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Expression of PE_PGRS 62 protein in *Mycobacterium smegmatis* decrease mRNA expression of proinflammatory cytokines IL-1β, IL-6 in macrophages

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Abstract The pathogenesis of tuberculosis causing *Mycobacterium bovis* is largely due to its successful entry and survival in macrophages. Previous research indicated that mycobacteria-specific PE_PGRS genes code for cell surface proteins which may have role in mediating interactions with macrophages. In this study, we expressed PE_PGRS 62 gene in a non-pathogenic fast growing *Mycobacterium smegmatis* strain and found that the recombinant *Mycobacterium smegmatis* decreased macrophages livability in a dosage-dependent manner and time-dependent manner, compared with parental strain containing the vector only. To explore whether PE_PGRS 62 modulates the gene expression profile of macrophages, we stimulated macrophages by the *M. smegmatis* strain expressing PE_PGRS 62 as well as the control strains, followed by real-time RT–PCR assay for the mRNA expression level of IL-1β, IL-6, and iNOS. The results showed that the expression of IL-1β, IL-6 in macrophages were down-regulated by stimulation with the *M. smegmatis* strain expressing PE_PGRS 62 compared to the control strains ($P < 0.05$). In contrast, there were no measurable differences in the expression of iNOS. Overall, we demonstrated that PE_PGRS 62 protein altered the immune environment of the host cells, which suggest that the pathogenic PE_PGRS 62 protein altering the immune mechanism maybe involved in the pathogenesis of mycobacterial disease.

Keywords *Mycobacterium* · PE_PGRS 62 gene · Macrophages · L-1β · IL-6

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Introduction

Mycobacterium bovis (*M. bovis*), the causative agent of tuberculosis in a range of animal species, is capable of infecting humans causing zoonotic TB through ingestion, inhalation, and less frequently, by contact with mucous membranes and broken skin. It is estimated that one-third of the world's population has been infected with tuberculosis (TB), and 5–10% of cases is caused by *M. bovis* infection [1, 2]. The high incidence of zoonotic TB, besides being a major economic problem, poses an increased threaten to human health in the world today.

As an intracellular pathogen, *M. bovis* locates in macrophages of the host, and multiply intracellularly and primarily in macrophages. Although Macrophages express several antimicrobial mechanisms to limit the growth of the intracellular infection, however, virulent *M. bovis* is still able to use its multiple cell surface receptors to gain entry to the macrophage and evade macrophage killing on it through the use of different mechanisms [3–5]. Thus, further understanding of the defenses mechanism of the bacterium is clearly needed to rationally develop more effective vaccines and drugs to control this devastating disease.

The PE family of *Mycobacterium* is a unique gene family of 100 genes found dispersed throughout the genome of *M. tuberculosis* [6, 7] and the genome of *M. bovis* [8]. The PE multigene family consists of two sub-families, the PE family (37 members in H37Rv), which code for

proteins of approximately 110 amino acids, and the PE_PGRS family (63 members in H37Rv) which contain a PE domain followed by a varied region rich in glycine and alanine-containing repeats [7, 9–11]. Evidence to date suggests that certain PE_PGRS proteins are found at the surface of mycobacteria and that they have some role in mediating interactions with macrophages [12–14]. Investigation of the function of the proteins expressed by the PE family is an area of intense interest for researchers studying the pathogenesis of tuberculosis [9, 15].

Previous studies have shown that differential expression of PE_PGRS proteins could have a role in the pathogenesis of tuberculosis and in altering the way the host responds to infection [11, 12, 16, 17]. Microarray analysis also indicate that certain PE_PGRS genes show variable expression patterns under conditions that mimic in vivo pathogenesis such as nutrient depletion, low pH or oxidative stress [9, 18, 19]. Using a frog model of *Mycobacterium marinum* infection, Ramakrishnan et al. [20] found that MAG 24 gene, a PE_PGRS homolog, was specifically up-regulated in granulomas, which was associated with adherence and persistence of mycobacteria. PE_PGRS 62 protein is encoded by MAG 24 homolog gene (Rv3812), and its function remains unknown. In this study, we have constructed the recombinant *M. smegmatis* expressing PE_PGRS 62, which is lacking in the parental strain, and investigated the effect of this recombinant *Mycobacterium smegmatis* on the variability and cytokine response of ANA-1 macrophages. This study is benefit to understand the immune mechanism of the mycobacterial disease.

Materials and methods

Mycobacterial culture

M. smegmatis ATCC607 and virulent *M. bovis* Beijing strain (bovis strain 93006),which is a wild-type strain isolated from tuberculosis lesions of a tuberculin skin test-positive cow in Beijing in 1953 and can make Guinea pig die at a dose of 20 ml by abdominal cavity injection, were derived from China Institute of Veterinary Drug Control (CVCC, China). Bacteria were grown at 37°C for 7 days to a logarithmic phase under shaking conditions in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 and 10% oleic acid-albumin-dextrose-catalase enrichment(OADC) (Difco West Molesey, UK).

Gene amplification, plasmids construction, and recombinant *M. smegmatis*

The full length Rv3812 gene was amplified by PCR using genomic DNA of M.bovis as previously described [21]. Primer sequences of PE_PGRS 62 were 5′-ATGGATCCGTGTCGTTCGTGGTCACAGTGC-3′(forward) and 5′-ATAAGCTTCTAAGCCGCCGGTTTGATTG-3′(reverse) with BamHI and EcoRI sites (underscored), respectively. The PCR fragments purified by E. Z. N. A.® Gel Extraction Kit (Omega Bio-tek, Doraville, GA, USA) were cloned into the shuttle expression vector PMV261 and the clones were confirmed by digestion of KpnI and SacI and sequencing. The recombinant plasmids were transformed into *M. smegmatis* by electroporation [22] and colonies were selected on 7H11 agar plates (Difco, Detroit, MI) containing 10% OADC (albumin-dextrose-catalase enrichment) (BBL Middlebrook, BD Microbiology Systems, Sparks, MD) and 50 μg/ml of Kanamycin (Sigma Chemical Co., St. Loius, MO) as previously described [12], and further cultured in 7H9 liquid media (Difco, Detroit, MI) with 10% OADC enrichment, 0.05% of Tween 80 and 50 μg/ml of Kanamycin. The expression of transformed genes in recombinant *M. smegmatis* was confirmed by PCR and stocks vials were frozen in 25% glycerol at −80°C. All recombinant strains show the same growth kinetics in axenic culture.

Cell cultures

The ANA-1 murine macrophages were acquired from the American Type Culture Collection, USA and cultured in RPMI-1640 (Invitrogen Life Technologies) medium containing 2 mM L-glutamine, 10 mM HEPES, supplemented with 10% fetal calf serum(Hyclone) and 0.05 mM β-mercaptoethanol, 100 U/ml penicillin and 100 U/ml streptomycin. The plates were coated with 0.1 mg/ml Poly-L-Lysine before the cells were grown.

Expression of recombinant plasmid in *M. smegmatis*

Both recombinant *M. smegmatis* and *M. smegmatis* parent strain were incubated at 42°C for 30 min as described [23], and the cells were collected by centrifugation at 10,000 rpm for 10 min. The cells were then washed twice with prechilled PBS and resuspended in prechilled PBS (1/50th of original volume). Next, 2 × SDS loading buffer (identical volume) was added to the cell suspension. The cell suspension was boiled at 95°C for 10 min and centrifugated at 10,000 rpm for 10 min, then the supernatants were harvested and proteins were analyzed by SDS–PAGE and Western blot.

In vitro infection with recombinant *M. smegmatis*

ANA-1 murine macrophage monolayers containing 1×10^6 monolayers were infected with *M. smegmatis* transformants using various multiplicities of infection

(MOI) and incubated for 4 h at 37°C under 5% CO2. After the time allowed for phagocytosis, cells were washed four times with sterile phosphate buffered saline (PBS) to remove extracellular bacteria and then incubated again with fresh RPMI-1640 medium plus 10% fetal calf serum for 24 and 48 h, each sample has four repeats in this experiment. For a control, macrophages infected with *M. smegmatis* parent strain were maintained under the same conditions. After incubation, 20 μl MTT [3,(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide] (promage) was added to each sample. After 4 h, dimethyl sulfoxide (DMSO) was added and the sample was kept at 37°C for 10 min. Then each sample was observed under an optical microscope, and the absorbance was measured at 490 nm.

RNA extraction and evaluation of the mRNA expression of IL-1β, IL-6, and iNOS by real-time RT–PCR

The ANA-1 murine macrophage cells were cultured in RPMI -1640 medium for 24 h (5% CO2 at 37°C), and then infected with *M. smegmatis* transformants as well as the control strains at a multiplicity of infection (MOI) 10:1 (bacteria-to-BMMO ratio) for 4 h at 37°C in 5% CO2. After 4 h incubation, the medium were discarded, and the cells were washed four times with PBS,then incubated again with fresh RPMI -1640 medium plus 10% fetal calf serum. At various time points (6, 12, 24, 30, and 50 h), the macrophage cells were scraped for harvesting and counting. RNA-Solv reagent (Omega Bio-tek, Doraville, GA, USA) was added to each sample. RNA was extracted according to the manufacturer's instructions and then treated with DNAase I (Takara, Kyoto, Japan). The DNAase-treated total RNA ($\sim$0.5 μg) was transcribed into cDNA with oligo(dT18) primer using the ImProm-IITM Reverse Transcription System (Promega, Madison, WI, USA). All these experiments were repeated three times.

The expression levels of the genes for IL-1β, IL-6, and iNOS were detected by realtime quantitative PCR using DNA Engine Opticon TM2 fluorescence detection system (MJ Research Inc.) and DyNAmoTM SYBR® Green qPCR Kit (MJ Research, Waltham, USA). The sequences of the PCR primers were 5′-GTTCCCATTAGACAACTGC-3′ (forward) and 5′-TGCCGTCTTTCATTACAC-3′ (reverse) for IL-1β, with a size of 229 bp; 5′-TGCCTTCTTGGG ACTGAT-3′ (forward) and 5′-CTGGCTTTGTCTTTCTT GTT-3′ (reverse) for IL-6, with a size of 384 bp; 5′-TG TGTCAGCCCTCAGAGTAC-3′ (forward) and 5′-CACT GACACTYCGCACAA-3′ (reverse) for iNOS, with a size of 312 bp; and 5′-TGCTGTCCCTGTATGCCTCTG-3′ (forward) and 5′-TTGATGTACCGCACGATTTCC-3′ (reverse) for housekeeping gene β-actin, with an amplification size of 223 bp.

Specific DNA fragments were amplified by PCR in a 25 μl reaction mixture containing 12.5 μl DyNAmoTM SYBR® Green qPCR mix and 0.5 μl primer pair at 20 pmol μl^{-1} each for IL-1β, IL-6, iNOS and, β-actin, 2 μl cDNA as template for each reaction. The expression level of each cytokine gene was measured by normalizing the quantity of IL-1β, IL-6, iNOS transcripts to that of β-actin transcripts using a relative standard curve method. The standard curves of the cycle threshold (Ct) values were obtained from amplification of serial dilutions (10–10^4 copies/well) of the purified recombinant plasmids. For each experimental sample, the amounts of IL-1β, IL-6, iNOS, and β-actin mRNA were determined from the respective standard curves, and the quantity of IL-1β, IL-6, iNOS mRNA was divided by that of β-actin mRNA, respectively, to obtain a normalized value for cytokine gene expression. All samples were analyzed in triplicate.

Statistical analysis

The experiments were performed in triplicate. Differences between groups were analyzed with a one-way ANOVA test using SPSS software (Statistical Package for the Social Sciences, version 13.0 for Windows; SPSS Inc., Chicago, IL, USA).Data are shown as mean ± SEM (standard error of the mean) and values of $P < 0.05$ were considered statistically significant.

Results

Expression of PE_PGRS 62 gene in *M. smegmatis*

The ORF of the PE_PGRS 62 gene in *M. bovis* is 1,515 bp and encodes 504 amino acids. In this study, PCR-amplified PE_PGRS 62 gene was cloned into shuttle plasmids PMV261. The recombinant plasmids were then transformed into *M. smegmatis* and accordingly recombinant *M. smegmatis* strains were obtained. Total proteins of recombinant *M. smegmatis* and *M. smegmatis* parent strain were gained after heat induction. SDS–PAGE confirmed that the expressed 60-kDa PE_PGRS 62 protein was present in the cell lysates of recombinant *M. smegmatis*, while no same band appeared in those of *M. smegmatis* parent strain (Fig. 1). This result was further confirmed by Western blot as described previously (Fig. 2). The data show that the PE_PGRS 62 protein from *M. bovis* was successfully expressed in *M. smegmatis* and thus could be used in further biological analysis.

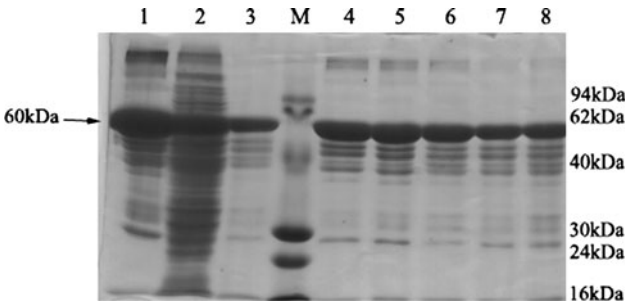

Fig. 1 Expression of PE_PGRS 62 in recombinant *M. smegmatis*. The 12% SDS–PAGE showed a 60-kDa protein expressed by recombinant *M. smegmatis* strains induced by heat shock. *Lane M*: Molecular weight protein marker; *lane 1*: recombinant *M. smegmatis* strains induced by heat shock at 42°C for 30 min; *lane 2*: the control strains induced by heat shock at 42°C for 30 min; *lane 3–8*: recombinant *M. smegmatis* strains induced by heat shock at 42°C for 60 min, 42°C for 90 min, 43°C for 30 min, 43°C for 60 min, 45°C for 30 min, 45°C for 60 min, respectively

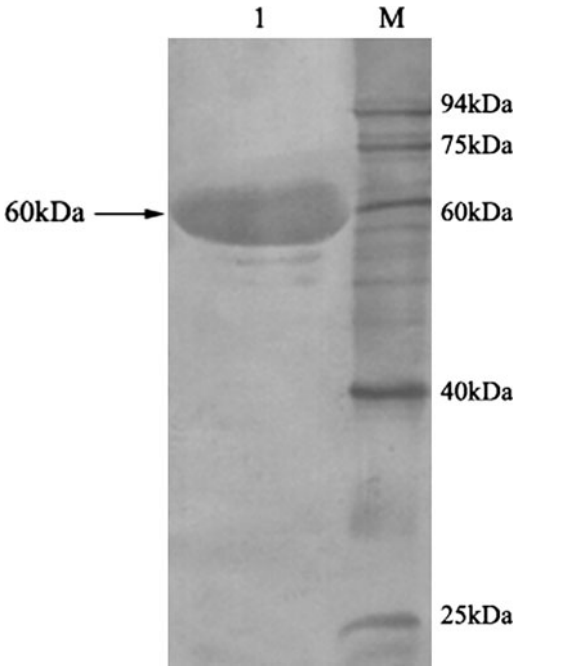

Fig. 2 Western blot assay. Lane M: Molecular weight protein marker; *lane 1*: PE_PGRS 62 protein expressed in recombinant *M. smegmatis* strains induced by heat shock at 42°C for 30 min

Effect of the recombinant *M. smegmatis* expressing PE_PGRS 62 on viability of murine macrophages

To test the effect of PE_PGRS 62 on macrophage viability, ANA-1 murine macrophages infected by recombinant *M. smegmatis* containing PE_PGRS 62 or the control strains at different MOI. MTT analysis at OD490 was carried out 24 and 48 h after infection. For 24 h infection at MOI of 5:1, 10:1, 20:1, 30:1, and 40:1, the viabilities of macrophages were 119.2, 95.5, 84.2, 77.5, and 75.1%, respectively, compared to the control strain (Fig. 3). And at 24 h, it showed no significant difference for the viabilities of macrophages at MOI of 5:1 and 10:1, indicated that the recombinant *M. smegmatis* containing PE_PGRS 62 can inhibit activity of murine macrophagesfor at MOI of 20:1,30:1 and 40:1 ($P < 0.05$). For 48 h infection, the macrophage viability was 79.1, 68.8, 56.1, 52.3, and 46.1%, respectively, compared to the control strain (Fig. 3). After 48 h of infection, inhibition of viability in murine macrophages occurred at all MOI when infected with recombinant *M. smegmatis* ($P < 0.05$).

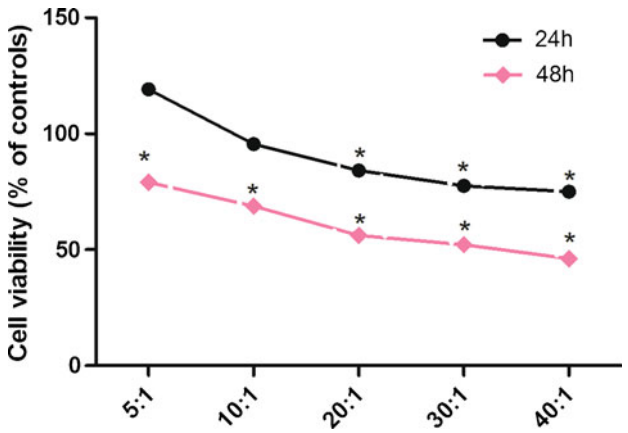

Fig. 3 ANA-1 murine macrophages viability analysis by MTT. MTT assay showed that recombinant *M. smegmatis* did not decrease the ANA-1 murine macrophages viability at MOI of 5:1 and 10:1 for 24 h of infection(119.2 and 95.5%, respectively), while significantly decreased the ANA-1 murine macrophages viability at MOI of 20:1, 30:1, and 40:1 ($P < 0.05$). For 48 h of infection, it showed significant decrease of macrophages viability at various MOI ($P < 0.05$). Each treatment was repeated three times and ANA-1 murine macrophages numbers were measured four times. Statistically significant differences comparing to values of control group are highlighted by *asterisks* (*)

mRNA expression of IL-1β, IL-6, and iNOS assayed by real-time RT–PCR

To investigate the effect of PE_PGRS 62 protein on IL-1β, IL-6, and iNOS mRNA expression, inflammatory cytokine mRNA expression was determined by quantitative RT–PCR. *M. smegmatis* containing the PE_PGRS 62 down-regulated mRNA levels of IL-1β (Fig. 4a) and IL-6 (Fig. 4b) significantly after 24 h of infection, and the down-regulation were maintained to 50 h of infection. While it showed no effect after 6 and 12 h of infection both for IL-1β, IL-6. However, there was an increase in mRNA level of iNOS in murine macrophage through the infection process, no differences were seen in macrophages infected with recombinant *M. smegmatis* containing PE_PGRS 62 compared with *M. smegmatis* parent strain (Fig. 4c).

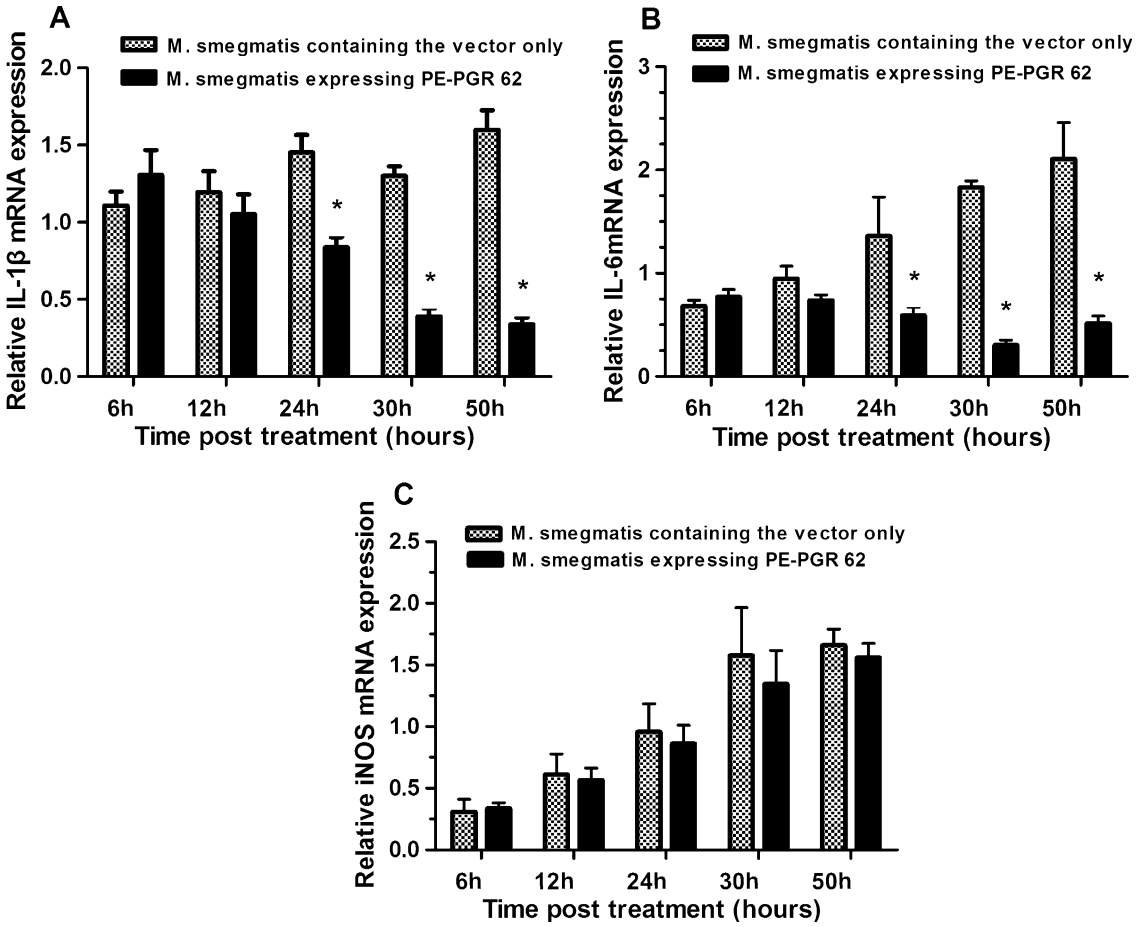

Fig. 4 Quantitative RT–PCR assay of mRNA expression of IL-1β, IL-6 and iNOS. Quantification of mRNA expression of IL-1β (**a**), IL-6 (**b**) and iNOS (**c**) after infection of ANA-1 murine macrophages with recombinant *M. smegmatis* strains. Cultured macrophages were infected at MOI of 10:1 with *M. smegmatis* containing PE_PGRS 62 or the control strains for different time ranging from 6, 12, 24, 30, and 50 h, as indicated in the figure. In each experiment, the relative mRNA expression measurement is an average of four values tested in duplicate at each time point. *Asterisks* (*) indicate a statistical significance between the *M. smegmatis* parent strain and the recombinant expressing PE_PGRS 62. SEM is presented as *error bars*

Discussion

M. tuberculosis is an intracellular pathogen, multiplies mainly inside mononuclear phagocytes. And it is evident that the outcome of exposure to *Mycobacterium tuberculosis* (in terms of disease symptoms) largely depends on the selective gene expression of tuberculosis bacilli during different phases of infection. Recent report has suggested that PE proteins, one group of genes of *M. tuberculosis* selectively expressed upon infection of macrophages, perhaps perform vital functions in pathogenesis of tuberculosis [6, 10].

As the member of PE family, Rv3812 is expressed by many strains of Mycobacterium bacilli and the up-regulation of Rv3812 gene correlating with persistence of *M. tuberculosis* in macrophages and host tissue has been observed by Ramakrishnan et al. [20]. Rv3812 is localized in the mycobacterial cell wall, mostly at the bacterial cell poles, where it is influence interaction with other cells [13, 14]. However, the biological significance or the exact nature of the roles of Rv3812 gene in providing survival benefits to *M. tuberculosis* infected macrophages from surrounding T cells in granulomas or in pathogenesis associated with infection remains the subject of considerable interesting and debate.

In this study, our data demonstrates that *M. smegmatis* recombinant strain expressing PE_PGRS 62 inhibited the viability of ANA-1 murine macrophages. This result was not observed in *M. smegmatis* strains containing the vector only. Therefore, the full length PE_PGRS containing the Gly-Ala rich PGRS domain appears to influence the macrophage phagocytization function and the ability to kill *M. bovis*. Since the *M. smegmatis* strain expressing PE_PGRS 62, as well as the control strains, do not aggregate as is commonly observed for pathogenic mycobacteria, it is unlikely that the observed results are due to differences in

bacterial aggregation. As the primary targets and a critical reservoir of the infection of mycobacteria, macrophages secret various kinds of cytokines to mediate the inflammation response. Here, our results also showed that the *M. smegmatis* strain expressing PE_PGRS 62 might have substantial impact on host cell immune response by influencing the cytokine production of macrophage.

Interleukin-1β (IL-1β), a major proinflammatory cytokine, is activated by processing upon assembly of the inflammasome, which is a specialized inflammatory caspaseactivating protein complex. Previous study has showed that Mtb prevents inflammasome activation and IL-1β processing [24]. We wonder if the persistence of Rv3812 gene up-regulation in granulomas could be related to the elicited IL-1β. Here in this study, we found that *M. smegmatis* strain expressing PE_PGRS 62 can significantly down-regulate the IL-1β mRNA expression level of macrophages compared with the control strains, which provide evidence that PE_PGRS 62 protein could decrease IL-1β mRNA expression to inhibit inflammatory responses.

Interleukin-6 (IL-6), commonly regarded as an indicator of general inflammation, is a pleiotropic cytokine that is produced by a variety of cells, including macrophages, T cells, endothelial cells, and fibroblasts, and also play a role in the initiation of T cell activation [25–28], and supporting effector T cell proliferation in vivo by suppressing regulatory T cells [29]. Our results show that the *M. smegmatis* strain expressing PE_PGRS 62 significantly decreased IL-6 mRNA expression level compared with the control strains, which suggests that PE_PGRS 62 protein had great impact on the inflammation response and influence infectivity and/or survival of mycobacteria within host cells by down-regulating IL-6 expression of macrophage.

Nitric oxide (NO) is a well-known antimicrobial mechanism employed by macrophages [30], and has been shown to plays a major role in the pulmonary host-defense mechanism [31, 32]. In inflammatory responses, NO is produced by the inducible form of NO synthase (iNOS), which is present mostly in inflammatory cells such as macrophages [21]. Here, we also examined the production of iNOS and our data demonstrated that no differences in *M. smegmatis* strain expressing PE_PGRS 62 versus the control strains. Thus, it appeared that iNOS did not participate in the cytokine responses inducing by PE_PGRS 62 protein.

Hence, constitutive expression of the PE_PGRS protein may be critical for the pathogen invasion through some specific mechanism. A number of publications have suggested the possible role of the PE_PGRS proteins in antigenic variability via alterations in its sequence variations in the Gly-Ala rich PGRS domains of the PE_PGRS genes [6, 7, 13]. Evidence has showed that certain PE_PGRS proteins are surface exposed [12–14] and that they can interact with extracellular components like fibronectin. This was also suggested by the fact that they elicited antibodies and mediated immune response [33–35]. Other studies have implicated PE_PGRS proteins of *M. tuberculosis* in pathogenesis and persistence [20].Overall, the data obtained in our study suggest that expression of the PE_PGRS 62 protein provided the non-pathogenic *M. smegmatis* with some specific properties including affecting the viability of macrophage and decreasing the mRNA expression levels of IL-1, IL-6, but no effect on iNOS. Therefore, this study suggests that a PE_PGRS protein delivered in a live *M. smegmatis* vehicle may be one of the critical factors to modify the cytokine response and increase persistence of mycobacterium within host cells. Further experiments are required to confirm if the infection course in mice will be altered when infected with the recombinant stain, and it will be also interest to determine the role of PE_PGRS family members on the pathogenesis caused by *M. tuberculosis*.

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