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Mycobacterium tuberculosis PE_PGRS17 Promotes the Death of Host Cell and Cytokines Secretion via Erk Kinase Accompanying with Enhanced Survival of Recombinant *Mycobacterium smegmatis*

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Tuberculosis (TB) remains a serious threat to global public health, largely due to the successful manipulation of the host immunity by its etiological agent *Mycobacterium tuberculosis*. The PE_PGRS protein family of *M. tuberculosis* might be a contributing factor. To investigate the roles of PE_PGRS17, the gene of *PE_PGRS 17* was expressed in nonpathogenic fast growing *Mycobacterium smegmatis*. We found that the recombinant strain survives better than the control in macrophage cultures, accompanied by more host cell death and a marked higher secretion of tumor necrosis factor-alpha by a recombinant strain compared with control. Blocking the action of Erk kinase by an inhibitor can abolish the above effects. In brief, our data showed that PE_PGRS 17 might facilitate pathogen survival and disserve the host cell via remodeling the macrophages immune niche largely consisting of inflammatory cytokines. This furnishes a novel insight into the immune role of this mycobacterium unique gene family.

Introduction

Tuberculosis (TB) remains a formidable infectious disease with global significance (Raviglione and others 1995). About one third of the world's population is latently infected with *Mycobacterium tuberculosis*, the etiological agent of TB. About 3 million people die of active TB annually. *M. tuberculosis* is one of the most successful intracellular pathogens, which can resist a variety of antimicrobial mechanisms, and even modulate the immunological niches of monocytes and macrophages (Huang and others 2010). The *M. tuberculosis* PE_PGRS family has been widely proposed as a molecular mantra mediating the evasion of host killing. This family is featured by the conserved N-terminal and variable C-terminal. The PE_PGRS protein family, with functions largely unknown, exclusively presents among pathogenic mycobacteria and is associated with the mycobacterial cell wall (Banu and others 2002; Singh and others 2008).

Several members of the PE_PGRS family have been heterologously expressed and functionally characterized. The PE_PGRS family can be functionally classified into 3 groups: (1) some cell wall-associated PE_PGRS proteins can change the cell structure such as the shape and size, as well as colony formation (Delogu and others 2004); (2) the PE_PGRS family contains proteins such as lipase necessary for *M. tuberculosis* multiplication and persistence within macrophages (Deb and others 2006; Talarico and others 2007); (3) some PE_PGRS proteins are well-established antigens, and actively engaged in the interaction with the host cells (Brennan and others 2001). Partially activated macrophages after *M. tuberculosis* infection seek to suppress the intracellular persistence and replication of the bacteria. However, virulent *M. tuberculosis* outwits the macrophages somehow (Shinnick and others 1995; Fenton and Vermeulen 1996; Behar and others 2010). Investigation of mechanisms underlying the interactions between macrophages and *M. tuberculosis* is crucial to understand the pathogenesis of *M. tuberculosis* and find better countermeasures.

The PE_PGRS 17 (Rv0978c) displays distinct antigenic attributes and is dependent on the pathological conditions (Bansal and others 2010). The characteristics of PE_PGRS 17 include the following 3 aspects: (1) PE_PGRS 17 is upregulated in *M. tuberculosis* within mouse macrophages, and upregulated more than 8-fold in the human brain microvascular endothelial cell (Schnappinger and others 2003; Jain and others 2006); (2) among all the proteins of the PE family, PE_PGRS 17 exhibits very high antigenicity (Narayana and others 2007); (3) PE_PGRS 17 is reactive to sera from tuberculosis patients and demonstrates much higher titration than other PE antigens (Narayana and others 2007).

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The rapid growth and readily expression of heterogenous genes from pathogenic mycobacteria enable the nonpathogenic *Mycobacterium smegmatis* a good surrogate for the pathogenic mycobacteria. We constructed the recombinant *M. smegmatis* strain expressing the PE_PGRS 17 protein, and investigated whether PE_PGRS17 can promote the survival of recombinant *M. smegmatis* in the differentiated human macrophage-like cell line U937. The cytokine responses such as the tumor necrosis factor (TNF)-α and interleukin-10 (IL-10) secreted by U937 were also assayed.

Materials and Methods

Strains and vectors

The sterilized MTB H37Rv strain was provided by Chongqing Pulmonary Hospital. The pMD19-T Simple Vector was purchased from TakaRa BIO Co., Ltd. The human monocytic U937 cell was purchased from the Conservation Center in Wuhan University, China. The strains of *Escherichia coli* and *M. smegmatis* mc^2 155, and pNIT (MYC) plasmid were preserved in the Institute of Modern Biopharmaceuticals.

Methods

Mycobacterial culture and recombinant DNA manipulations. *M. smegmatis* mc^2 155 (Msmeg) and the derivative strains were cultured at 37°C in the Middlebrook 7H9 broth with 0.05% Tween-80, and plated in 7H10 supplemented with 0.5% bovine serum albumin and 0.15% sodium chloride (Pandey and others 2009). *E. coli* was grown in Luria-Bertani media according to the protocol instruction. Kanamycin was added to the required concentration at 20 μg/mL.

Gene amplification, plasmids construction, and recombinant M. smegmatis. The open reading frame of *PE_PGRS 17* (*Rv0978c*) was amplified by polymerase chain reaction (PCR) using genomic DNA of the sterilized MTB H37Rv strain as previously described. The primer sequence of *Rv0978c* was 5′-GGGAATTCATGTCGTTTGTCAACGTG-3′ (forward) and 5′-TGGGATCCGCTGATTACCGACAC-3′ (reverse) with *Eco*RI and *Bam*HI sites (underscored), respectively. The purified PCR fragments were cloned into the shuttle expression vector pNIT (MYC) pretreated with the same enzymes to yield pNIT (MYC)-*Rv0978c*. The plasmids pNIT (MYC) and pNIT (MYC)-*Rv0978c* were introduced into *M. smegmatis* by electroporation (Snapper and others 1990), and colonies were selected on 7H10 agar plates, and subsequently cultured in 7H9 liquid media. The success of the gene transformed in recombinant *M. smegmatis* was confirmed by sequencing.

Expression of recombinant plasmid in M. smegmatis. The recombinant *M. smegmatis* strains were cultured in the 7H9 liquid medium supplemented with 0.05% Tween-80, 0.5% bovine serum albumin, and 0.15% sodium chloride at 37°C. When OD_{600} reaches 0.8–1.0, the inducer ε-caprolactam was added to a final concentration of 28 mM and incubating for 24 h (Pandey and others 2009). An aliquot of 2 mL was centrifuged with 12,000 r/min for 1 min and the cell pellet was washed twice with prechilled phosphate-buffered saline (PBS) and resuspended in 200 μL prechilled PBS for ultrasonication. About 36 μL of the 20× sodium dodecyl sulfate (SDS)-loading buffer was subsequently added to 60 μL cell suspension with 4 μL β - mercaptoethanol. The cell suspension was boiled at 100°C for 10 min, followed by centrifugation with 12,000 r/min for 10 min. The proteins were then analyzed by SDS-polyacrylamide gel electrophoresis and Western Blot.

Cell cultures. The human monocytic U937 cells were cultured in the RPMI-1640 (Hyclone) medium containing 2 mM L-glutamine, 10 mM HEPES, supplemented with 10% fetal calf serum (Hyclone), 100 U/mL of penicillin, and 100 U/mL of streptomycin. U937 cells can be converted from a nonadherent, weak phagocytic form to an adherent, active phagocytic state after the stimulation of phorbol esters and other agents. In our study, monocytic U937 cells were differentiated into macrophages by incubating with Phorbol Myristate Acetate (Sigma) at the final concentration of 100 ng/mL for 48 h before infection. The cell line of U937 is an established model to study the interaction between the macrophage and intracellular pathogens (Bosque and others 1998; Wei and others 2000; Song and others 2003).

Measurement of the viability of the infected macrophages by lactate dehydrogenase (LDH) release assay. After differentiation into macrophages, U937 cells containing 2×10^6 monolayers were infected with recombinant *M. smegmatis* strains at a multiplicity of infection (MOI) of 10:1 bacteria/cell, and incubated for 4 h at 37°C under 5% CO_2. After the duration allowed for phagocytosis, cells were washed 3 times with sterile PBS, and then hygromycin was added to a final concentration of 100 μg/mL to remove extracellular bacteria. After 2 h, cells were incubated again with a fresh RPMI-1640 medium plus 10% fetal calf serum for 6, 24, and 48 h. Each sample has 3 repeats in this experiment, and similar results were obtained in 3 independent experiments. Macrophages infected with *M. smeg*-pNIT (MYC) were set as control. Up to indicated incubating time, the macrophages were harvested by scraping, and the LDH activity in the culture supernatants was assayed with CytoTox 96®Non-Radioactive Cytotoxicity Assay (Promega). The percentage of LDH release was calculated according to the formula: the percentage of cell death = 100 × experimental LDH release (OD_{490})/maximal LDH release (OD_{490}). A value of maximal LDH release was obtained from the culture supernatants of macrophages without infection lysed with 1% (v/v) Triton X-100.

Measurement of the intracellular survival rate of recombinant M. smegmatis by CFU assay. The U937 cells were infected by *M. smegmatis* transformants as well as the control strains at an MOI 10:1 bacteria/cell for 4 h at 37°C in 5% CO_2. After 4-h incubation, the media were discarded, and the cells were washed 3 times with PBS, and treated with hygromycin. After 2 h, cells were incubated again with the fresh RPMI – 1640 medium plus 10% fetal calf serum. At different time points postinfection (24, 48, 72 h), the infected macrophages were washed by the sterile PBS buffer for 3 times at each time point. The sediments were collected through centrifugation at 250 *g*, 4 min. Cells were lysed by adding 1 mL of 1% TritonX-100 for 10 min, and then diluted serially (10-fold in PBS). About 10-μL aliquots from each dilution were plated on the Middlebrook 7H10 plate. The plates were incubated at 37°C, and the CFU were determined after 72 h.

Evaluation of the expression of TNF-α and IL-10 by ELISA. Culture supernatants were harvested 6, 12, 24, and 30 h after infection of U937 cells with *Msmeg*-pNIT (MYC) or *Msmeg*-pNIT (MYC)-*Rv0978c*, respectively. The concentrations of TNF-α and IL-10 in the culture supernatants were determined by ELISA kits (eBioscience).

Measurement of enzyme activities involved in the signaling of TNF-α and IL-10 secretion. U937 cells were pretreated with

20 mM U0126 (a MEK1/2 inhibitor; Sigma) and with 10 mM SB202190 (a p38 inhibitor; Sigma) for 1 h, respectively, and then infected by recombinant *M. smegmatis*. The concentrations of the inhibitors were optimized to give the best MEK1/2 and p38 activities inhibition. After 4-h incubation, the media were discarded, and the cells were washed 4 times with PBS, and subsequently treated with hygromycin at the indicatied concentration. Cells were then incubated again with a fresh RPMI -1640 medium plus 10% fetal calf serum.

After 24 h, the macrophage cells were harvested by scraping. The RNA-Solv reagent (Invitrogen) was added to each sample. RNA was extracted according to the manufacturer's instructions and then treated with DNAase I (Takara). The expression levels of the cytokine genes for TNF-α and IL-10 were detected by one-step semi-quantitative PCR (Tiangen). The sequences of TNF-α are 5′-CGCTCCCCAAG AAGACAG-3′ (forward) and 5′-TGAAGAGGACCTGGG AGT-3′ (reverse) with a size of 375 bp; For IL-10, 5′-GCAC AGCTCCAAGAGAAAGGCATCT-3′ (forward) and 5′-GAA TCCCTCCGAGACACT-3′ (reverse) with a product of 625 bp; For β-actin, 5′-CGGCTCCGGCATGTGCAA-3′ (forward) and 5′-ATGTCACGCACGATTTCC-3′ (reverse) and the product is 600 bp.

Culture supernatants of the infected U937 cells were harvested at 24 h after infection, and ELISA assays were performed to determine the changes of cytokine concentrations to pinpoint the particular MAPK involved.

Statistical analysis. Significance analysis was performed by SPSS software (Statistical Package for the Social Sciences, version 16.0; SPSS, Inc.). Statistical significance was defined as a P value < 0.05.

Results

The expression of PE_PGRS17 in M. smegmatis

We generated 2 recombinant *M. smegmatis* strains—*Msmeg*-pNIT (MYC) and *Msmeg*-pNIT (MYC)-*Rv0978c*— to investigate the effects of PE_PGRS17 on the responses of U937 cells during infection. The recombinant strain was engineered to express the PE_PGRS17 protein from a recombinant pNIT (MYC)-*Rv0978c* vector, while the control strain contained the vector only. Total proteins of recombinant *M. smegmatis* were gained after ε-caprolactam induction for 24 h. The presence of the expected 36-kDa protein in the recombinant *M. smegmatis* total cell lysates was confirmed by Western blotting with the anti-MYC antibody, while absence in the *M. smegmatis* control strain (Fig. 1). The data showed that the PE_PGRS17 protein from *M. tuberculosis* was successfully expressed in *M. smegmatis*, and thus can be used for further analysis.

The survival of M. smegmatis *expressing PE_PGRS 17 in U937 cell*

To test whether PE_PGRS 17 can promote the survival of the pathogen within host macrophages, U937 cells were infected with 2 engineered recombinant *M. smegmatis* strains at the MOI of 10:1, respectively. At 24, 48, and 72 h after infection, CFU assays showed that compared with the strain containing the vector only, *M. smegmatis* expressing the PE_PGRS 17 protein presented with greater numbers of bacteria residing within macrophages for 3 days (Fig. 2A). Whether this is due to the enhanced replication or augmented survival of the bacterium within macrophages remains to be determined. No *in vitro* growth kinetic difference can be found for both recombinant strains (Fig. 2B).

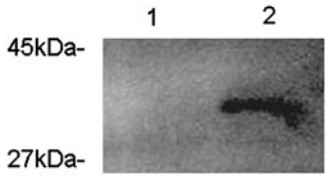

FIG. 1. Expression of PE_PGRS17 in recombinant *Mycobacterium smegmatis* revealed by Western Blotting. The 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis exhibited a 36-kDa protein, the sum of a 31-kDa expected protein, and 5-kDa MYC tag expressed by recombinant *M. smegmatis* strains induced by ε-caprolactam. Lane 1: the protein of PE_PGRS 17 expressed in recombinant *M. smegmatis* strains induced by ε-caprolactam for 24 h. Lane 2: recombinant *M. smegmatis* strains containing the vector only.

Viability of U937 cell after infection with M. smegmatis *expressing PE_PGRS 17*

To determine whether PE_PGRS17 can affect cell death, U937 cells were infected with recombinant *Msmeg*-pNIT (MYC) and *Msmeg*-pNIT (MYC)-*Rv0978c* at the MOI of 10:1, respectively. LDH assay at OD_{490} was performed at 6, 24, and 48 h after infection. The results showed that for 6-h infection, both groups of macrophages released comparable

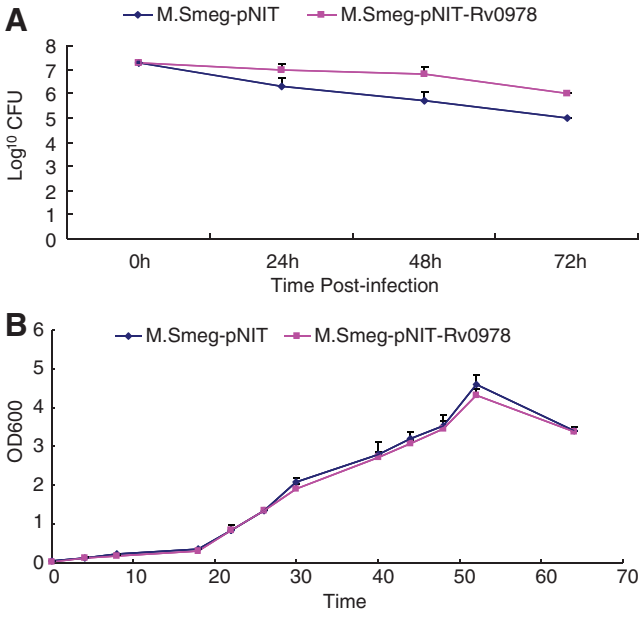

FIG. 2. Survival of recombinant *M. smegmatis* strains after infection of U937 at an MOI of 10:1. **(A)** U937 cells were infected with recombinant *M. smegmatis* strains containing vector only (◆), PE_PGRS 17 (■) at the MOI of 10. At 24, 48, and 72 h after infection, the macrophages were washed and lysed. Lysates containing the live bacteria were diluted gradually and then plated on 7H10 agar plates to determine CFU. **(B)** Growth of 2 recombinant *M. Smegmatis* strains containing vector only (◆), PE_PGRS 17 (■) at 37°C in 7H9 liquid media were assayed by OD_{600} values. Similar results were obtained in three independent experiments.

low amounts of LDH. At 24 and 48 h, macrophages infected by *M. smegmatis* containing PE_PGRS 17 showed higher LDH release amounts than the control group with appreciable difference: 22.7% and 31.1% for 24 h, 82.7% and 92.7% for 48 h. In each time point, the former value represented the control group and the latter one was for the experimental group, indicating that PE_PGRS17 could induce cell death at the MOI of 10 (Fig. 3).

Cytokines secretion in U937 cells infected with M. smegmatis *expressing PE_PGRS 17*

To explore the role of PE_PGRS 17 in the virulence of *M. tuberculosis*, we investigated the effect of PE_PGRS 17 on the U937 cytokine production. U937 cells were infected with *Msmeg*-pNIT (MYC) and *Msmeg*-pNIT (MYC)-*Rv0978c*, and the concentrations of cytokines in the culture supernatants were determined at 6, 12, 24, and 30 h by ELISA assay. We found that the infected U937 cells can produce TNF-α and IL-10 (Fig. 4). Previous studies (Post and others 2001; Roach and Schorey 2002; Lee and Schorey 2005) showed that soluble TNF-α can be detrimental to mycobacteria by contributing to destructive inflammation and cell death. In our results, the secretion of TNF-α by macrophages infected with *M. smegmatis* expressing PE_PGRS 17 was higher than the control group (Fig. 4A). In addition to TNF-α, no noticeable difference as to the production of IL-10 was tested by macrophages infected with *M. smegmatis* expressing PE_PGRS17 and the control (Fig. 4B). Taken together, these results suggested that PE_PGRS17 might interfere with the cytokine production.

ERK might mediate the effect of PE_PGRS17 on TNF-α release

To study the signaling pathway underlying the effect of PE_PGRS17 on TNF-α release, we first investigate whether the activation of mitogen-activated protein kinases (MAPKs)

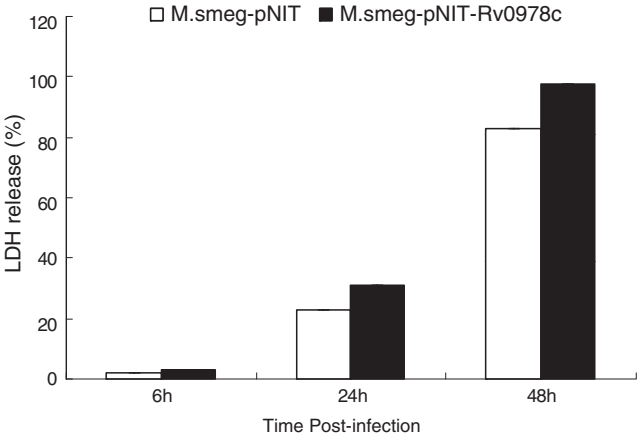

FIG. 3. Death of U937 macrophages infected with recombinant *M. smegmatis*. U937 macrophages were infected with *Msmeg*-pNIT (MYC)-*Rv0978c* (black bars) and *M. smeg*-pNIT (MYC) (white bars) at an MOI of 10, respectively. At 6-, 24-, and 48-h postinfection, culture supernatants were harvested. The release of LDH was estimated by testing its activity in the culture supernatants. The results showed that recombinant *M. smegmatis* containing PE_PGRS 17 increased U937 macrophages death at the MOI 10:1 in a time-dependant manner. Each treatment was repeated three times.

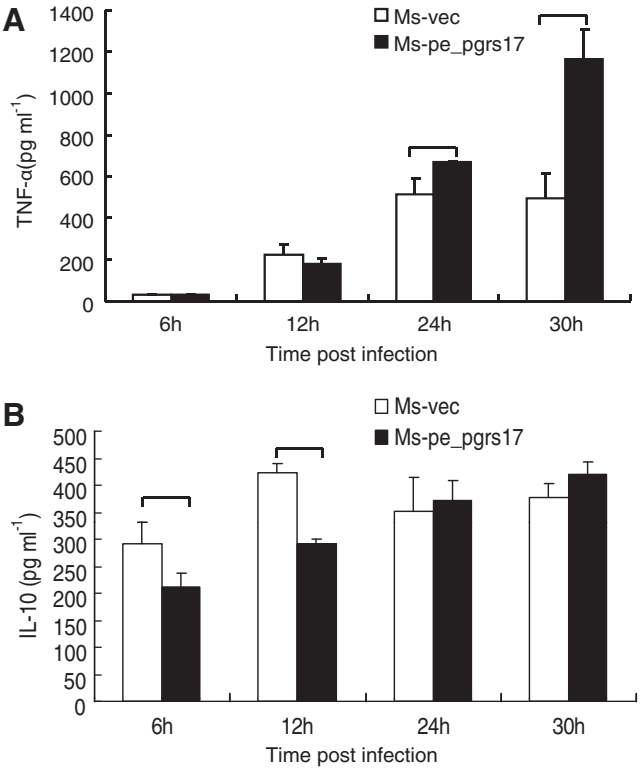

FIG. 4. Secretion of the tumor necrosis factor (TNF)-α and interleukin-10 (IL-10) in U937 infected with recombinant *M. smegmatis* strains measured by ELISA. **(A, B)** U937 were infected at an MOI of 10:1 with *M. smegmatis* containing vector only (□), *PE_PGRS 17* (■) and incubated for different time duration. Culture supernatants were collected at indicated intervals and used to quantify the production of TNF-α and IL-10 by the ELISA kit. **(A)** The secretion of TNF-α is significantly higher by macrophages infected with *M. smegmatis* expressing PE_PGRS 17 than the control group at 24 h and 30 h post-infection. **(B)** The release of IL-10 in macrophages is much higher by the control group at 6 h after infection. Each treatment was repeated three times.

might be affected in macrophages infected with *Msmeg*-pNIT (MYC)-*Rv0978c*. Reverse transcription-PCR (RT-PCR) and ELISA assays were performed to assess the levels of TNF-α and IL-10 in the infected macrophages with the addition of the inhibitors of p38 and MEK1/2. Both ELISA and semiquantitative PCR assays showed that the secretion of TNF-α was significantly inhibited (Fig. 5). These results indicated that PE_PGRS17-promoted TNF-α release was mediated by ERK.

Discussion

PE_PGRS proteins represent a unique *M. tuberculosis* family consisting of 61 members (Brennan and Delogu 2002). The PE_PGRS family has a highly conserved N-terminal domain (PE) containing 110 amino acid residues. The C-terminal of the family is a PGRS domain encoded by a polymorphic GC-rich repeated sequence, with variable sizes, sequences, and repeat copy numbers (Tian and Jian-ping 2010). A highly polymorphic PGRS domain might be engaged in the antigenic variation and immune evasion. The PE_PGRS family members exclusively present in the mycobacterium, mostly within pathogenic mycobacteria (Singh and others 2008), such as *M. tuberculosis*, *Mycobacterium bovis*,

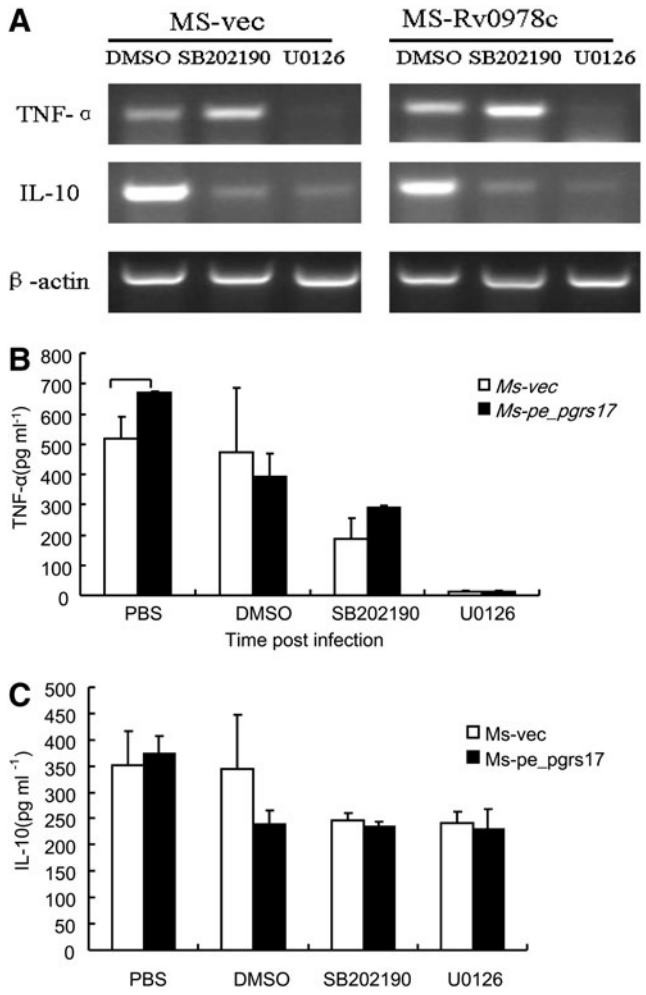

FIG. 5. The secretion of TNF-α and IL-10 in U937 cells infected with recombinant *M. smegmatis* strains measured by ELISA and reverse transcription-polymerase chain reaction (RT-PCR). **(A)** U937 cells were infected at an MOI of 10:1 with *M. smegmatis* containing vector only (□), *PE_PGRS 17* (■) and incubated for 24 h. Culture sediments were used to extract RNA, followed by semiquantitative RT-PCR to demonstrate the transcriptional levels of TNF-α and IL-10. **(B, C)** Culture supernatants were collected at 24 h after infection and used to quantify the production of TNF-α and IL-10 by the ELISA kit.

Mycobacterium ulcerans, and *Mycobacterium marinum.* It is tempting to speculate that PE_PGRS proteins may have a role in the pathogenesis and persistence of mycobacterial disease and that there may be a specific role for this family in influencing host cell responses to infection (Tian and Jianping 2010). While some members have been studied, the function of most PE_PGRS proteins remains unknown. Therefore, selective expression of specific PE_PGRS proteins *in vitro* can facilitate the understanding of their roles in *M. tuberculosis*. In this study, the recombinant *M. smegmatis* strain expressing PE_PGRS 17 instead of *M. smegmatis* strains containing the vector only showed enhanced survival within U937 macrophages *in vitro*. It is fascinating to expect a similar role for PE_PGRS 17 during *M. tuberculosis* infection. The variable transcriptional levels of PE-PGRS 17 with exposure to hypoxia, DNA damaging agents, and clinical isolates (www.ncbi.nlm.nih.gov/geoprofiles?term=PE-PGRS17) also suggested its important roles. Intensive study of this family might provide novel insights into TB pathogenesis and inform new strategies for better prevention and therapy.

TNF-α exerts a key role in the innate and adaptive immunity against tuberculosis (Basu and others 2006), such as the induction of apoptosis and activation of a series of antimicrobial responses (Stenger 2005). Elevated levels of TNF-α during avirulent mycobacteria infection presumably can facilitate the hosts to eliminate the invading bacteria, resulting in poor survival of nonpathogenic *M. smegmatis* within macrophages. On the contrary, virulent mycobacteria can thwart the clearance effort mounted by the host and maintain a favorable niche within the host (Roach and others 2002). However, a delicate timing and balance with other cytokines are crucial for the proper function of TNF-α. In our study, significant greater amounts of TNF-α were released from U937 macrophage cultures infected with *M. smegmatis* expressing PE_PGRS 17 compared with the control. The increased secretion of TNF-α is accompanied by greater levels of macrophage necrosis infected with *M. smegmatis* expressing PE_PGRS 17 after 24 h of infection, through measurement of the level of released LDH, an assessment of cytotoxicity and cell necrosis. We also found that the survival rate of *M. smegmatis* expressing PE_PGRS 17 is higher than the control groups during the detected time length. The induction of macrophages necrosis might be imposed by high levels of TNF-α, and could promote the bacteria spreading to infect fresh cells for new niches. This might underlie the superior survival of virulent mycobacteria within the host in contrast with the avirulent mycobacteria. However, the role the death of infected macrophages invested during *M.tuberculosis* infection remains controversial to date. It might be stage and niche specific. Understanding the strategy *M.tuberculosis* employed to regulate TNF-α induction within the host is important to address this dispute.

IL-10 is a cytokine with pleiotropic effects in immunoregulation and inflammation, and also important in anti-inflammatory function during *M. tuberculosis* infection. IL-10 is primarily produced by monocytes and, to a lesser extent, lymphocytes and is normally secreted at a later stage in the immune response toward a pathogen compared with other cytokines (Redpath and others 2001). IL-10 can downregulate the production of many cytokines, including interferon gamma (IFN-γ) and TNF-α. Being an immunosuppressive cytokine, IL-10 is supposed to favor the intracellular survival of *M.tuberculosis*. However, the cases regarding IL-10-mediated immunoregulatory function of macrophages are quite controversial. Dheenadhayalan and others (2006) reported that the increased production of IL-10 promoted human macrophage necrosis and poor survival of intracellular bacteria. On the other hand, Haruaki Tomioka and coworkers reported that IL-10 was not effective in downregulating the antibacterial functions of macrophages both *in vitro* and *in vivo* (Sano and others 1999). In our study, we found the levels of IL-10 were significantly lower in macrophages infected with recombinant strains expressing PE_PGRS 17 at 6- and 12-h postinfection (Fig. 4B). However, the level of macrophage necrosis between the 2 groups at the indicated time was the same. In-depth appreciation of the discrepancy between macrophage necrosis and enhanced *M. smegmatis* survival needs further investigation, particularly the signaling events mediating the 2 outcomes

Briefly, our data suggest that the expression of the PE_PGRS 17 might provide the nonpathogenic *M. smegmatis*

with some novel properties, including affecting the viability of macrophages and increasing the expression levels of TNF-α. Further studies in mice with this recombinant or the PE_PGRS 17 deletion mutants of *M. tuberculosis* will shed more light on this important molecule. Further studies on how this gene is regulated within the host, their expression level, and receptors are warranted to clarify the underlying mechanism.

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Author Disclosure Statement

No competing financial interests exist.

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