic molecules, will be the key for better TB control.

69 members of PPE multigene antigen family have been found exclusively among mycobacteria [3, 4]. PPE family shares a conserved N-terminal around 110 and 180 amino acid residues and a significant variable C-terminal with both sequences and sizes [5]. The PPE was named after their N-terminal Pro(P)-Pro(P)-Glu(E) residues [6]. Versatile roles have been proposed for this highly flexible C-terminal domain, such as the cell homeostasis, intracellular survival advantage, and replication under certain niches [3, 7, 8]. PPE family can be further subdivided into four subfamily based on their C-terminal motifs. The first subfamily characterized by the motif Gly-Xaa-Xaa-Ser-Val-Pro-Xaa-Xaa-Trp, constitutes the "PPE-SVP" subfamily, and the second group is "PPE-PPW" including the highly conserved Gly-Phe-Xaa-Gly-Thr and Pro-Xaa-Xaa-Pro-Xaa-Xaa-Trp motifs at its C terminal. The major polymorphic tandem repeat PPE subfamily contains multiple C-terminal repeats of the motif Asn-Xaa-Gly-Xaa-Gly-Asn-Xaa-Gly. The fourth PPE subfamily proteins have low homology at the C terminal [3, 9].

The predicted or demonstrated [7, 10-12] cell wall associated or secreted characteristics of many PPE proteins suggested a role in subverting host immune response [13, 14]. PPE family protein PPE34 [11], PPE41 [7] and PPE38 [15] were experimentally demonstrated to be cell surface exposed. PPE38 can modulate the host innate immune response via manipulating the production of TNF- α and IL-6 [15]. PPE-SVP subfamily protein Rv1808 has 49% similarity with *Mycobacterium avium subsp. paratuberculosis* PPE MAP1152, which is immunogenic and reactive against all positive tuberculosis patients' sera [16]. The up-regulation of Rv1808 under nutrition starvation [17] suggested a role in the persistence of mycobacteria. The N-terminal signal sequence of Rv1808 is highly similar to that of two PPE proteins: PPE18 [18] and PPE25 [19], which regulated host innate immunity via phosphorylation the suppressor of cytokine signaling 3 protein (SOCS3) and interference of phagosome-lysosome fusion, respectively.

In this study, in order to investigate the roles of Rv1808, the gene of Rv1808 was expressed in nonpathogenic fast growing *M. smegmatis*. We found Rv1808 prolonged the survival of the *M. smegmatis* within host macrophage, and slightly accelerated cell death of infected macrophage. The cell wall-associated Rv1808 protein physically interacted with TLR2 and induced the expression of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines TNF- α , IL-6 via MAPK and NF- κ B signaling pathways.

Materials and Methods

The expression of Rv1808 protein

The open reading frame encoding *M. tuberculosis* Rv1808 protein was amplified by PCR from H37Rv genomic DNA using the forward primer 5'ATAGGATCCTGGTTGGACTTCG3' containing a *Bam*HI site (underlined) and the reverse primer 5' TATAAGCTTGCCTTATCCGGCC 3' containing a *Hind* III site (underlined). The PCR product of approximately 1200bp was cloned into *Mycobacteria-Escherichia coli* shuttle plasmid pNITmyc and bacterial expression vector pET28 vector (Invitrogen) in-frame with a six N-terminal his-tag for protein expression and purification. The sequence verity was confirmed by DNA

sequencing. *Escherichia coli* cells were grown in Luria-Bertani (LB) broth or on agar at 37°C supplemented with the following antibiotics as appropriate: kanamycin at 50 µg/ml. *Escherichia coli* BL21 cells carrying recombinant plasmids were induced with 1mM IPTG (isopropyl β-D-thiogalactopyranoside) and the bacteria were incubated to 200 r.p.m. for 4 h at 37°C. Cells were then pelleted by centrifugation, re-suspended in urea buffer [8 M urea, 100 mM NaH₂PO₄, 10mM Tris-HCl (pH 8.0)] and disrupted by sonication. The suspension was centrifuged, and the supernatant was filtered through Ni-nitrilotriacetic acid (Ni-NTA) columns (Bio-Rad). The column was washed with urea buffer at pH 8.0 and urea buffer at pH 6.0, and finally the bound protein was eluted with urea buffer at pH 4.5. The His-tagged Rv1808 protein gradually dialysed at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20% glycerol, 0.5 M NaCl, 0.1% Triton X-100, various amounts of urea (6,4,2,1 and 0M) which was changed every hour. The final dialysis was done in storage buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20% glycerol, 240 mM NaCl, 0.1% Triton X-100 and 3 mM EDTA, and aliquots of the purified proteins were stored at -70°C. Protein concentrations were determined by the Bradford procedure.

Detection of myc-tagged and his-tagged Rv1808

Recombinant *M. smegmatis* strains were cultured to an OD600 of 0.6–1.0 in 25 ml Middlebrook 7H9 liquid medium or 50 ml Sauton medium in the presence of 25 mg/ml kanamycin. Bacterial pellets were harvested, washed three times with ice-cold PBS and re-suspended in 1×PBS buffer. Bacterial cells were disrupted and supernatants were collected after centrifugation at 12000×g for 15 min at 4°C. For preparation of his-tagged Rv1808, *Escherichia coli* BL21 cells carrying recombinant plasmids were induced with isopropyl 1mM IPTG for 4h at 37°C. Polyhistidine tagged recombinant protein was purified with Ni-nitrilotriacetic acid (Ni-NTA) columns (Bio-Rad) according to manufacturer's recommendation as described in 2.1. Samples were subjected to SDS-PAGE, and the his-tagged or myc-tagged Rv1808 protein was detected by Western blotting using mouse anti-his antibody and anti-myc antibody respectively. For stimulation experiment, in order to remove endotoxin contamination, the Rv1808 protein was incubated with 10% v/v polymyxin B-agarose (Sigma-Aldrich) for 1h at 4°C as described earlier [20]. The preparation had a very low endotoxin content (<0.05 EU/ml) as measured by the E-toxate (Limulusamebocyte lysate) kit (Sigma-Aldrich).

Cell culture

The human monocytic cell line U937, 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 100µg/ml streptomycin (all from Invitrogen) at 37°C in a 5% CO₂ humidified incubator. Macrophages were seeded at 2 × 10⁶, cells/well in 6-well tissue culture plates. Cells were incubated with Rv1808 protein (5µg/ml). Human monocytic U937 cells were differentiated to macrophages by incubation with 100ng/ml PMA (Sigma-Aldrich) for 48 h.

M. smegmatis infection of macrophages

Macrophages were seeded at 1×10⁶ cells per well in 12-well tissue culture plates or at 5×10⁵ cells per well in 24-well tissue culture plates. Cells were infected with Ms_Rv1808 or Ms_pNITmyc at an m.o.i. of 10. Four hours after infection, hygromycin was added to give a final concentration of 1ug/ml. At 6, 24, 48 h and 72h after infection, LDH activity in the culture supernatants was assayed with LDH cytotoxicity detection kit(Takara Bio). Percentage of LDH release was calculated following this formula: % release=100 x (experimental LDH release-spontaneous LDH release) / (maximal LDH release – spontaneous LDH release). A value of maximal LDH release was obtained from culture supernatants of macrophages that were lysed with 1 % (v/v) Triton X-100. Macrophages were washed and lysed in PBS containing 0.1% (v/v) Triton X-100 for 3 times. Lysates were plated on Middlebrook 7H10 agar plates containing 25 mg kanamycin/ml and the number of intracellular bacteria was enumerated.

Localization of the Rv1808 protein

Recombinant *M.smegmatis* strains harboring the Rv1808_pNITmyc(Ms_Rv1808) and pNITmyc(Ms_pNITmyc) constructs were grown and subjected to cell fractionation using the method previously described [15]. Briefly, the cells were lysed by sonication, and cell debris and unlysed cells were removed by centrifugation at 3,000×g for 5 min. The supernatant was subjected to ultracentrifugation at 27,000×g

Table 1. PCR primers for cytokines

Gene	Sequence (5'–3')	Length
TNF- α (P1)	CGCTCCCAAGAAGACAG	375bp
TNF- α (P2)	TGAAGAGGACCTGGGAGT	
IL-6(P1)	TTCCTCTCTGCAAGAGACT	432
IL-6(P2)	TGTATCTCTCTGAAGGACT	
IL10(P1)	AGACTCTGCTTCCTGATTGC	400bp
IL10(P2)	GTGAGTGTCCCTGCTGGTC	
β -actin(P1)	CGGCTCCGGCATGTGCAA	600bp
β -actin (P2)	ATGTCACGCACGATTTC	

for 30min at 4°C. The pellet from this centrifugation step was considered the cell wall fraction, and the supernatant was considered the combined cell membrane and cytosol fractions. Equal amounts of protein from each fraction were subjected to Western blotting using monoclonal anti-bodies against myc-tag and his-tag (control) (Sigma) for analyses of Rv1808 and GroEL expression, respectively.

RNA extraction and assay for cytokines

PMA-differentiated U937 macrophages were infected with Ms_Rv1808 or Ms_pNITmyc at an m.o.i. of 10. At 6, 24, 48 h, total RNA was extracted with RNA extraction kit (TIANGEN) for assaying cytokines production. In addition, PMA-differentiated U937 macrophages were incubated either with 5 μ g/ml endotoxin-free Rv1808 protein or treated with proteinase K(PK). Culture supernatants were harvested after macrophages incubated with endotoxin-free Rv1808 protein for 6h. Concentrations of cytokines in the culture supernatants were determined with commercially available ELISA kits for TNF- α , IL-6, IL-10 and IL-1 β (eBioscience). Total RNA was extracted with RNA extraction kit (TIANGEN). RNA (200ng) was reversely transcribed into cDNA and subsequently PCR was performed in equal reaction volume. RT-PCR detection of TNF- α , IL6 and IL-10 gene, the PCR was performed at an annealing temperature of 58°C with gene specific PCR pair primer (Table 1). The amplification conditions for TNF- α , IL-6, IL-10 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 25 cycles, the equal volumes of amplified products for TNF- α (375bp), IL6(432bp) and IL-10(400bp) and β -actin (600bp) were resolved by electrophoresis on 2% agarose gels and visualized by Gold ^{view} staining.

Interaction between Rv1808 and TLR2

The human U937 cells were washed briefly with ice-cold PBS, and cell lysates were prepared in 1 $\times$ RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml each of aprotinin, leupeptin, pepstatin, 1 mM Na₃VO₄, 1mM NaF). Lysates from U937 cells were incubated with his-tagged Ni²⁺-NTA beads or Rv1808-bound Ni²⁺-NTA-agarose at 4°C for overnight with shaking or 37°C for 3 hours. The beads were washed and boiled in 5 $\times$ Laemmli buffer for 5 min, and proteins were separated by 12% SDS-PAGE, transferred onto a nylon membrane, and immunoblotted with anti-TLR2 antibody and anti-his antibody to detect the existence of his-Rv1808-bound TLR2.

Pharmacological inhibition of signaling

All the pharmacological inhibitors were obtained from Sigma, reconstituted in sterile DMSO (Sigma), and used at the following concentrations: p38 signaling inhibitor SB 202190, ERK signaling inhibitor PD98059, JNK signaling inhibitor SP600125 and NF- κ B signaling inhibitor N-p-Tosyl-L-phenylalanine chloromethyl ketone. DMSO at 0.1% concentration was used as the vehicle control. In experiments with inhibitors, the cells were treated with a given inhibitor for 1h prior to by treatment with Rv1808. Cells were then stimulated with purified Rv1808 protein. Culture supernatants were harvested and assayed for cytokine activity.

Statistical analysis

The experiments were performed in triplicate. Differences between groups were analyzed with a one-way ANOVA test using SPSS software (Statistical Package for the Social Sciences, version 13.0 for Windows;

Fig. 1. The expression of Rv1808 protein. (A) PCR amplification of Rv1808 encoding gene approximately 1200bp. The detection of the expression of 45 kD Rv1808 in *E.coli* BL21 strain by commassie blue staining (B) and Western blotting (C). (D) The expression of myc-tagged Rv1808 protein in *M. smegmatis*.

SPSS Inc., Chicago, IL, USA). Data are shown as mean $\pm$ SEM (standard error of the mean) and values of $P < 0.05$ were considered statistically significant.

Results

The expression of M. tuberculosis Rv1808

In this study, we generated recombinant *E.coli* and *M. smegmatis* strains to investigate the effect of Rv1808 on the macrophage response to bacterial infection. The Rv1808 gene (1200bp) was amplified from *M. tuberculosis* H37Rv genome (Fig. 1A) and used to construct recombinant *E.coli* BL21 and MS_Rv1808. The *E.coli* BL21_PET28_Rv1808 strains were engineered to express a 6 his-tagged Rv1808 protein from a recombinant vector. *E.coli* BL21_PET28 strains harboured the vector alone serve as control. SDS-PAGE and Western blotting confirmed that the Rv1808 protein (approximately 45kD) was successfully produced from *E.coli* BL21 strains using IPTG as inducer (Fig. 1B and C). The Ms_Rv1808 strain was engineered to express a myc-tagged Rv1808 protein from a recombinant pNITmyc vector, while the Ms_pNITmyc strain harboured the vector alone. Both Ms_Rv1808 and Ms_pNITmyc, which were grown in Middlebrook 7H9 medium in the presence of kanamycin, expressed the Rv1808 gene. However, only Ms_Rv1808 expressed the Rv1808 gene (Fig. 1D).

Rv1808 enhanced the survival of M. smegmatis within macrophages

Non-pathogenic *M. smegmatis* can not multiply inside macrophages, and the number of intracellular bacteria decreased gradually after infection of macrophages *in vitro*. To determine whether Rv1808 can enhance the intracellular survival of recombinant bacteria within macrophages, we compared the survival kinetics of Ms_Rv1808 and Ms_pNITmyc in macrophages. The results showed significant difference in the number of bacteria between Ms_Rv1808 and Ms_pNITmyc up to 72 h after infection (Fig. 2A). These results suggested that the presence of Rv1808 can enhance the intracellular survival of *M. smegmatis* within macrophages.

Rv1808 predisposed the macrophages to death

Infection of macrophages with *M. tuberculosis* can induce necrosis, defined by cell lysis. *M. tuberculosis* might manipulate host cell death to cause disease. Alternatively, infection can result in macrophages apoptosis to maintain an intact plasma membrane, to diminish pathogen viability and enhance host immunity [21]. To determine whether Rv1808 can alter the death of macrophages infected with *M. smegmatis*, PMA-differentiated macrophages were infected with Ms_Rv1808 or Ms_pNITmyc, the amount of LDH released into the culture supernatant was determined. We found that macrophages infected with Ms_Rv1808 released

Fig. 2. Prolonged existence of Recombinant *M. smegmatis* inside macrophages (A) Intracellular survival of recombinant *M. smegmatis* in macrophages (B) The death of macrophages infected with recombinant *M. smegmatis*. Macrophages were infected with *Ms_Rv1808* or *Ms_pNITmyc* at an m.o.i. of 10. At 6, 24, 48 and 72h after infection, the macrophages were washed and lysed. Lysates were diluted and plated on Middlebrook 7H10

agar plates containing 25 mg kanamycin/ml-1 to determine the c.f.u. Culture supernatants were harvested. Release of LDH, a measure of macrophage cell death, was estimated by assaying LDH activity in the culture supernatants. Data are shown as means $\pm$ SD of triplicate wells. Similar results were obtained in three independent experiments. *, $p < 0.05$.

Fig. 3. Rv1808 is associated with the cell wall. Recombinant *M. smegmatis* strains expressing myc-tagged Rv1808 were subjected to fractionation experiments, and the presence of the Rv1808 protein in different fractions was detected by Western blotting using anti-myc antibody.

The expression of cytosolic GroLe in each fraction was also detected by anti-his antibody as a control.

more LDH than infected with the *Ms_pNITmyc*, but there is no significant difference between these two strains (Fig. 2B).

Rv1808 was associated with cell wall and surface exposed.

Several PE/PPE proteins have been found to be associated with the cell wall or secreted into the extracellular medium [10, 11, 15]. Gpos-PLoc identified that Rv1808 protein localized to extracellular (<http://www.csbio.sjtu.edu.cn/bioinf/Gpos/>). Therefore, we hypothesized that Rv1808 might localize in the cell wall, which would provide an explanation for the observed phenotypes associated with the alteration of cell surface properties. To test this hypothesis, we made constructs with the myc-tagged Rv1808 in *M. smegmatis*. GroEL serve as a control and as a marker for cytoplasmic protein of *M. smegmatis*. Recombinant *M. smegmatis* strains expressing myc-tagged Rv1808 and *Ms_pNITmyc* were subjected to cell fractionation experiments, followed by Western blotting analysis. The Rv1808 protein was detected in the cell wall fraction (Fig. 3), indicating that Rv1808 is associated with the cell wall. As expected, GroEL was detected only in the cytoplasm of *M. smegmatis* (Fig. 3).

Rv1808 interacted with Toll-like receptor 2

Pattern recognition receptors (PRRs) are proteins expressed by innate immune system. PRRs recognized mycobacteria pathogen-associated molecular patterns, such as cell wall

Fig. 4. Rv1808 associates with TLR2. Cell lysates from U937 cells were incubated with Rv1808 immobilized on Ni-NTA beads, and bead-bound proteins were immunoblotted for the presence of TLR2. Western blotting shows the existence of Rv1808 protein by anti-his antibody.

Fig. 5. The production of pro-inflammatory cytokines by macrophages co-incubated with Rv1808 proteins. PMA-differentiated U937 macrophages (2×10^6 /well/2ml) were incubated with Rv1808 (5 μ g/ml). After 6h, supernatants were collected and assayed for the amounts of IL-10(A), IL-6(B), TNF- α (C) and IL-1 β (D) cytokines (mean $\pm$ SD) by EIA. Results are the mean $\pm$ SD of three different experiments. ***, $p < 0.001$.

associated antigens. Toll-like receptors (TLRs) are one of the pivotal PRRs for host immunity against invading pathogens, including mycobacteria [22-24]. Previous data showed that several mycobacteria antigens interacted with TLR1, TLR2, TLR4, and TLR6 and culminate in differential host immune responses [20, 25-28]. Whether Rv1808 can interact with TLR2 remains to be determined. To address this question, we firstly analyzed whether Rv1808 associated with TLR2, which was reported to interact with another two PPE family proteins [20, 29]. The result demonstrated that Rv1808 physically associated with TLR2 (Fig. 4), suggesting a role of Rv1808 as TLR2 agonist.

Rv1808 protein triggered the secretion of immunomodulatory cytokines

The outcome of mycobacteria infection is largely determined by the interplay between the innate and the acquired immunity. Macrophage can secrete multiple cytokines to prime the anti-inflammatory response. Mycobacteria-infected macrophages or monocytes secrete both pro-inflammatory cytokines including TNF- α , IL-1, IL-6, and IL-12 and anti-

Fig. 6. Semi-quantitative RT-PCR analysis of the mRNA amount of cytokines. Macrophages were incubated with Rv1808 (5 µg/ml). At 6h after infection, macrophages were washed and total RNA was extracted. Equal amounts of total RNA were subjected to RT-PCR in equal reaction volumes. DNA bands were visualized by Goldview™ staining. Similar results were obtained in three independent experiments. Results are the mean ± SD of three different experiments. *, $p < 0.05$.

inflammatory cytokines including IL-4 and IL-10 [30, 31]. These cytokines are essential for the recruitment of monocytes and lymphocytes from the bloodstream to the infected foci, to limit the inflammatory response, and engaging in subsequent granuloma formation and the outcome of mycobacteria infections.

In order to explore whether Rv1808 can subvert the early immune responses, PMA-differentiated U937 macrophages were incubated with endotoxin-free (< 0.05 U/ml) Rv1808 recombination protein and human monocytic cell line U937 were used. The PMA-differentiated U937 macrophages were treated with the recombinant Rv1808 (5 µg/ml). After 6h, the culture supernatants were collected to measure various cytokines production by EIA. Interestingly, macrophages incubated with the Rv1808 protein secreted significantly larger amounts of the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines, including TNF-α and IL-6 than the control. No differences in other inflammatory proteins, including IL-1β, can be found (Fig. 5). The transcriptional levels of these cytokines were confirmed by RT-PCR analyses (Fig. 6). In general, both the transcription and translation of macrophages IL-10, TNF-α and IL-6 were enhanced by Rv1808 protein. These results suggested that Rv1808 might play a role in modulating host cytokines production.

Rv1808 specifically induced the release of immunomodulatory cytokines

To preclude possible non-specific effects of Rv1808 protein on the release of above mentioned cytokines, BSA proteins were included as negative control. The PMA-differentiated U937 macrophages were treated with various concentrations of the recombinant Rv1808 protein, culture supernatants were collected to measure various cytokines by ELISA. As previous data from two PE/PPE proteins, PE_PGRS33 [32] and PPE18 [20], the recombinant Rv1808 protein specifically stimulated the release of IL-10, TNF-α and IL-6 from macrophage in a dose-dependent manner, while there was no difference in inflammatory cytokine levels when incubation with non-related BSA protein (Fig. 7).

Fig. 7. The expression of IL10, TNF- α and IL-6 is Rv1808 protein dosage dependent. PMA-differentiated U937 macrophages (2×10^6 /well/2ml) were incubated with various concentrations of Rv1808 (5 μ g/ml). After 6h, supernatants were collected and assayed for levels of IL-10(A), IL6(B), TNF- α (C) and IL-1 β (D) cytokines (mean $\pm$ SD) by EIA.

In addition, semi-quantitative RT-PCR analysis revealed a marked decrease of IL-10, TNF- α and IL-6 mRNA when Rv1808 protein was treated by proteinase K. According the manufacturer's instruction, proteinase K was inactivated at 65°C for 40min after treatment of Rv1808 for 1h. ELISA assay demonstrated the same results (Fig. 8), suggesting a specific effect of Rv1808 on the release of IL-10, TNF- α and IL-6.

MAPK and NF- κ B signaling were involved in the Rv1808-mediated induction of cytokines

The mechanism underlying the simultaneous activation of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines TNF- α and IL-6 by Rv1808 was fascinating. The delicate balance between MAPK (p38 MAPK, ERK 1/2) and NF- κ B within macrophages was partly responsible for the relative levels of pro-inflammatory and anti-inflammatory cytokines [33-36]. To determine whether the effect of Rv1808 on the cytokines involved the crosstalk between MAPK and NF- κ B signaling pathway, specific signaling inhibitors were used in our experiments. The link between the MAPKs activation and PPE18 induction of cytokines was examined by treating the cells with p38 MAPK inhibitor (SB203580) [33, 34], ERK1/2 inhibitor (PD98059 [37-39], JNK inhibitor (SP600125) [40], JAK/STAT inhibitor (AG490) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (NF- κ B inhibitor) [29] for 1 hour before incubation with Rv1808 protein. Cells were pretreated with SB203580 or PD98059 or SP600125 or NF- κ B inhibitor before exposure to Rv1808 protein. The inhibitors pretreatment significantly decreased the induction of IL-10, TNF- α and IL-6 (Fig. 9). However, no change can be found among these cytokines when the cell was treated by JAK/STAT inhibitor (AG490). These results indicate that the simultaneously activation of MAPK (Erk, JNK, p38 MAPK) and NF- κ B is essential for Rv1808-mediated IL-10, TNF- α and IL-6 induction.

Discussion

Tuberculosis (TB), although largely a curable disease, remains a major cause of morbidity and mortality worldwide. Control of the global TB epidemic has been impaired by

Fig. 8. The recombinant Rv1808 protein specifically up-regulated TNF- α , IL6 and IL10 in macrophages. Semi-quantitative RT-PCR analysis of cytokines mRNA in total RNA extracted from PMA-differentiated U937 macrophages incubated with Rv1808(5 mg/ml) and Rv1808 (5 mg/ml) treated with protease K. The mRNA level was normalized to β -actin mRNA. The levels of TNF- α , IL-6, and IL-10 were analyzed by EIA. Results are mean $\pm$ SD of three different experiments. *, $p < 0.05$, ***, $p < 0.001$.

the dubious efficacy of sole available BCG vaccine and lack of an effective novel vaccines [41, 42], the emergence of drug-resistant *M. tuberculosis*, and the shortage of sensitive and rapid diagnostics [43]. In addition, the complex and incompletely characterized immune response to *M. tuberculosis* further compounded this scenario. Antigen presenting cells are crucial for the first-line host defense and bridge the innate and adaptive immunity. The initial encounter of macrophages with *M. tuberculosis* will elicit many cellular reactions, including the production of pro-inflammatory and anti-inflammatory cytokines [44-47]. Many features of the PPE family, such as the almost exclusive distribution among pathogenic *Mycobacterium* and absence among the non-pathogenic species, predicted cell wall association, imply a role in the host immune responses [7, 10-12].

The clinical outcome of mycobacteria infection depends largely on the pathogen capacity to manipulate the macrophage initial response. Necrosis or apoptosis of the *M. tuberculosis* infected macrophages have distinct consequences. Apoptosis is associated with diminished pathogen viability and enhanced immunity [21], while necrosis allows exit from macrophages and spread of the bacilli, a new approach to evade host immunity. Our results have shown that recombinant Ms_Rv1808 had no effect on the macrophages cell death but promoted the survival of recombinant *M. smegmatis* within macrophage, supposing that the enhanced survival of recombinant *M. smegmatis* might not be mediated by necrosis as there

Fig. 9. The production of cytokines involves ERK1/2, JNK, p38 MAPK-dependent pathway. PMA-differentiated macrophages were treated with specially pharmacological inhibitors of ERK1/2 (U0126, 10 μ M and PD98059, 15 μ M), p38 MAPK (SB202190, 20 μ M), JNK (SP600125, 10 μ M), or DMSO (vehicle control) for 1 h prior to treatment with Rv1808 for 6 h, and the level of secreted TNF- α , IL-6 and IL-10 was analyzed. Data are presented as mean $\pm$ S.E. from three independent experiments. ***, $p < 0.001$.

is no significant difference of the expression of IL-1 β between Ms_Rv1808 and Ms_pNITmyc infected macrophage.

To our knowledge, several *M. tuberculosis* PPE family proteins have been demonstrated to be able to activate the inflammatory cytokines [20, 29, 32, 47]. Rv1196 was reported to directly interact with the macrophage TLR2 to induce anti-inflammatory cytokine IL-10 through activating the p38 MAPK signaling [20], contrasting with the effect of PE_PGRS family protein PE_PGRS33 which triggers a pro-inflammatory response by the production of TNF- α [32, 47]. The cytokines and chemokines produced by macrophages and dendritic cells are crucial for the outcome of *M. tuberculosis* infection [48]. A rather paradox we found is that Rv1808 specifically up-regulated both anti-inflammatory cytokine IL-10 and inflammatory cytokines IL-6 and TNF- α . The underlying mechanism of this seemingly contradictory up-regulation remains unknown. Our speculation was that the ratio of different cytokines might be crucial for the outcome of the infection. However, it remains elusive about the identity of the specific cytokine(s) and their ratio. It was shown earlier that the effect of many other PPE family proteins on cytokines and chemokines production was mediated by the toll-like receptor 2 [49, 50]. Consistent with that, the affinity column pull downs using Rv1808 immobilized on Ni-NTA beads followed by immunoblotting with anti-TLR2 antibody confirmed the physical interaction between Rv1808 and TLR2. Other mediators affecting the surface interaction or physiological function of Rv1808 is yet to be defined.

We further defined the TLR2 downstream signaling events that mediated the effect of Rv1808. The TLR2-mediated downstream signaling usually associates with mitogen-activated protein (MAP) kinases, ubiquitous eukaryotic serine/threonine protein kinases involved in a wide range of cellular functions from proliferation to differentiation and programmed cell death [51]. At least three distinct MAP kinases families exist in mammalian cells: the p42/44 extracellular signal-regulated kinase (ERK) MAP kinase, c-Jun NH2-terminal

kinases (JNKs), and p38 MAP kinase. The active heterodimer p50/p65 of nuclear factor- κ B (NF- κ B) is crucial for immunological events by inducing the expression of multiple genes involved in the inflammatory response [52-55]. In cardiac myocytes, the stimulation of p38 MAPK by MKK6 activates the transcription factor nuclear factor NF- κ B for further trigger of IL-6 gene expression and release [56]. In addition, ERK1/2, p38 MAPK and NF- κ B pathways were involved in the IL-6 secretion [57]. Mycobacteria can activate MAPK, including ERK1/2 and p38 MAPK [33, 52, 58-61]. *M. avium*-Induced TNF- α and IL-10 production by human macrophages were differentially regulated at the level of mitogen-activated protein kinase activity [44]. P38 instead of ERK activity is necessary for IL-10 secretion [34, 38], whereas ERK instead of p38 activity is indispensable for the production of TNF- α [35, 36]. Song et al. (2003) demonstrated that both ERK and p38 MAPKs were essential for *M. tuberculosis* H37Rv-induced TNF- α production, whereas the activation of the p38 MAPK pathway alone was sufficient for *M. tuberculosis* H37Rv-induced IL-10 production [62]. The impaired expression of ERK1/2 and p38MAPK is associated with the down-regulation of TNF- α , IL-6, and IL-10 in *M. abscessus* lung disease [61]. Administration of the specific inhibitor of NF- κ B (N-p-Tosyl-L-phenylalanine chloromethyl ketone) [63] confirmed the biochemical cross-talk between the MAPK and NF- κ B pathways in Rv1808-induced production of IL10, IL-6 and TNF- α in U937 cells.

Blockage by specific inhibitors further supported a role of MAP kinases (ERK1/2, JNK and p38 MAPK) in the secretion of TNF- α , IL-6, and IL-10 by human macrophages in response to *M. tuberculosis* Rv1808. The production of TNF- α , IL-6 and IL-10 was significantly reduced when the cells were treated SB203580 (p38 MAPK inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) and N-p-Tosyl-L-phenylalanine chloromethyl ketone (NF- κ B inhibitor). We found that p38, ERK, JNK and NF- κ B are critically important in the *M. tuberculosis* PPE protein Rv1808-induced activation and subsequent IL-10, TNF- α and IL-6 production by human macrophages.

The disproportionate production of pro-inflammatory and inflammatory cytokines might lead to the dissolution of granulomas and the dissemination of the pathogens. In the mouse model of infection, TNF- α is required for the control of acute *M. tuberculosis* infection [64] and plays a critical role in granuloma formation [30]. IL-6 has been shown to have both pro- and anti-inflammatory properties in the host response to *M. tuberculosis* and is important for the initial innate response to the pathogen [19, 65]. Excessive pro-inflammatory responses can be deleterious to the host. In active tuberculosis (TB), emerging evidence suggests that high TNF- α level can accelerate disease progression [66]. The pro-inflammatory cytokines, including TNF- α and IL-6, present at the foci [67], are partially responsible for systemic manifestations such as cachexia and wasting in tuberculosis patients [68, 69]. IL-10 is an important regulator of myeloid cells, potentially inhibiting the capacity of monocytes/macrophages to secrete inflammatory cytokines as well as down-regulating their capacity to serve as accessory cells [70].

Conclusion

The most interesting inference from this study is that the effect of Rv1808 on the production of TNF- α , IL-6 and IL-10, crucial cytokines implicated in macrophage inhibition and response to IFN- γ , depended on TLR2 signaling. Previous study showed that *M. avium* elicited macrophage-dependent orchestration of pro-versus anti-inflammatory events occurred at the level of MAP kinase activity [33], including p38 MAPK and ERK. The receptor and early signaling events involved in *M. tuberculosis* Rv1808-induced activation of human macrophages involved TLR2 and downstream p38MAPK, JNK, ERK, and NF- κ B signaling. *M. tuberculosis* Rv1808 elicited, macrophage TLR2 mediated secretion of IL-10, TNF- α and IL-6 might be an integral part of the feedback circuit that limits the inflammatory. In this study, we found Rv1808 related to cell wall and enhanced both pro-inflammatory (TNF- α , IL-6) and anti-inflammatory cytokines (IL-10) may related the enhanced survival of

recombinant *M.smegmatis* within macrophage. This revealed some new aspect of the role of *M.tuberculosis* PPE family and furthered our understanding of the interaction between *M.tuberculosis* and host. The relations between these cytokines and how they enhanced the survival of recombinant *M.smegmatis* remain to be determined.

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