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

Sylvia Daim, Ikuo Kawamura, Kohsuke Tsuchiya, Hideki Hara, Takeshi Kurenuma, Yanna Shen, Sita R. Dewamitta, Shunsuke Sakai, Takamasa Nomura, Huixin Qu and Masao Mitsuyama

Department of Microbiology, Kyoto University Graduate School of Medicine, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

Correspondence

Ikuo Kawamura

ikuo_kawamura@mb.med.kyoto-u.ac.jp

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.

Received 9 September 2010

Accepted 10 January 2011

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- κ B, nuclear factor kappa B; TNF- α , 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 the *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* DH5 α 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% (v/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 Rosenkrands & Andersen (2001). When required, 25 μ g 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) thioglycollate (Eiken Chemical). Cells were washed, seeded in tissue culture plates and cultured for 2 h in 5% CO₂ at 37 °C in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Adherent cells were used as peritoneal exudate macrophages after non-adherent cells had been removed by washing. In other experiments, bone marrow cells were collected from the tibiae of mice. Cells were cultured for 5–7 days in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 20 μ g gentamicin 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'-TTACTAGTcaccatcacACCTTCCCGAT-3' containing an *SpeI* site (underlined) and three His codons (lower-case letters), and reverse primer 5'-CCGTAAGCTTCTTCAACGTTTAACTGACC-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'-TGAATTCATGcatcaccatcacacACC-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 μ g 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 μ g 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 μ g total RNA in a 40 μ l reaction mix containing reverse transcriptase buffer, 150 ng random primers, 2 μ l 10 mM dNTP mix, 2 μ l 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'-TGTTGGACTGGTTCATCTCG-3' (forward) and 5'-CAGTCT-TGTTGCTTTGCTGG-3' (reverse), product size 500 bp; and (ii) aminoglycoside phosphotransferase (*aph*) gene – 5'-AGGTAGC-GTTGCCAATGATG-3' (forward) and 5'-CTCACCGAGGCAGTT-CCATA-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 μ g 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 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 μ m syringe filter and concentrated to approximately 150 μ l 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 $\times$ 10⁶ cells per well in 12-well tissue culture plates or at 3 $\times$ 10⁵ 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 μ g 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 μ g 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

was assayed with an LDH cytotoxicity detection kit (Takara Bio). The percentage of LDH release was calculated as: percentage release = $100 \times (\text{experimental LDH release} - \text{spontaneous LDH release}) / (\text{maximal LDH release} - \text{spontaneous LDH release})$. 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-1 β (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 tenfold, and a PCR was performed in an equal reaction volume using a KOD-Plus enzyme kit and the following primer pairs: (i) *tnf* gene – 5'-CATGAGCACAGAAAGCATGATCCG-3' (forward) and 5'-TCTGGGCCATAGAACTGATGAGAG-3' (reverse), product size 230 bp; (ii) *IL-6* gene – 5'-TTCCTCTCTGCAAGAGACT-3' (forward) and 5'-TGTATCTCTCTGAAGGACT-3' (reverse), product size 432 bp; and (iii) *Actb* gene – 5'-TGGAATCCTGTGGCA-TCCATGAAAC-3' (forward) and 5'-TAAACGCGAGCTCAGTAA-CAGTCCG-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 $\mu\text{g ml}^{-1}$) 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- κ B), 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- κ B, 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.

Fig. 1. Expression of *M. tuberculosis* PPE37 by *M. smegmatis* mc² 155. (a) Ms_ppe37 and Ms_vec were grown at 37 °C in Middlebrook 7H9 liquid medium to an OD₆₀₀ of 0.6–1.0. Total bacterial RNA was isolated and subjected to RT-PCR to detect expression of the *ppe37* and *aph* genes. (b) Lysates were prepared from bacterial cells that were cultured as in (a) and subjected to Western blot analysis to detect His-tagged PPE37 protein using mouse anti-penta-His antibody. (c) Lysate and culture filtrate fractions were prepared from Ms_ppe37 grown at 37 °C in Sauton medium to an OD₆₀₀ of 0.6–1.0. Samples were diluted and subjected to Western blot analysis (left panel) as in (b), as well as to Coomassie blue staining (right panel), after SDS-PAGE. (d) Growth of Ms_ppe37 (○) and Ms_vec (●) at 37 °C in Middlebrook 7H9 liquid medium was monitored by determining OD₆₀₀ at intervals of 3 h. Similar results were obtained in two independent experiments. Ori, Original undiluted sample; 1/2 and 1/4, samples diluted twofold and fourfold, respectively; M, protein marker.

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

Fig. 2. Intracellular survival of recombinant *M. smegmatis* in macrophages. Peritoneal exudate macrophages (a) or bone marrow-derived macrophages (b) were infected with Ms_ppe37 (white bars) or Ms_vec (black bars) at an m.o.i. of 20. At 6, 24 and 48 h after infection, the macrophages were washed and lysed. Lysates were diluted and plated on Middlebrook 7H10 agar plates containing 25 µg kanamycin ml⁻¹ to determine the number of c.f.u. Data are shown as means ± SD of triplicate wells. Similar results were obtained in three independent experiments.

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

Fig. 3. Assay of cell death in macrophages infected with recombinant *M. smegmatis*. Peritoneal exudate macrophages were infected with Ms_ppe37 (white bars) or Ms_vec (black bars) at an m.o.i. of 20. At 6, 24 and 48 h after infection, culture supernatants were harvested. Release of LDH as 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.

infected with Ms_ppe37 or Ms_vec for 24 h, and the concentrations of cytokines in the culture supernatants were determined. Consistent with the cytokine profiles reported elsewhere for macrophages infected with *M. smegmatis* (Lee & Schorey, 2005; Post *et al.*, 2001; Roach & Schorey, 2002), peritoneal exudate macrophages infected with Ms_vec or Ms_ppe37 also produced TNF- α , IL-6, IL12p70 and IL-1 β (Fig. 4a–d). However, we discovered that Ms_ppe37 induced a significantly lower level of these cytokines in infected macrophages compared with Ms_vec. We repeated the infection experiment in bone marrow-derived macrophages, and measured the production of TNF- α and IL-6. Similar to infection in peritoneal exudate macrophages, Ms_ppe37 also induced a significantly lower level of TNF- α and IL-6 in the infected bone marrow-derived macrophages compared with Ms_vec (Fig. 4e, f). RT-PCR analyses showed that the expression of TNF- α mRNA in macrophages infected with Ms_ppe37 was delayed compared with the expression in macrophages infected with Ms_vec. The difference in the expression level was observed until 9 h after infection and thereafter became comparable (Fig. 4g). Although expression of IL-6 mRNA occurred much later, from 12 h after infection, the mRNA levels were also lower in macrophages infected with Ms_ppe37 than in macrophages infected with Ms_vec. However, despite the differential cytokine response, flow cytometric analysis revealed no significant differences in the expression levels of MHC-I, MHC-II, CD80, CD86 and CD40 between macrophages infected with Ms_ppe37 and macrophages infected with Ms_vec (Fig. 5). Taken together, these results suggested that PPE37 might possess a functional property that interferes with the pro-inflammatory cytokine

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- κ B, ERK and p38 in macrophages infected with *M. smegmatis*

NF- κ B 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- κ B 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- κ B p65 subunit (Lee & Schorey, 2005). The altered TNF- α and IL-6 mRNA expression shown in Fig. 4(g) therefore raised the possibility that NF- κ B 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- κ B 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- κ B, 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 & Schorey, 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- κ B, 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-1 β were produced at significantly lower concentrations by macrophages infected with Ms_ppe37 compared with

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-1 β (d) were determined. Data are shown as means $\pm$ 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- κ B, 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- κ B, 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- κ B 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- κ B 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- κ B 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- κ B 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- κ B 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- κ B 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- κ B 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.psорт.org/psортb/>). 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- κ B 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- κ B 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 $\pm$ 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- κ B. However, TLR2 is not the only candidate receptor with the possibility of interacting with PPE37, as the common activation of the MAPK and NF- κ B 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*.

ACKNOWLEDGEMENTS

This study was supported by a grant-in-aid for scientific research on priority areas from the Ministry of Education, Science, Culture and

Sports of Japan, grants-in-aid for scientific research (B and C), a grant-in-aid for young scientists (B) from the Japan Society for the Promotion of Science, a grant-in-aid for research on emerging and re-emerging infectious disease from the Ministry of Health, Labour and Welfare of Japan, and a grant-in-aid from the Waksman Foundation of Japan.

REFERENCES

- Adindla, S. & Guruprasad, L. (2003). Sequence analysis corresponding to the PPE and PE proteins in *Mycobacterium tuberculosis* and other genomes. *J Biosci* **28**, 169–179.
- Akira, S., Yamamoto, M. & Takeda, K. (2003). Role of adapters in Toll-like receptor signalling. *Biochem Soc Trans* **31**, 637–642.
- Behar, S. M., Divangahi, M. & Remold, H. G. (2010). Evasion of innate immunity by *Mycobacterium tuberculosis*: is death an exit strategy? *Nat Rev Microbiol* **8**, 668–674.
- Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H. & Kompala, D. S. (1990). Plasmid-encoded protein: the principal factor in the “metabolic burden” associated with recombinant bacteria. *Biotechnol Bioeng* **35**, 668–681.
- Chapin, K. C. & Lauderdale, T.-L. (2007). Reagents, stains, and media: bacteriology. In *Manual of Clinical Microbiology*, 9th edn, pp. 334–364. Edited by P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry & M. A. Pfaller. Washington, DC: American Society for Microbiology.
- Choudhary, R. K., Mukhopadhyay, S., Chakhaiyar, P., Sharma, N., Murthy, K. J. R., Katoch, V. M. & Hasnain, S. E. (2003). PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. *Infect Immun* **71**, 6338–6343.
- Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S. & other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Collart, M. A., Baeuerle, P. & Vassalli, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κ B-like motifs and of constitutive and inducible forms of NF- κ B. *Mol Cell Biol* **10**, 1498–1506.
- Divangahi, M., Desjardins, D., Nunes-Alves, C., Remold, H. G. & Behar, S. M. (2010). Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis*. *Nat Immunol* **11**, 751–758.
- Elbek, O., Uyar, M., Aydin, N., Börekçi, S., Bayram, N., Bayram, H. & Dikensoy, Ö. (2009). Increased risk of tuberculosis in patients treated with antitumor necrosis factor alpha. *Clin Rheumatol* **28**, 421–426.
- Faggioli, L., Costanzo, C., Donadelli, M. & Palmieri, M. (2004). Activation of the interleukin-6 promoter by a dominant negative mutant of c-Jun. *Biochim Biophys Acta* **1692**, 17–24.
- Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W. & Bloom, B. R. (1995). Tumor necrosis factor- α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* **2**, 561–572.
- Gardy, J. L. & Brinkman, F. S. L. (2006). Methods for predicting bacterial protein subcellular localization. *Nat Rev Microbiol* **4**, 741–751.
- Gey van Pittius, N. C., Sampson, S. L., Lee, H., Kim, Y., van Helden, P. D. & Warren, R. M. (2006). Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (*esx*) gene cluster regions. *BMC Evol Biol* **6**, 95.
- Gordon, S. V., Eiglmeier, K., Brosch, R., Garnier, T., Honore, N., Barrell, B. & Cole, S. T. (1999). Genomics of *Mycobacterium tuberculosis* and *Mycobacterium leprae*. In *Mycobacteria Molecular Biology and Virulence*, pp. 93–109. Edited by C. Ratledge & J. Dale. Oxford: Blackwell Science.
- Gutierrez, M. G., Mishra, B. B., Jordao, L., Elliott, E., Anes, E. & Griffiths, G. (2008). NF- κ B activation controls phagolysosome fusion-mediated killing of mycobacteria by macrophages. *J Immunol* **181**, 2651–2663.
- Jacobs, M., Togbe, D., Fremont, C., Samarina, A., Allie, N., Botha, T., Carlos, D., Parida, S. K., Grivennikov, S. & Nedospasov, S. (2007). Tumor necrosis factor is critical to control tuberculosis infection. *Microbes Infect* **9**, 623–628.
- Jo, E.-K., Yang, C.-S., Choi, C. H. & Harding, C. V. (2007). Intracellular signalling cascades regulating innate immune responses to *Mycobacteria*: branching out from Toll-like receptors. *Cell Microbiol* **9**, 1087–1098.
- Khan, N., Alam, K., Nair, S., Valluri, V. L., Murthy, K. J. R. & Mukhopadhyay, S. (2008). Association of strong immune responses to PPE protein Rv1168c with active tuberculosis. *Clin Vaccine Immunol* **15**, 974–980.
- Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, N. R. & Nedospasov, S. A. (1999). Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol* **162**, 4045–4052.
- Larsen, M. H. (2000). Some common methods in mycobacterial genetics. In *Molecular Genetics of Mycobacteria*, pp. 319–320. Edited by G. F. Hatfull & W. R. Jacobs, Jr. Washington, DC: American Society for Microbiology.
- Lee, S.-B. & Schorey, J. S. (2005). Activation and mitogen-activated protein kinase regulation of transcription factors Ets and NF- κ B in mycobacterium-infected macrophages and role of these factors in tumor necrosis factor alpha and nitric oxide synthase 2 promoter function. *Infect Immun* **73**, 6499–6507.
- Liebermann, T. A. & Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF- κ B transcription factor. *Mol Cell Biol* **10**, 2327–2334.
- Lin, P. L., Plessner, H. L., Voitenok, N. N. & Flynn, J. L. (2007). Tumor necrosis factor and tuberculosis. *J Invest Dermatol Symp Proc* **12**, 22–25.
- Manca, C., Tsenova, L., Barry, C. E., III, Bergtold, A., Freeman, S., Haslett, P. A. J., Musser, J. M., Freedman, V. H. & Kaplan, G. (1999). *Mycobacterium tuberculosis* CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. *J Immunol* **162**, 6740–6746.
- Nair, S., Ramaswamy, P. A., Ghosh, S., Joshi, D. C., Pathak, N., Siddiqui, I., Sharma, P., Hasnain, S. E., Mande, S. C. & Mukhopadhyay, S. (2009). The PPE18 of *Mycobacterium tuberculosis* interacts with TLR2 and activates IL-10 induction in macrophage. *J Immunol* **183**, 6269–6281.
- Post, F. A., Manca, C., Neyrolles, O., Ryffel, B., Young, D. B. & Kaplan, G. (2001). *Mycobacterium tuberculosis* 19-kilodalton lipoprotein inhibits *Mycobacterium smegmatis*-induced cytokine production by human macrophages in vitro. *Infect Immun* **69**, 1433–1439.
- Rao, K. M. K. (2001). MAP kinase activation in macrophages. *J Leukoc Biol* **69**, 3–10.
- Roach, S. K. & Schorey, J. S. (2002). Differential regulation of the mitogen-activated protein kinases by pathogenic and nonpathogenic mycobacteria. *Infect Immun* **70**, 3040–3052.
- Roach, S. K., Lee, S.-B. & Schorey, J. S. (2005). Differential activation of the transcription factor cyclic AMP response element binding protein (CREB) in macrophages following infection with pathogenic and nonpathogenic mycobacteria and role for CREB in tumor necrosis factor alpha production. *Infect Immun* **73**, 514–522.

- Rodriguez, G. M., Gold, B., Gomez, M., Dussurget, O. & Smith, I. (1999). Identification and characterization of two divergently transcribed iron regulated genes in *Mycobacterium tuberculosis*. *Tuber Lung Dis* 79, 287–298.
- Rodriguez, G. M., Voskuil, M. I., Gold, B., Schoolnik, G. K. & Smith, I. (2002). *ideR*, an essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infect Immun* 70, 3371–3381.
- Romano, M., Rindi, L., Korf, H., Bonanni, D., Adnet, P. Y., Jurion, F., Garzelli, C. & Huygen, K. (2008). Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). *Vaccine* 26, 6053–6063.
- Rosenkrands, I. & Andersen, P. (2001). Preparation of culture filtrate proteins from *Mycobacterium tuberculosis*. In *Mycobacterium tuberculosis Protocols*, pp. 205–215. Edited by T. Parish & N. G. Stoker. Totowa, NJ: Humana Press.
- Schnappinger, D., Ehrh, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D. & other authors (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* 198, 693–704.
- Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H. & other authors (1991). New use of BCG for recombinant vaccines. *Nature* 351, 456–460.
- Surewicz, K., Aung, H., Kanost, R. A., Jones, L., Hejal, R. & Toossi, Z. (2004). The differential interaction of p38 MAP kinase and tumor necrosis factor- α in human alveolar macrophages and monocytes induced by *Mycobacterium tuberculosis*. *Cell Immunol* 228, 34–41.
- Tundup, S., Pathak, N., Ramanadham, M., Mukhopadhyay, S., Murthy, K. J. R., Ehtesham, N. Z. & Hasnain, S. E. (2008). The co-operonic PE25/PPE41 protein complex of *Mycobacterium tuberculosis* elicits increased humoral and cell mediated immune response. *PLoS ONE* 3, e3586.
- Voskuil, M. I., Schnappinger, D., Rutherford, R., Liu, Y. & Schoolnik, G. K. (2004). Regulation of the *Mycobacterium tuberculosis* PE/PPE genes. *Tuberculosis (Edinb)* 84, 256–262.
- Wang, J., Qie, Y., Zhang, H., Zhu, B., Xu, Y., Liu, W., Chen, J. & Wang, H. (2008). PPE protein (Rv3425) from DNA segment RD11 of *Mycobacterium tuberculosis*: a novel immunodominant antigen of *Mycobacterium tuberculosis* induces humoral and cellular immune responses in mice. *Microbiol Immunol* 52, 224–230.
- Wolfe, F., Michaud, K., Anderson, J. & Urbansky, K. (2004). Tuberculosis infection in patients with rheumatoid arthritis and the effect of infliximab therapy. *Arthritis Rheum* 50, 372–379.
- Zhang, Y., Broser, M. & Rom, W. N. (1994). Activation of the interleukin 6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF- κ B. *Proc Natl Acad Sci U S A* 91, 2225–2229.