

The PPE25 (Rv1787) - PE18 (Rv1788) - PPE26 (Rv1789) gene cluster encodes an interacting protein pair and is involved in immune evasion by *Mycobacterium tuberculosis*

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Short Report

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Abstract

Mycobacterium tuberculosis (*M. tb*), the causative agent of human tuberculosis, encodes multiple virulence factors to subvert host immune responses. One such class of proteins is encoded by the multigenic *PE_PPE* family, which accounts for 10% of its coding potential. A number of these genes occur in clusters, of which the *PPE25*(*Rv1787*)-*PE18*(*Rv1788*)-*PPE26*(*Rv1789*) locus alone is organised in a *PPE-PE-PPE* arrangement. We establish here that this cluster is co-operonic in *M. tb* and for the first time identify, *PPE25::PPE26* as an interacting protein pair encoded by this cluster. Recombinant *M. smegmatis* strains expressing *PPE25*, *PE18*, and *PPE26* exhibited enhanced survival in THP-1 macrophages, with infected cells displaying increased levels of the anti-inflammatory cytokine *IL-10* and reduced levels of the pro-inflammatory cytokine *IL-12*. Macrophages infected with recombinant *M. smegmatis* expressing *PPE26* showed increased phosphorylation of the MAP kinase p38, consistent with the known TLR2 binding activity of *PPE26*. In contrast to strains expressing the individual cluster genes, the recombinant expressing the entire *PPE25-PE18-PPE26* operon showed no change in intra-macrophage CFUs, suggestive of an inhibitory role for the *PPE25::PPE26* complex in CFU enhancement. Taken together, our findings implicate the *PPE25-PE18-PPE26* cluster in playing an immune evasion role in the pathophysiology of *M. tb*.

Introduction

Mycobacterium tuberculosis (*M. tb*), the causative agent of human tuberculosis, possesses the ability to thrive and persist within macrophages by altering their immediate antimicrobial actions and evading host immunity [1]. Mycobacteria have highly hydrophobic and impenetrable cell walls, and therefore require highly specific and efficient mechanisms to secrete the effectors needed for immune evasion. Their genomes encode an atypical type VII secretion apparatus, commonly known as the ESX system, and also contain transporters known in other bacteria [2]. A growing body of evidence has revealed that the multigenic *PE_PPE* proteins, which comprise close to 10% of the coding potential of the *M. tb* genome, are either secreted from or are associated with the mycobacterial cell envelope, and are linked to antigenic diversity, immune evasion, and mediating host-pathogen interactions [3]. Our understanding of the molecular basis of their functioning however, remains limited.

Interestingly, the 12 gene ESX-5 locus contains two clusters of members of the *PE_PPE* family of proteins, including a three gene segment encoding *PPE25* (*Rv1787*), *PE18* (*Rv1788*), and *PPE26* (*Rv1789*) [4, 5]. The *M. tb* Δ *PPE25-PE19* mutant containing a deletion in 5 *PE_PPE* genes encoded in the *esx5* locus was shown to be avirulent in immunocompetent mice. This strain however, remained strongly immunogenic and showed a greater protective efficacy against *M. tb* infection than BCG [6]. This 5-gene deletion strain also showed reduced virulence in murine macrophages, and in a SCID mouse model of *M. tb* infection [5]. Several bacterial processes, including replication, vacuole acidification, and phagolysosome fusion in macrophages, were affected when the gene homologous to *PPE25* was deleted from *Mycobacterium avium* [7]. *PPE25* has been used as a strong T-cell epitope for a DNA vaccine against *M. tb* [8]. Moreover, the expression of the *PPE25*, *PE18* and *PPE26* was observed to be

upregulated under conditions of hypoxia/ dormancy, and NO stress [9, 10]. Also, PPE25 and PPE26 were shown to influence cytokine secretion and increase the survival of *M. smegmatis* in mouse macrophages [11]. Taken together, these observations strongly suggest a significant role for this gene segment in the pathophysiology of *M. tb*, leading us to functionally evaluate this cluster for its contribution to *M. tb* pathogenesis.

Materials and methods

Bacterial strains, media and growth conditions

M. smegmatis mc²6, *M. smegmatis* mc²155, and *M. tb* H37Rv were cultivated in Middlebrook, 7H9 broth and 7H10 agar (Difco) containing albumin dextrose complex (5 g BSA, 2 g glucose, and 0.85 g NaCl/L), 0.5% (v/v) glycerol, and 0.05% (v/v) Tween 80. Luria-Bertani medium was used to cultivate *E. coli* strains - both *E. coli* and mycobacteria were cultured by shaking at 37°C. When required, 200 µg/ml ampicillin and 50 µg/ml kanamycin (for *E. coli*) or 15 µg/ml kanamycin (for mycobacteria) were added to the culture medium.

Sub-cellular localisation and Proteinase K sensitivity assay

In order to determine the subcellular location of these proteins, the *M. tb* PPE25, PE18 and PPE26 ORFs were cloned between *Bam*HI and *Eco*RI sites of pJEX55 [12]. Samples to determine the subcellular localisation of these proteins were prepared as described in [13]. Fractionation of *M. smegmatis* strains expressing c-myc-tagged PPE25, PE18 and PPE26 was done as mentioned in [14] The individual fractions were separated using denaturing SDS PAGE, and the fusion proteins were detected by western blotting with anti-c-myc antibodies (sc40, Santa Cruz).

Expression of PPE25, PE18, and PPE26 in *M. smegmatis*

The *M. tb* PPE25, PE18, PPE26 ORFs were amplified using gene-specific primers (Table S1) and cloned between the *Bam*HI and *Eco*RI sites of pMV261 [15]. These constructs were then transformed into *M. smegmatis* for overexpression.

Expression and purification of PPE25 and PPE26

The full-length *M. tb* PPE25 and PPE26 ORFs were cloned as C-terminal 6xHis-tagged fusion in pET22b, and N-terminal Glutathione-S-Transferase (GST) tagged fusion in pGEX6p1 respectively. Following sequence confirmation, the constructs were transformed into *E. coli* BL21 (DE3). For induction, cultures were allowed to grow to an OD of 0.5 and induced with 0.5 mM IPTG. His-tagged PPE25 was purified using Ni-NTA affinity chromatography and GST-tagged PPE26 was purified using glutathione agarose beads. The purified proteins were used for pull-down experiments.

In vitro growth kinetics

To functionally characterise *M. tb* PPE25, PE18, PPE26, their ORFs were amplified from *M. tb* H37Rv genomic DNA using gene-specific primers (Table S1), cloned between the *Bam*HI and *Eco*RI sites of pMV261, and transformed into *M. smegmatis*. The growth kinetics of the recombinant *M. smegmatis* strains were performed as described in [13]

Macrophage infection

THP-1 culture and all infections, were performed as described in [13]. Briefly, THP-1 monocytes were cultured at 37° C in 5% CO₂ in RPMI 1640 medium supplemented with 10% (v/v) Fetal bovine serum and antibiotics. For the infection experiments, monocytes were seeded at a density of 2x10⁶/well for 24 h, differentiated with 5 ng/ml PMA, and then infected 72 h later. Exponentially growing recombinant *M. smegmatis* were washed and resuspended in antibiotic-free RPMI medium and single cell suspensions were prepared by repeated passage through 26 1/2 gauge needles (5–6 times). Based on preliminary MOI optimization, cells were infected with each strain at an MOI of 1:100. Post-infection CFU counts were determined by lysing infected cells after 2 h of incubation with bacteria (T0 counts). Extracellular bacteria were eliminated by incubation with complete RPMI containing gentamycin. Infected cells were lysed with 0.1% Triton X-100 and plated on Middlebrook 7H10 agar at the designated time points to determine CFU counts. For each experiment, macrophages infected with *M. smegmatis* expressing the empty vector were used as controls.

Real-time RT-PCR analysis

The expression profiles of PPE25, PE18 and PPE26 in *M. tb* and in recombinant strains of *M. smegmatis* expressing pMV261-PPE25, pMV261-PE18 and pMV261-PPE26 as a function of growth, were determined using real-time transcript measurements as described in [13], using *gene*-specific primers (Table S1). Gene-specific transcript levels in *M. smegmatis* were normalised to the *sigA* transcript in each sample, and in *M. tb* to the 16s rRNA transcript. In *M. smegmatis*, the relative fold change in transcript levels at each time point was calculated with respect to the levels at 4 h. In the *M. tb* expression profiles, the relative fold change in transcript at each OD point was calculated with respect to the levels at OD 0.8. Quantitation of host cytokines and *iNOS2* transcript levels was performed as described in [13], using primers specific to *iNOS2*, *IL-10*, *IL-12* (Table S1). The mRNA levels were normalised to the transcript levels of GAPDH and β -actin. Relative fold change was calculated with reference to macrophages infected with *M. smegmatis* expressing the empty vector.

Western blotting

The levels of total and phosphorylated p38 in infected THP-1 macrophages were determined by preparing cell lysates in RIPA lysis buffer. SDS-PAGE and western blotting were performed as described

in [13]. The signals obtained were subjected to densitometric quantification using ImageJ, and the P-p38 to total p38 ratio was normalised to β -actin. The normalised values in control samples (EV) were assigned a value of 1, and the fold change in the levels of these proteins in the test samples (PPE25, PE18, PPE26) were derived with respect to the control.

Co-transcriptional analysis

Total RNA extracted from exponentially growing *M. tb* H37Rv was used to assess the transcriptional state of the *PPE25-PE18-PPE26* cluster. RNA treated with DNase I was used as a template for cDNA synthesis with the primer *PPE26* R1 (Table S1). The cDNA was utilised for PCR amplification with gene-specific primer combinations (+ RT). The analysis includes the appropriate negative controls (-RT).

Co-immunoprecipitation

To perform *in vivo* pull-down assays, C-terminal c-myc-tagged PPE25 was generated by cloning its ORF into pJEX55, and a C-terminal 6x-His-tagged PPE26 was generated by cloning its ORF in pSCW54 [16], using the primers listed in Table S1. Recombinant *M. smegmatis* strains were generated by co-transforming the plasmid combinations pJEX55 + pSCW54, pJEX55-*PPE26* + pSCW54, pJEX55 + pSCW54-*PPE25*, and pJEX55-*PPE26* + pSCW54-*PPE25* by electroporation. Following this, co-immunoprecipitation assays were performed as described in [17].

Mycobacterial Protein Fragment Complementation (MPFC) assay

To assess the interaction of PPE25-PE18, PE18-PPE26 and PPE25-PPE26 *in vivo*, *PPE25*, *PE18* and *PPE26* were cloned into the *E. coli* - *Mycobacterium* shuttle vectors pUAB400 and pUAB300 respectively. Both constructs were co-transformed in *M. smegmatis* mc²155, and the interaction assay was performed as described [18]. Appropriate positive (pUAB100 + pUAB200) and negative (pUAB100 + pUAB400, pUAB400 + pUAB300) controls were included in the assay.

In silico analyses

All *M. tb* sequences were obtained from the Mycobrowser database (<https://mycobrowser.epfl.ch/>). The Dense Alignment Surface (DAS) method [19] and Kyte & Doolittle [20] algorithms were used to identify transmembrane domains and regions of hydrophobicity respectively.

Results

PPE25, PE18 and PPE26 are co-operonic and the PPE25 and PPE26 gene products interact

The *PPE25-PE18-PPE26* cluster[16] is the only one of 40 clusters of PE/PPE genes in the **M. tb** genome that has a *PE* gene directly flanked by two *PPE* genes, in a tandem arrangement [21]. Its predicted co-

operonic status, is based on the short spacing between the genes, with 78 bp separating *PPE25-PE18*, and 13 bp separating *PE18-PPE26* (Fig. 1A). To confirm this prediction, RT-PCR experiments were carried out with RNA isolated from log phase grown **M. tb** H37Rv using primer pairs (Table S1) intended to amplify ORF and junction-specific areas of the cluster (Fig. 1A). The sizes of the amplicons obtained (*PPE25*-200 bp, *J1*-200 bp, *PE18*-200 bp, *J2*-200bp, *PPE26*-200 bp), and the absence of amplification products in the -RT control lanes, establish that this gene cluster is transcribed as a mono-cistronic message (Fig. 1B). This observation was further validated by examining the expression levels of the three genes as a function of growth in *M. tb* H37Rv. All the genes were observed to be coordinately expressed (Fig. S1), confirming the operonic status of this cluster. It is well known that genes expressed in a single operon have related functions, and their products are likely to physically interact with each other. To test this possibility, we performed a pairwise screen for interaction between *PPE25* and *PPE26* using the M-PFC assay [18]. In this screen, the test genes were expressed as murine dihydrofolate reductase domain F [1, 2] and F [3] fusions, and then co-transformed into **M. smegmatis** mc²155. Co-transformants for the *PPE25-PPE26* pair were observed to be resistant to 20 µg/ml trimethoprim (Fig. 1C), indicating that *PPE25* and *PPE26* interact *in vivo*; no growth was observed in the negative control samples. To confirm this interaction, we performed an *in vivo* pull-down in *M. smegmatis*, which lacks this gene pair. *PPE25* and *PPE26* were shown to co-precipitate in this experiment (Fig. 1D), proving that the two proteins indeed interact *in vivo*; equal loading was ensured for each sample (Figure. S2). This interaction could not be tested **in vitro** since *PPE25* cloned with a C-terminal 6xHis tag could be expressed but not purified because of its extremely poor solubility (Fig. S3A). However, we were able to partially purify an N-terminal GST-tagged *PPE26* (Fig. S3B).

Recombinant strains of M. smegmatis expressing PPE25, PE18, and PPE26, show a survival advantage in macrophages

To investigate the functions of *PPE25*, *PE18*, and *PPE26* in mycobacterial pathophysiology, we used *M. smegmatis* as a surrogate model, the genome of which lacks homologues for the vast majority of PE-PPE proteins [22], including *PPE25*, *PE18*, and *PPE26*. First, recombinant *M. smegmatis* strains expressing c-myc-tagged *PPE25*, *PE18* and *PPE26* were subjected to Proteinase K treatment and sub-cellular fractionation to determine the subcellular location of each protein. Each fraction's loading was ensured to be equal (Fig. S4 A, B, C) before their spatial distribution was analysed. Western blotting showed that all three proteins were localised to the cell envelope, and their signals were greatly reduced in the cell wall-associated fractions in the Proteinase K-treated samples as compared to those that were untreated (Fig. S4 D, E, F). This showed that *PPE25*, *PE18*, and *PPE26* were exposed at the cell surface, enabling this model to evaluate their function as modulators of pathogenicity *via* their interactions with the host. Their cell-envelope localisation was consistent with the computationally predicted presence of transmembrane and hydrophobic stretches in these proteins (Fig. S5A, B). Next, we investigated the possible functions of the proteins in modulating host-pathogen interactions using constitutively expressed *PPE25*, *PE18*, and *PPE26* in *M. smegmatis*. Over-expression of these genes did not cause growth defects *in vitro*, as shown by growth profiling of *M. smegmatis* strains expressing *PPE25*, *PE18*, *PPE26*, the entire operon (*PPE25-PE18-PPE26*), and the empty vector (Fig. S6A, B). Real-time RT-PCR

analysis of recombinant *M. smegmatis* strains using gene-specific primers (Table S1) revealed that transcript levels for the three genes were similar, indicating that their expression did not change with growth (Fig. S6C, D, E, F). When we infected THP-1 macrophages with these recombinant strains, we observed that the strains expressing *PPE25*, *PE18*, and *PPE26*, showed a survival advantage over the empty vector containing control strain (Fig. 2A). This elevation in CFU counts, was consistent with the reduction in transcript levels of *iNOS2*, which is primarily responsible for control of bacillary load in this model (Fig. 2B). In contrast to this, the strain expressing the full operon did not show a change in CFU counts compared to the empty vector control (Fig. 2A), which correlated with the absence of change in levels of the *iNOS2* transcript in these cells (Fig. 2B).

PPE25 and PPE26 modulate the macrophage innate immune response

The levels of numerous cytokines involved in the control of *M. tb* infection [23] were assessed to evaluate the immunomodulatory potential of *PPE25*, *PE18*, and *PPE26*. Among the anti-inflammatory and pro-inflammatory cytokines we measured, IL-10 and IL-12 showed changes in their expression patterns in THP-1 macrophages infected with the recombinant *M. smegmatis* strains. Macrophages infected with recombinants expressing *PPE25*, *PPE26*, and the entire operon, showed a significant upregulation in transcript levels of the anti-inflammatory cytokine IL-10, but the same was not observed in cells infected with the *PE18* expressing strain (Fig. 2C). Also, the transcript levels of IL-12, a critical pro-inflammatory cytokine, was seen to be significantly down-regulated in macrophages infected with all the recombinant strains (Fig. 2D). Since the interaction of *PPE26* with macrophage TLR2 had been described earlier [24], we proceeded to verify if signalling through this receptor was being activated by assessing the phosphorylation status of p38, a marker for TLR2 activation, in the lysates of THP-1 macrophages infected with recombinant *M. smegmatis* expressing *PPE25*, *PE18*, *PPE26*, and the entire operon. An induction in p38 phosphorylation was observed in macrophages infected with *M. smegmatis* recombinants expressing *PPE26*, and the entire operon, but not in cells infected with recombinants expressing *PE18* or *PPE25* alone (Fig. 3A, B). This finding validated the observations of Su *et al.* [24], and also suggested a possible mechanistic avenue for the above observations.

Discussion

In order to survive in the face of a robust host immune response, pathogenic mycobacteria have developed a wide variety of adaptive strategies [13, 25]. This study set out to investigate the involvement of the *PPE25* (*Rv1787*) - *PE18* (*Rv1788*) - *PPE26* (*Rv1789*) gene cluster in ESX-5-associated *M. tb* pathogenicity, by assessing its functional characteristics. In addition to their short intergenic spacing, the strong association between the expression patterns of the cluster genes in a dataset exhibiting global transcriptional responses of *M. tb* to inhibitors of metabolism [26, 27], led us to hypothesise that these genes are operonic, a premise that we confirmed using co-transcriptional and growth-dependent expression analyses. Our finding that *PPE25* and *PPE26* physically interact was not surprising given the commonality of interaction between the products of co-ordinately expressed genes. This also represents

only the third identification of PPE-PPE interaction, providing credence to our earlier observation of interaction between PPE50 and PPE51 [28], a finding that had not been reported earlier. *M. smegmatis* strains expressing *PPE25*, *PPE26*, and *PE18* showed enhanced intra-macrophage survival compared to the control strain, with the increase in CFUs correlating with a decrease in *iNOS2* levels. Surprisingly, the recombinant strain expressing the entire operon did not show improved survival compared to controls, indicating a possible antagonistic role for the PPE25-PPE26 heterodimer in the independent CFU control function of the two proteins. *iNOS2* levels did not differ from control levels in this scenario, explaining the lack of CFU enhancement. A recent report describing studies on the *PPE25-PE18-PPE26* locus demonstrated PE18::PPE26 interaction, and identified a role for this interaction in cytokine responses and antigen presentation [29], adding another facet to the functional role of this cluster in the pathobiology of *M.tb*.

Cytokine measurements following infection of THP-1 macrophages with recombinant strains expressing the cluster genes individually or as a whole, showed that both PPE25 and PPE26 could mediate the intra-macrophage lowering of *IL-12* levels and an increase in *IL-10* expression in macrophages. Consistent with the established TLR2 binding activity of PPE26 [24], THP-1 macrophages infected with recombinant strains *M. smegmatis* expressing *PPE26* & *PPE25-PE18-PPE26* showed enhanced p38 phosphorylation. It is highly likely that the modulation of IL-10 and IL-12 by these proteins is mediated through the TLR2-p38-MAPK axis.

Our findings implicate the ESX-5 associated *PPE25-PE18-PPE26* gene cluster in *M. tb* pathogenesis via the modulation of pro- and anti-inflammatory cytokine expression, and by facilitating its intracellular survival. Evidence from this research also suggests that the PPE proteins possess unique functions in their monomeric form, and may have possible opposing functions when they heterodimerise with other PPE proteins (Fig. 3C) - an observation we also made with the PPE50-PPE51 protein pair in the context of TLR1 binding [28]. Although the use of *M. smegmatis* as a surrogate model provided valuable insights into the functional roles of these proteins, our ongoing studies in virulent *M. tb* will aim to validate the findings in the context of the native pathogen. While the precise mechanism of this phenomenon remains to be elucidated, it represents a significant expansion in the immune-regulatory arsenal of *M. tb*, and highlights the enormous versatility this pathogen possesses in evading immune responses, to establish and persist in its human host.

Declarations

Conflict of interest

The authors declare that there are no conflicts of interest.

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Author contributions

T. R. R. designed the study. R. P. M., K. P., and K. C. performed the experiments. T. R. R., R. P. M., K. P., and K. C. analysed the data. T. R. R. wrote the manuscript.

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Figures

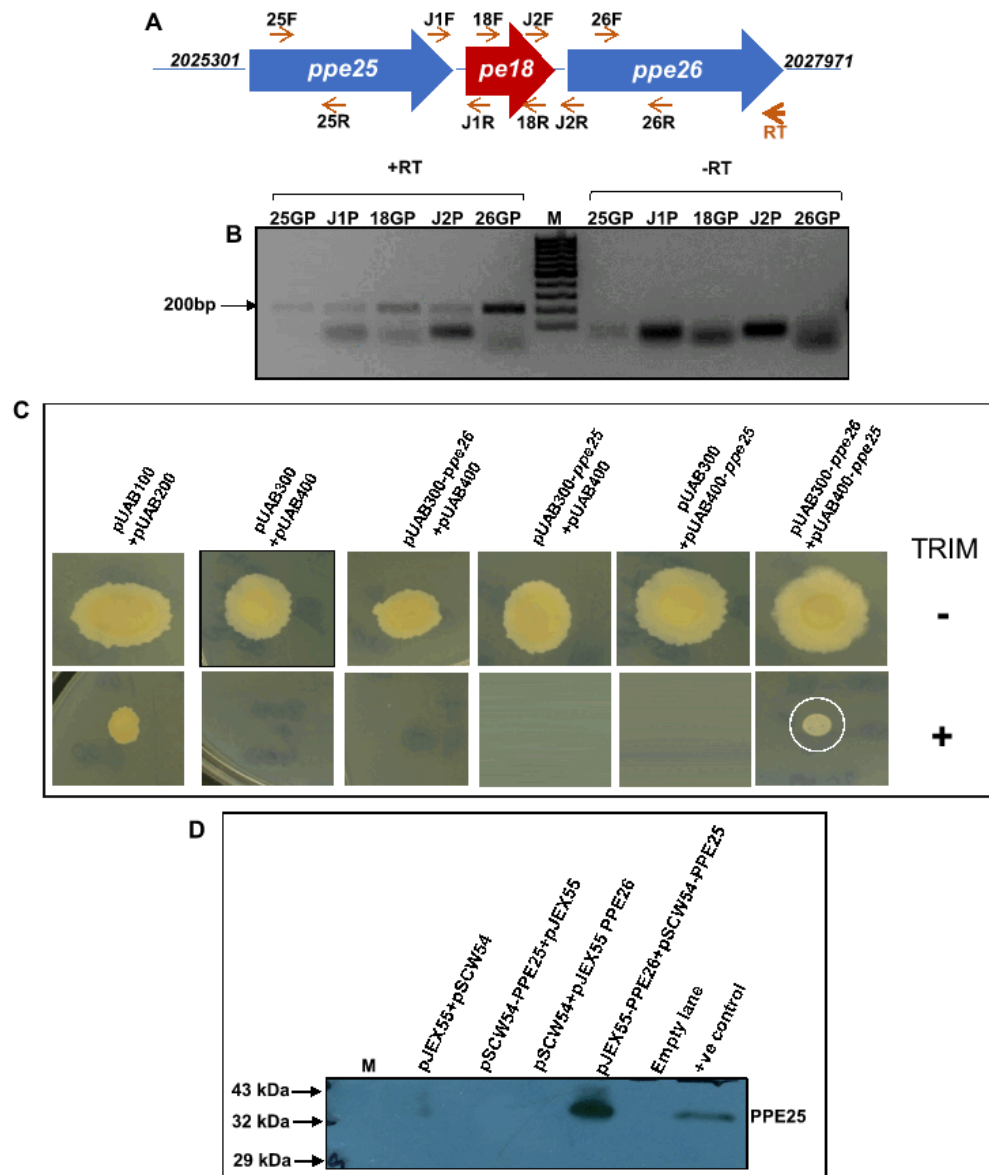


Fig. 1: Analyses of operonic organisation and protein-protein interactions in the *PPE25-PE18-PPE26* gene cluster. A) Genomic organisation of the *PPE25-PE18-PPE26* region of the *M. tb* genome shown schematically to highlight the positions of the gene (G) and junction (J) specific primers used in the experiment. (B) RT-PCR products amplified from the *PPE25-PE18-PPE26* cluster: 25GP - 25F+25R, J1P - J1F+J1R, 18GP - 18F+18R, J2p - J2F+J2R, 26GP - 26F+26R; The bands below 100 bp represent primer dimers; M-100 bp ladder. (C) M-PFC analysis of interaction between PPE25 and PPE26. The image depicts the growth pattern of *M. smegmatis* co-transformants on Middlebrook 7H11 agar plates with (+) or without (-) 20 µg/ml trimethoprim (TRIM). The circled sample marks positivity for PPE25::PPE26 interaction. (D) Immunoblot of PPE25::PPE26 co-IP reactions from *M. smegmatis* expressing PPE26-c-myc and PPE25-6xHis. An anti-c-myc antibody was used to pull-down the complex, and the blot was probed with an anti-His antibody. All findings reported are based on at least two biological replicates.

Figure 1

See image above for figure legend.

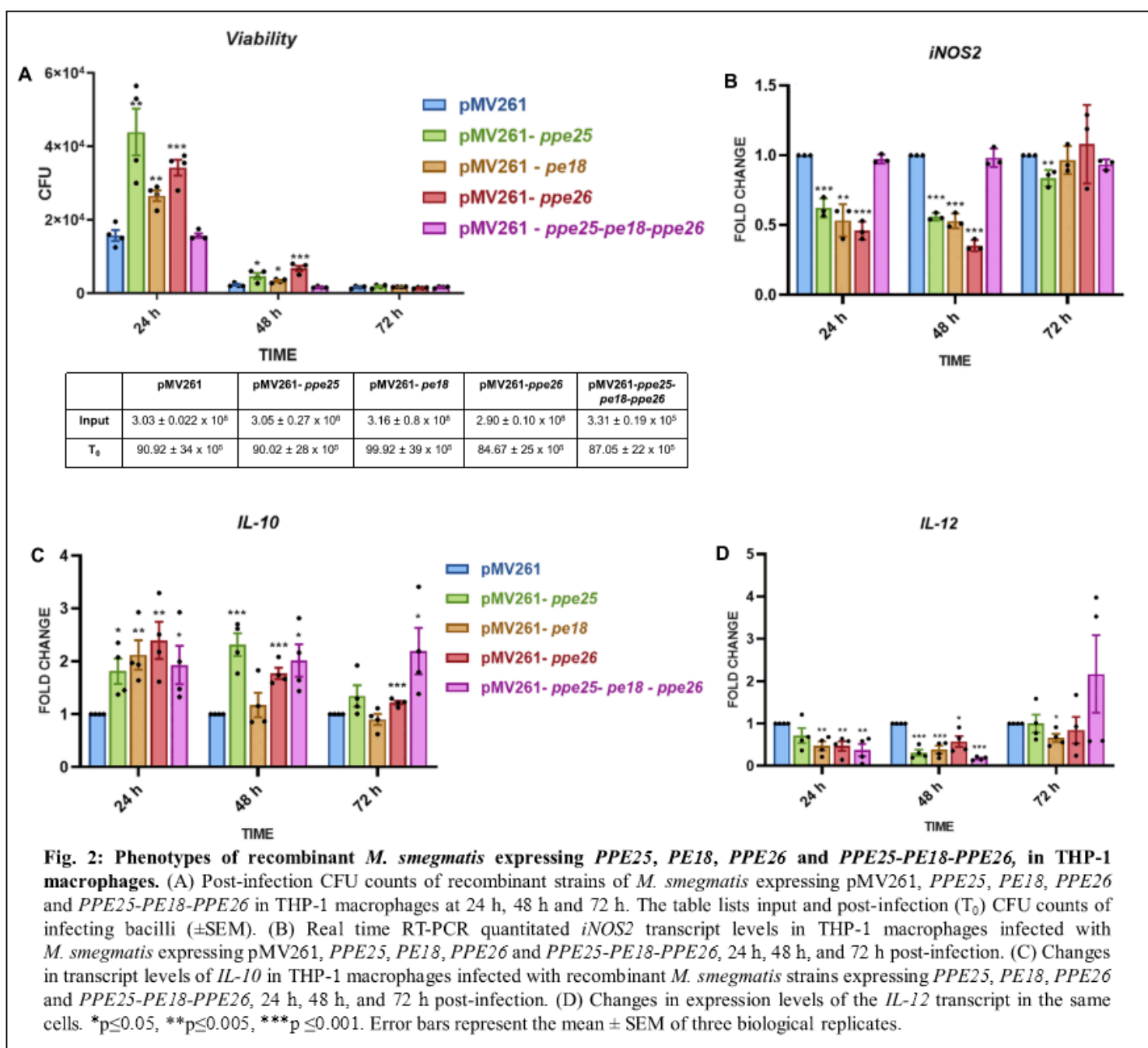


Figure 2

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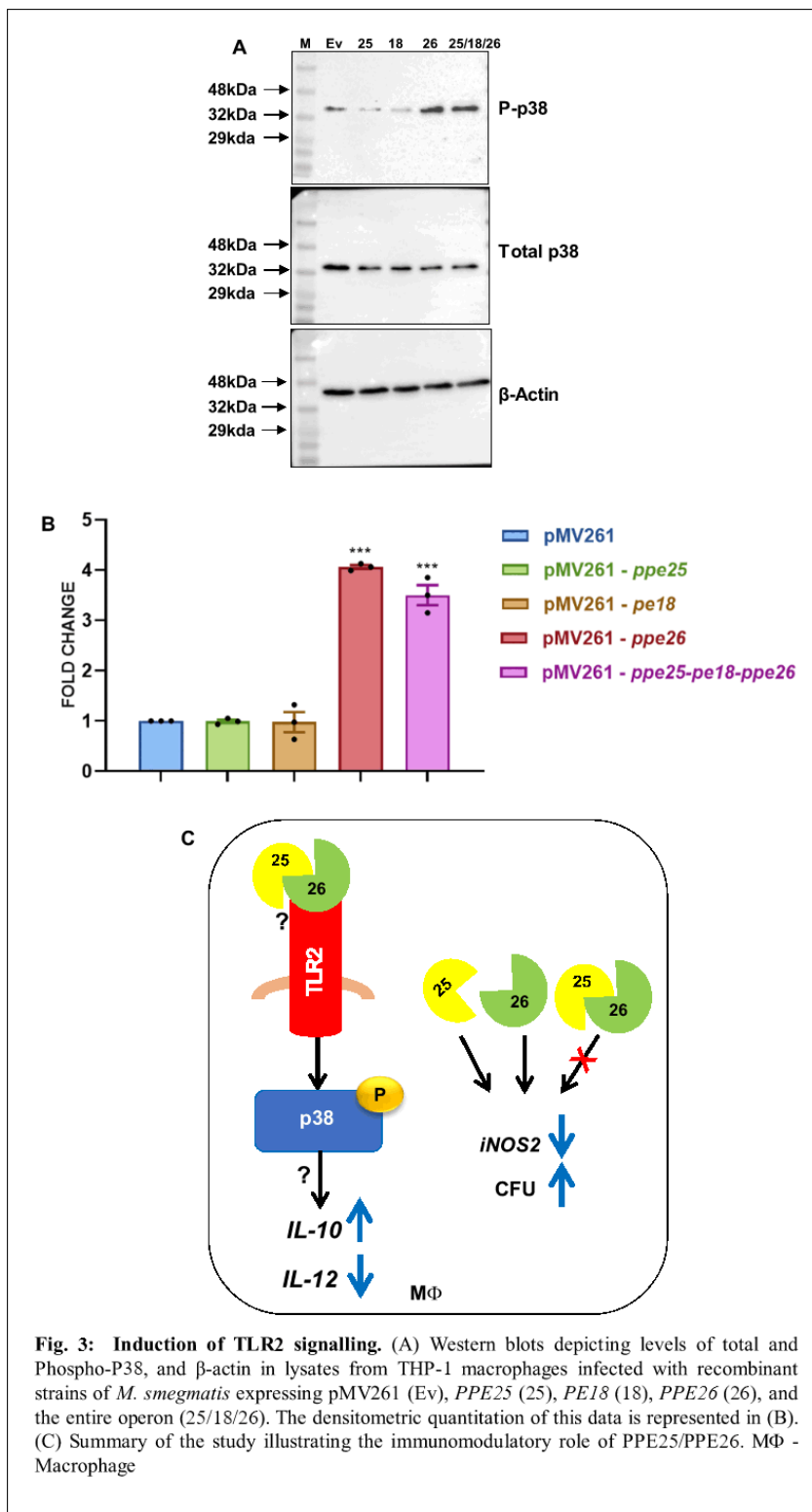


Figure 3

See image above for figure legend.

Supplementary Files

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