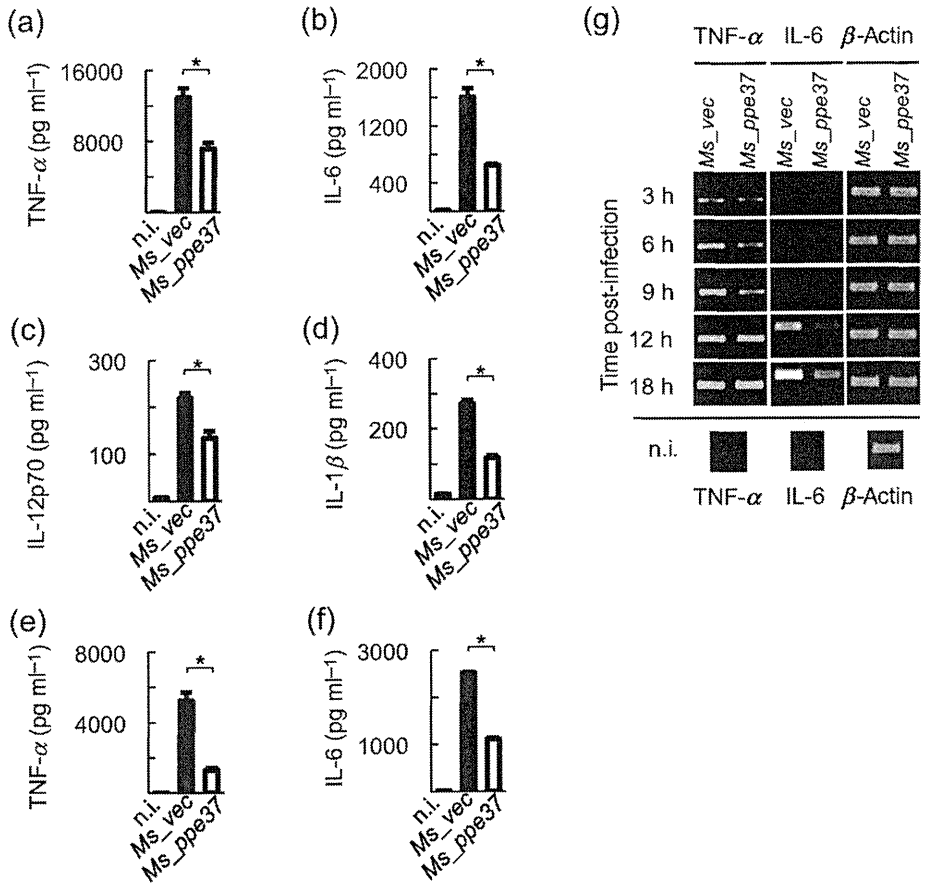

Fig. 4. Production of pro-inflammatory cytokines by macrophages infected with recombinant *M. smegmatis*. Peritoneal exudate macrophages (a–d) or bone marrow-derived macrophages (e, f) were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. Culture supernatants were harvested after 24 h of infection and the concentrations of TNF-α (a, e), IL-6 (b, f), IL-12p70 (c) and IL-1β (d) were determined. Data are shown as means ± SD of triplicate wells. *, $P<0.05$ by Student's two-tailed *t*-test. Similar results were obtained in three independent experiments. (g) Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. At 3, 6, 9, 12 and 18 h 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 ethidium bromide staining after equal volumes of PCR mixture had been electrophoresed. Similar results were obtained in two independent experiments. n.i., No infection.

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

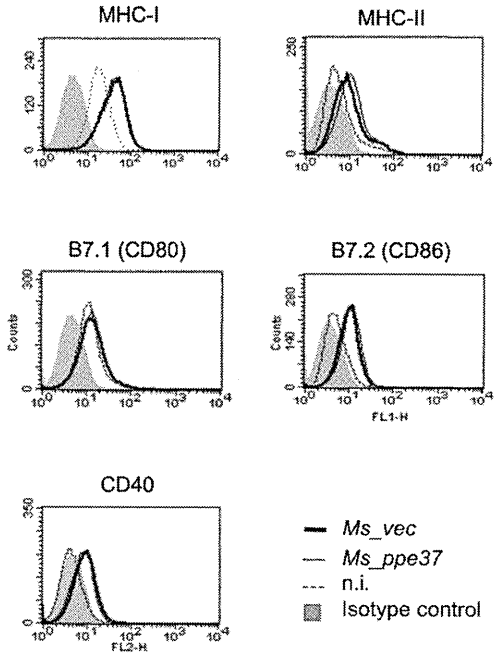

Fig. 5. Expression of cell-surface markers on macrophages infected with recombinant *M. smegmatis*. Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. After 24 h of infection, the macrophages were harvested and the expression levels of MHC-I, MHC-II, CD86, CD80 and 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.

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-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 position 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.

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

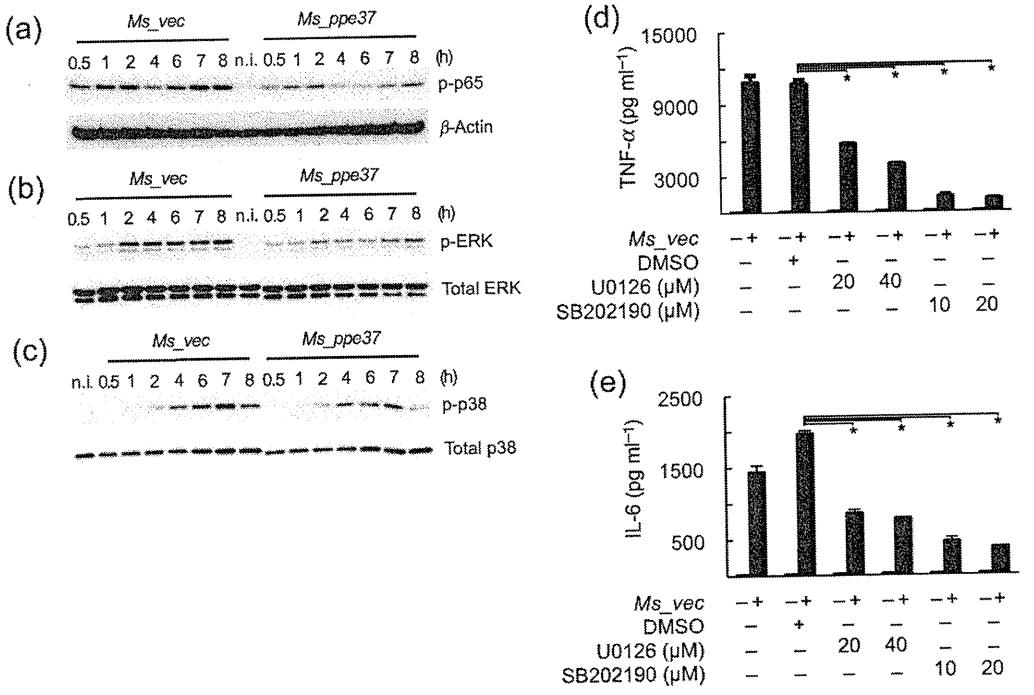

Fig. 6. Phosphorylation of the NF-κB p65 subunit, ERK and p38 in macrophages infected with recombinant *M. smegmatis*. (a–c) Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. Macrophages were washed and lysed after 0.5, 1, 2, 4, 6, 7 and 8 h of infection. Lysates were subjected to Western blot analyses to detect the phosphorylated NF-κB p65 subunit (p-p65) (a), phosphorylated ERK (p-ERK) (b) and phosphorylated p38 (p-p38) (c) with specific antibodies. Detection of β-actin, total ERK and total p38 indicated equal protein loading. Similar results were obtained in three independent experiments. n.i, No infection. (d, e) Peritoneal exudate macrophages were treated with U0126 (a MEK1/2 inhibitor) or SB202190 (a p38 inhibitor) at the indicated concentrations. Treatment with DMSO served as a control for the inhibitor treatments. After 1 h, the macrophages were infected with Ms_vec at an m.o.i. of 20. Culture supernatants were harvested after 24 h of infection and the concentrations of TNF-α (d) and IL-6 (e) in the culture supernatants were determined. Data are shown as means ± SD of triplicate wells. *, $P<0.05$ by Student's two-tailed *t*-test. Similar results were obtained in three independent experiments. −, No infection or treatment; +, with infection or treatment.

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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