Expression of the *Mycobacterium tuberculosis* PPE37 protein in *Mycobacterium smegmatis* induces low tumour necrosis factor alpha and interleukin 6 production in murine macrophages

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PPE37 is a member of the *Mycobacterium tuberculosis* proline-proline-glutamic acid (PPE) multigene family. Its expression is upregulated in bacteria that are phagocytosed by macrophages and is enhanced even more in bacteria isolated from the lungs of infected mice. This raises the possibility that PPE37 may play a role in the virulence of *M. tuberculosis* and led to this investigation of the function of PPE37. Recombinant bacterial strains, one expressing the *M. tuberculosis* PPE37 protein (*Ms*_ppe37) and another harbouring the vector alone (*Ms*_vec) were generated from the non-pathogenic *Mycobacterium smegmatis*. These bacterial strains were used to infect peritoneal exudate and bone marrow-derived macrophages. It was found that, despite the comparable intracellular survival between the two recombinant *M. smegmatis* strains, *Ms*_ppe37 induced a significantly lower level of tumour necrosis factor alpha and interleukin 6 in the infected macrophages compared with *Ms*_vec. Western blot analyses revealed that the activation levels of nuclear factor kappa B, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase and MAPK/p38 were lower in macrophages infected with *Ms*_ppe37 than in macrophages infected with *Ms*_vec. These results suggest that PPE37 may have a potential role in interfering with the pro-inflammatory cytokine response of infected macrophages.

**INTRODUCTION**

The existence of the proline-proline-glutamic acid (PPE) multigene family was revealed when the decoding of the *Mycobacterium tuberculosis* genome was completed (Cole et al., 1998). Members of this gene family were found to share a highly conserved N-terminal sequence of approximately 180 aa that also contained the conserved PPE motif. In contrast, their C-terminal regions were highly heterogeneous in both sequence and length (Cole et al., 1998). The _ppe_ genes are not found outside the genus *Mycobacterium* and are highly distributed among the pathogenic species of mycobacteria (Gey van Pittius et al., 2006). For these reasons, they are speculated to contribute to the pathogenicity of *M. tuberculosis*. A possible functional role has been proposed for the PPE proteins, in which they serve as a source of antigenic variation that promotes antigenic diversity in *M. tuberculosis* (Cole et al., 1998). Indeed, the PPE proteins reported to date are immunogenic, eliciting either humoral or T-cell immune responses (Choudhary et al., 2003; Khan et al., 2008; Romano et al., 2008; Tundup et al., 2008; Wang et al., 2008).

One of the PPE proteins, PPE37, may have a role in the virulence of *M. tuberculosis*. It has been reported that expression of the _ppe37_ gene is upregulated in *M. tuberculosis* during infection of murine bone marrow-derived macrophages (Schnappinger et al., 2003; Voskuil et al., 2004). Moreover, in *M. tuberculosis* isolated from the lungs of infected mice, expression of the _ppe37_ gene is enhanced even more (Schnappinger et al., 2003). Earlier studies have shown that an iron-dependent transcriptional regulator that is critical for proper iron homeostasis in *M. tuberculosis* regulates the expression of _ppe37_ (Rodriguez et al., 1999, 2002). When *M. tuberculosis* was exposed to iron-limiting conditions in _in vitro_ culture, the expression of _ppe37_ increased greatly (Rodriguez et al., 2002; Schnappinger et al., 2003). In addition, _in vitro_ exposure of *M. tuberculosis* to nitrosative and oxidative growth conditions also increased the expression of _ppe37_ (Schnappinger et al., 2003; Voskuil et al., 2004). Both of

**Abbreviations:** ERK, extracellular signal-regulated kinase; IL, interleukin; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; NF-HB, nuclear factor kappa B; TNF-a, tumour necrosis factor alpha; TLR, Toll-like receptor.
these conditions are reported to mimic the macrophage phagosomal environment that contains *M. tuberculosis* (Schnappinger et al., 2003). From these results, it seems that PPE37 is required for the adaptation of *M. tuberculosis* to the intracellular niche in macrophages.

In the present study, as a first step towards evaluating the possible role of PPE37 in the virulence of *M. tuberculosis*, we took advantage of the lack of *ppe* genes in *Mycobacterium smegmatis* and generated two recombinant bacterial strains using this non-pathogenic bacterium. Unlike the *M. tuberculosis* genome, which contains 69 *ppe* ORFs, the *M. smegmatis* genome contains only 2. Furthermore, none of the *M. smegmatis* *ppe* genes are orthologues of the *M. tuberculosis* *ppe37* gene (Gey van Pittius et al., 2006). We cloned the *ppe37* gene from *M. tuberculosis* strain H37Rv and expressed the gene in *M. smegmatis* strain mc2 155 (Ms_{ppe37}). The ability of Ms_{ppe37} to survive inside macrophages was assessed in *in vitro* infection of mouse macrophages. In addition, the effect of PPE37 on the macrophage cytokine response was also investigated.

**METHODS**

**Bacterial strains and growth conditions.** *Escherichia coli* DH5α was routinely grown in Lennox LB medium for use in DNA cloning procedures. *M. tuberculosis* strain H37Rv and *M. smegmatis* strain mc2 155 were grown at 37 °C in Middlebrook 7H9 liquid medium or on Middlebrook 7H10 agar (Difco) supplemented with 0.5% (w/v) albumin fraction V, 0.2% (w/v) glucose, 0.5% (w/v) glycerol and 0.05% (v/v) Tween 80. For the preparation of culture filtrate fraction, recombinant *M. smegmatis* Ms_{ppe37} was grown in Sauton medium as described by Rosenkranz & Andersen (2001). When required, 25 μg kanamycin ml⁻¹ was also added.

**Macrophages.** C57BL/6 female mice of 7 to 9 weeks old (Japan SLC) were used in experiments according to protocols approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine. Peritoneal exudate cells were harvested from mice 3–4 days after intraperitoneal injection with 2.5 ml 3% (w/v) mouse peritoneal exudate cells were washed three times with ice-cold PBS and resuspended in extraction buffer containing 20 mM Tris/HisCl (pH 6.8), 4 mM EDTA, 0.6% SDS and 1% Triton X-100. Lysates were plated on Middlebrook 7H10 agar containing 25 μg kanamycin ml⁻¹ and 100 ng mouse macrophage colony-stimulating factor ml⁻¹ (R&D Systems). After removal of non-adherent cells, adherent cells were used as bone marrow-derived macrophages.

**Generation of recombinant *M. smegmatis* expressing PPE37.** Chromosomal DNA was isolated from *M. tuberculosis*, and the *ppe37* gene was PCR-amplified with the use of forward primer 5′-TTACTAGTtgcactacacACCTCCCGGAT-3′ containing an Spel site (underlined) and three His codons (lower-case letters), and reverse primer 5′-CGTGAAAGTCCTTCACAGTTTAATCGGACC-3′ containing a HindIII site (underlined). The PCR product of approximately 1.5 kb was cloned into the pEGFP vector (Clontech Laboratories), generating the recombinant plasmid pEGFP-his3ppe37. Three additional His codons were introduced at the 5′ end of the his3ppe37 sequence with the use of the same reverse primer and a second forward primer, 5′-TGAATTCATGatccatcactacctagc ACC-3′, containing an EcoRI site (underlined) and the His codons (lower-case letters). The resultant PCR product containing six consecutive His codons was inserted in frame into the cloning site of pMV261, a mycobacterial expression vector (Stover et al., 1991), generating pMV261-his6ppe37. The recombinant plasmid or empty pMV261 was electroporated into *M. smegmatis* mc2 155 according to standard procedures (Larsen, 2000). Recombinant *M. smegmatis* expressing 6His-tagged PPE37 (Ms_{ppe37}) and *M. smegmatis* harbouring empty pMV261 alone (Ms_{vec}) were selected on Middlebrook 7H10 agar containing 25 μg kanamycin ml⁻¹.

**Detection of ppe37 gene expression in recombinant *M. smegmatis*.** Recombinant *M. smegmatis* strains were cultured until they reached an OD₆₀₀ of 0.6–1.0 in 100 ml Middlebrook 7H9 liquid medium in the presence of 25 μg kanamycin ml⁻¹. Total bacterial RNA was isolated using Sepasol RNA I Super (Nacalai Tesque). All RNA samples were treated with DNase I (Promega) and subjected to PCR to test for the complete removal of genomic DNA. cDNA was synthesized from 1 μg total RNA in a 40 μl reaction mixture containing reverse transcriptase buffer, 150 ng random primers, 2 μl 10 mM dNTP mix, 2 μl 0.1 M DTT and 400 U SuperScript III reverse transcriptase (Invitrogen). PCR was performed with a KOD-Plus enzyme kit (Toyobo) and the following primer pairs: (i) *ppe37* gene – 5′-TGGTGACTGGTACACCTCG-3′ (forward) and 5′-CATCTTGTGGTCTGCTG-3′ (reverse), product size 500 bp; and (ii) aminoglycoside phosphotransferase (*aph*) gene – 5′-AGGATGGTGTCGCGCACTGATG-3′ (forward) and 5′-CTCACGGAGCGATTCCATA-3′ (reverse), product size 540 bp.

**Detection of His-tagged PPE37.** 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 μg kanamycin ml⁻¹. Bacterial pellets were harvested, washed three times with ice-cold PBS and resuspended in extraction buffer containing 20 mM Tris/HCl (pH 6.8), 4 mM EDTA, 0.6% SDS and protease inhibitor cocktail (Nacalai Tesque). Bacterial cells were disrupted and supernatants were collected after centrifugation at 20,000 g for 20 min at 4 °C. For the preparation of the culture filtrate fraction, the culture supernatant of bacteria grown in Sauton medium was harvested by centrifugation at 2000 g for 15 min at 4 °C. The supernatant was filtered through a 0.2 μm syringe filter and concentrated to approximately 150 μl using a centrifugal filter with a cut-off value of 5 kDa (Millipore). Samples were subjected to SDS-PAGE, and the His-tagged PPE37 protein was detected by Western blotting and mouse anti-penta-His antibody (Qiagen). Chemiluminescent images were captured with a luminescent image analyser LAS-4000mini (Fujifilm).

**M. smegmatis infection of macrophages.** Macrophages were seeded at 1 × 10⁶ cells per well in 12-well tissue culture plates or at 3 × 10⁶ cells per well in 24-well tissue culture plates. Cells were infected with Ms_{ppe37} or Ms_{vec} at an m.o.i. of 20. At this m.o.i., the resulting infection rate was greater than 80% as estimated in preliminary infection assays from microscopy evaluation of slides stained according to the Kinyon method (Chapin & Lauderdale, 2007). Four hours after infection, gentamicin was added to give a final concentration of 5 μg ml⁻¹. At 6, 24 and 48 h after infection, macrophages were washed and lysed in PBS containing 0.1% (v/v) Triton X-100. Lysates were plated on Middlebrook 7H10 agar plates containing 25 μg kanamycin ml⁻¹ and the number of intracellular bacteria was enumerated.

**Assay for lactate dehydrogenase (LDH) release.** Culture supernatants were harvested after infection of macrophages with Ms_{ppe37} or Ms_{vec} for 6, 24 or 48 h. LDH activity in the culture supernatants

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was assayed with an LDH cytotoxicity detection kit (Takara Bio). The percentage of LDH release was calculated as: percentage release = (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.

Assay for cytokine production. Culture supernatants were harvested after infection of macrophages with Ms_ppe37 or Ms_vec for 24 h. The concentrations of cytokines in the culture supernatants were determined using commercially available ELISA kits for tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), IL-1β (eBioscience) and IL-12p70 (Endogen). In some experiments, after infection of macrophages with Ms_ppe37 or Ms_vec for 3, 6, 9, 12 and 18 h, total RNA was extracted with a Nucleospin RNA II kit (Macherey-Nagel). RNA (250 ng) was treated with RNase-free DNase (Promega) and subsequently reverse transcribed into cDNA using a SuperScript VILO cDNA synthesis kit (Invitrogen). cDNAs were diluted ten-fold, and a PCR was performed in an equal reaction volume using a KOD-Plus enzyme kit and the following primer pairs: (i) msf gene – 5’-CATGAGCACAGAAAGCATGATCCG-3’ (forward) and 5’-TCTGGGCATAAGAATGAGAG-3’ (reverse), product size 230 bp; (ii) IL-6 gene – 5’-TTCTCTCTGAAAAGACTGACT-3’ (forward) and 5’-TGATCTTCTGAGAGACT-3’ (reverse), product size 432 bp; and (iii) actb gene – 5’-TGGAATCTGTGGCTGCACCCTAGACGAA-3’ (forward) and 5’-AAATCGCTGCAATAGCAGTCCCC-3’ (reverse), product size 350 bp. Equal volumes of the PCR mixtures were electrophoresed in 1.5% agarose gel, and DNA bands were visualized with ethidium bromide (2 μg ml⁻¹) staining.

Flow cytometric analysis. Macrophages were harvested after a 24 h infection with Ms_ppe37 or Ms_vec and treated with anti-CD16/ CD32 mAb (clone 93; eBioscience) for 10 min. This was followed by a 20 min incubation on ice with one of the following phycoerythrin-conjugated antibodies against: major histocompatibility complex class I (MHC-I; clone 28-14-8), MHC-II (clone M5/114.15.2), B7.1 (CD80 clone 16-10A1), B7.2 (CD86, clone GL1) or CD40 (clone 1C10), or with an isotype control antibody (all from eBioscience). The intensity of each cell-surface marker was analysed on a FACScalibur flow cytometer equipped with CellQuest software (BD Biosciences).

Assay for nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinase (ERK) and p38 phosphorylation. Macrophages were infected with Ms_ppe37 or Ms_vec for 0.5, 1, 2, 4, 6, 7 or 8 h. After infection, macrophages were lysed in buffer containing 10 mM Tris/HCl (pH 6.8), 1% (v/v) NP-40 and proteinase/phosphatase inhibitor cocktail (Nacalai Tesque). Cell lysates were harvested and subjected to SDS-PAGE. Phosphorylated and unphosphorylated ERK and p38, as well as the phosphorylated p65 subunit of NF-κB, were detected in lots with specific antibodies (Cell Signaling Technology). β-Actin was detected with anti-β-actin antibody (Sigma-Aldrich). Chemiluminescent images were captured with a luminescent image analyser LAS-4000mini. In another experiment, macrophages were pre-treated with 20 or 40 μM U0126 (a MEK1/2 inhibitor; Cell Signaling Technology) or with 10 or 20 μM Calbiochem SB202190 (a p38 inhibitor; EMD Biosciences). On the basis of preliminary experiments, the inhibitors were used at the concentrations required to inhibit ERK and p38 activities. One hour after treatment, the macrophages were infected with Ms_ppe37 or Ms_vec. The culture supernatants were harvested 24 h after infection, and ELISA was performed to determine the concentrations of TNF-α and IL-6.

Statistical analysis. Data were analysed using Student’s two-tailed t-test. Statistical significance was defined as a P value <0.05. Error bars represent SD.

RESULTS

Ms_ppe37 constitutively expresses M. tuberculosis PPE37 protein

In this study, we generated two recombinant M. smegmatis strains to investigate the effect of PPE37 on the macrophage response to bacterial infection. The Ms_ppe37 strain was engineered to express a 6His-tagged PPE37 protein from a recombinant pMV261 vector, whilst the Ms_vec strain harboured the vector alone. The pMV261 vector contains the kanamycin resistance gene aph for selection of transformed bacteria (Stover et al., 1991). Both Ms_ppe37 and Ms_vec, which were grown in Middlebrook 7H9 medium in the presence of kanamycin, expressed the aph gene. However, only Ms_ppe37 was able to express the ppe37 gene (Fig. 1a). Furthermore, Western blot analysis with anti-penta-His antibody detected a protein band representing PPE37 in the total cell lysate prepared from Ms_ppe37 but not from Ms_vec (Fig. 1b). These results confirmed that the transformation was successful and that ppe37 gene expression was detectable only in M. smegmatis that had been electroporated with the recombinant vector. In addition, Western blot analysis also revealed that a protein band representing PPE37 was detectable in the total cell lysate but not in the culture filtrate fractions prepared from Ms_ppe37 grown in Sauton medium (Fig. 1c). This was not due to the absence of proteins in the culture filtrate fraction, as Coomassie blue staining revealed the presence of many protein bands in both the culture filtrate and the cell lysate fractions. From the result in Fig. 1(c), it could be suggested that PPE37 is not a secretory protein. We also compared the growth kinetics of Ms_ppe37 and Ms_vec in Middlebrook 7H9 medium, as excess production of recombinant protein is known to exert a metabolic burden on recombinant bacteria, sometimes reducing the growth of these cells (Bentley et al., 1990). We observed no marked difference in the growth kinetics (Fig. 1d), indicating that expression of the ppe37 gene did not influence the growth of Ms_ppe37.

PPE37 does not contribute to the intracellular survival of M. smegmatis in macrophages

M. smegmatis is inherently unable to multiply inside macrophages, and the number of intracellular bacteria decreased gradually after infection of macrophages in vitro. In order to determine whether PPE37 facilitated the intracellular survival of these bacteria in macrophages, we compared the survival kinetics of Ms_ppe37 and Ms_vec in peritoneal exudate macrophages by conducting a gentamicin protection assay. The results showed no significant difference in the number of bacteria between Ms_ppe37 and Ms_vec up to 48 h after infection (Fig. 2a). Similar results were also observed in bone marrow-derived macrophages (Fig. 2b). These results suggested that the presence of PPE37 was not able to enhance the intracellular survival of M. smegmatis in macrophages.
PPE37 does not affect macrophage cell death during infection with *M. smegmatis*

One of the consequences of infecting host cells with *M. tuberculosis* is cell death, with the possibility that *M. tuberculosis* manipulates host-cell death as one of the mechanisms of pathogenicity. Recently, emerging new evidence prompted the proposal of a new model on the interaction between *M. tuberculosis* and its host cell (Behar et al., 2010). This model suggests that, as a pathogenic strategy, virulent *M. tuberculosis* inhibits apoptosis whilst actively inducing necrosis in the infected host cell. The outcome of this strategy is a reduction in the efficiency of cross-presentation of mycobacterial antigens leading to the impairment in the initiation of T-cell immunity (Behar et al., 2010; Divangahi et al., 2010).

In this experiment, we thus wanted to determine whether PPE37 was able to affect the death of macrophages infected with *M. smegmatis*. Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec, and the amount of LDH released into the culture supernatant (an indicator of cell death) was determined. The result showed that macrophages infected with Ms_ppe37 or Ms_vec released a comparable amount of LDH (Fig. 3). In addition, microscopic examination also revealed no differences in the morphological features of the infected macrophages within the time period of infection (data not shown). Taken together, these results suggested that macrophage cell death was unaffected by PPE37 during infection with *M. smegmatis*.

**Ms_ppe37 induces a lower level of pro-inflammatory cytokines in infected macrophages**

To deduce the potential role of PPE37 in the virulence of *M. tuberculosis*, we investigated the effect that PPE37 might have on the immune response of infected macrophages. The aspect of the immune response that we examined first was cytokine production. Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec, and the amount of pro-inflammatory cytokines was determined. The result showed that macrophages infected with Ms_ppe37 or Ms_vec released a comparable amount of cytokines (Fig. 4). In addition, microscopic examination also revealed no differences in the morphological features of the infected macrophages within the time period of infection (data not shown). Taken together, these results suggested that macrophage cell death was unaffected by PPE37 during infection with *M. smegmatis*.
production of macrophages infected with *M. smegmatis* but does not affect the expression of surface markers on these macrophages.

**PPE37 alters the activation levels of NF-κB, ERK and p38 in macrophages infected with *M. smegmatis***

NF-κB is a major transcription factor responsible for the expression of both TNF-α and IL-6 mRNAs (Collart *et al.*, 1990; Faggioli *et al.*, 2004; Kuprash *et al.*, 1999; Libermann & Baltimore, 1990; Zhang *et al.*, 1994). It has been reported that NF-κB activation is needed to induce the expression of TNF-α and IL-6 mRNAs in macrophages infected with *M. smegmatis* (Gutierrez *et al.*, 2008). In addition, a previous study showed that *M. smegmatis* infection is also able to induce phosphorylation of the NF-κB p65 subunit (Lee & Shorey, 2005). The altered TNF-α and IL-6 mRNA expression shown in Fig. 4(g) therefore raised the possibility that NF-κB activation might be altered in macrophages infected with Ms_ppe37. To clarify this possibility, Western blot analysis was performed and the level of phosphorylated NF-κB p65 subunit in the infected macrophages was assessed. It was observed that Ms_ppe37 induced a relatively lower level of p65 phosphorylation in the infected macrophages compared with Ms_vec (Fig. 6a).

In addition to NF-κB, it has been shown that the expression of TNF-α mRNA in macrophages infected with *M. smegmatis* also requires the activation of p38 and ERK (Lee & Shorey, 2005; Roach *et al.*, 2005). Pharmacological inhibition experiments confirmed the requirement for ERK and p38 activities in the production of TNF-α and IL-6 in macrophages infected with Ms_vec (Fig. 6d, e). These results (Figs 4g and 6d, e) thus led us to investigate whether the activation of these mitogen-activated protein kinases (MAPKs) might also be affected in macrophages infected with Ms_ppe37. Western blot analysis was performed and the levels of phosphorylated ERK and p38 in the infected macrophages were assessed. Fig. 6(b, c) showed that Ms_ppe37 also induced a relatively lower level of ERK and p38 phosphorylation in infected macrophages compared with Ms_vec. The lower transcriptional activation of the TNF-α and IL-6 genes in macrophages infected with Ms_ppe37 therefore might have been due to a lower level of activation of NF-κB, ERK and p38. Taken together, these results further argue for the possibility of PPE37 interfering with the pro-inflammatory cytokine response of macrophages infected with *M. smegmatis*.

**DISCUSSION**

In the present study, the results suggested the possibility that the *M. tuberculosis* PPE37 protein might interfere with the pro-inflammatory cytokine response in infected macrophages. We found that TNF-α, IL-6, IL-12p70 and IL-1β were produced at significantly lower concentrations by macrophages infected with Ms_ppe37 compared with
macrophages infected with Ms_vec. The differential cytokine levels were due to lower transcriptional activation of the cytokine genes, which probably resulted from reduced activation of NF-κB, ERK and p38.

To the best of our knowledge, PPE18 is the only other PPE protein that has been reported to exhibit the property of interfering with the pro-inflammatory cytokine response in infected macrophages (Nair et al., 2009). In the study by Nair et al. (2009), phorbol myristate acetate-differentiated THP-1 macrophages were infected with either a recombinant M. smegmatis strain that expressed PPE18 or a control strain that harboured the vector alone. It was shown that IL-12p40 production was significantly lower in macrophages after infection with the PPE18-expressing strain than after infection with the control strain. Nair et al. (2009) concluded that the decrease in the level of IL-12p40 was due to the anti-inflammatory activity of IL-10. A significantly higher production of IL-10 was concurrently found in macrophages after infection with the PPE18-expressing strain. In contrast to our study, we observed very low levels of IL-10 and found no significant difference in the concentration of IL-10 after infection with Ms_vec and Ms_ppe37 (data not shown). Using a purified recombinant protein, Nair et al. (2009) showed that PPE18 stimulated the macrophages to secrete IL-10 by binding to Toll-like receptor 2 (TLR2). A consequence of this binding was an early and sustained activation of p38 MAPK, which has been shown to be critical for the induction of IL-10. Similarly, our findings also implied the involvement of MAPKs. However, our study suggests that the mechanism by which PPE37 might interfere with the pro-inflammatory cytokine response in infected macrophages involves reduced transcriptional activation of the...
macrophages were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. After 24 h of infection, the macrophages were harvested and the expression levels of MHC-I, MHC-II, CD86, CD80 and CD40 were analysed by flow cytometry. Grey-shaded areas represent the basal fluorescent intensity in macrophages stained with isotype control IgG2a. n.i., No infection.

Fig. 5. Expression of cell-surface markers on macrophages infected with recombinant M. smegmatis. Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. After 24 h of infection, the macrophages were stained with isotype control IgG2a. n.i., No infection.

The other factor that may have contributed to the distinct features in the effect of PPE18 and PPE37 on IL-10 production and the pattern of MAPK activation may stem from differences in the responses between mouse peritoneal macrophages and the human monocytic leukaemia cell line THP-1. Differences in MAPK activation are found to differ considerably depending on the cell type used (Rao, 2001). This has led to the assertion that signalling events associated with MAPK activation cannot be extrapolated from one cell type to another (Rao, 2001). In addition to cell type, it has also been reported that the level of cell maturity also affects the activation of MAPK. Indeed, it was shown that, upon infection with M. tuberculosis, the kinetics of p38 MAPK activation in human alveolar macrophages was faster than in human blood monocytes (Surewicz et al., 2004).

Our study showed that the phosphorylation levels of ERK, p38 and NF-κB p65 were lower in macrophages infected with Ms_ppe37. This suggests that PPE37 may be interfering with or inhibiting the activation of these molecules. How does PPE37 achieve this, considering that ERK, p38 and NF-κB p65 are three different proteins, each associating with three different signalling pathways? A possible mechanism as to how PPE37 might inhibit or interfere with the activation of ERK, p38 and NF-κB p65 is by inhibiting or interacting with a molecule that is involved in the common activation of these three different proteins. Although the MAPK and NF-κB signalling pathways are distinct, they are not mutually exclusive. For example, they are known to share some common stretches of the signalling pathways when the TLRs are stimulated (Akira et al., 2003). Among the TLRs, TLR2 is most frequently involved in the recognition of various pathogen-associated molecular patterns isolated from Mycobacterium spp. (Jo et al., 2007). Therefore, in the innate immune response to Mycobacterium spp. including M. smegmatis, the activation of MAPKs and NF-κB may occur most commonly through the stimulation of TLR2. In general, the stimulation of most TLRs results in the recruitment of the adaptor protein MyD88 to the receptor complex, where it promotes the subsequent interaction of IL-1R-associated kinase with TNF receptor-associated factor 6 (TRAF6). The signalling pathways from TRAF6 then branch out, with one leading to the MAPK pathway and another to the NF-κB pathway (Akira et al., 2003). This thus makes it very tempting to speculate on the possibility that PPE37 interacts with one of these molecules, including TLR2, that are involved in the common activation of the MAPK and NF-κB signalling pathways. Although the results shown in Fig. 1(c) suggested that PPE37 is not a secretory protein, computational analysis of the amino acid sequence predicted the subcellular localization of PPE37 to be on the bacterial cytoplasmic membrane (Gardy & Brinkman, 2006; http://www.psort.org/psortb/). In line with this, as TLR2 is a cell-surface receptor molecule, it may be more likely to interact with PPE37 than with other molecules in the TLR2 signalling pathways that are involved in the common

cytokine genes. This in turn is probably due in part to reduced activation of NF-κB, ERK and p38.

Although both PPE18 and PPE37 exhibit the similar property of interfering with the pro-inflammatory cytokine response in infected macrophages, there appear to be differences in the mechanisms. This is indicated by the discrepancy in the production of IL-10 and also in the pattern of MAPK activation. One of the possible contributing factors may be attributed to a difference in the intrinsic properties of PPE37 and PPE18. Analysis of their amino acid sequences has led to further classification of PPE37 and PPE18 into the PPE-PPW and PPE-SVP subfamilies, respectively (Adindla & Guruprasad, 2003; Gey van Pittius et al., 2006; Gordon et al., 1999). PPE proteins of the subfamily PPE-PPW are characterized by a conserved 44 aa residue region in the C terminus, which comprises highly conserved Gly-Phe-X-Gly-Thr and Pro-X-X-Pro-X-Trp sequence motifs (Adindla & Guruprasad, 2003; Gey van Pittius et al., 2006). Members of the PPE-SVP subfamily, on the other hand, contain the motif Gly-X-X-Ser-Val-Pro-X-X-Trp between positions 300 and 350 in their amino acid sequence (Gey van Pittius et al., 2006; Gordon et al., 1999). A systematic functional comparison has yet to be made, but these amino acid sequence motifs may confer distinct properties on the respective PPE proteins.
activation of MAPKs and NF-κB. However, TLR2 is not the only candidate receptor with the possibility of interacting with PPE37, as the common activation of the MAPK and NF-κB signalling pathways is not limited to this receptor alone.

In the context of M. tuberculosis infection, the possible role of PPE37 in interfering with the pro-inflammatory cytokine response in infected macrophages might also be applicable. Manca et al. (1999) reported that infection of human monocytes with M. tuberculosis clinical isolate CDC1551 induced a higher level of TNF-α, IL-6 and IL-12 than infection with the M. tuberculosis laboratory strain H37Rv. It may be possible that this vigorous pro-inflammatory cytokine response induced by CDC1551 was due in part to the loss of PPE37 function. Comparative genome analysis between CDC1551 and H37Rv has revealed that the ppe37 gene is deleted from the genome of CDC1551 (Gey van Pittius et al., 2006).

In conclusion, the present study suggests that the M. tuberculosis PPE37 may have a role in interfering with the pro-inflammatory cytokine response in macrophages infected with M. smegmatis. It is established that pro-inflammatory cytokines such as TNF-α (Elbek et al., 2009; Flynn et al., 1995; Jacobs et al., 2007; Lin et al., 2007; Wolfe et al., 2004) are critical to host immune responses in containing M. tuberculosis infection. Subversion and modulation of the host inflammatory response can thus be an advantageous pathogenic strategy for M. tuberculosis. In light of the possible role of PPE37 suggested by our study, a hypothesis of the possible contribution of PPE37 to such a pathogenesis strategy is presented. Our results thus provide a basis to investigate and characterize further the role of PPE37 in the context of M. tuberculosis infection. Future studies that are needed include the construction and testing of knockout genes in M. tuberculosis.

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