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Mycobacterium tuberculosis PE25/PPE41 protein complex induces necrosis in macrophages: Role in virulence and disease reactivation?

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ABSTRACT

Necrotic cell death during TB infection is an important prerequisite for bacterial dissemination and virulence. The underlying mechanisms and the bacterial factors involved therein are not well understood. The *Mycobacterium tuberculosis* (*M. tuberculosis*) co-operonic PE25/PPE41 protein complex, similar to ESAT-6/CFP-10, belonging to the PE/PPE and ESAT-6 families of genes has co-expanded and co-evolved in the genomes of pathogenic mycobacteria. We report a novel role of this highly immunogenic PE25/PPE41 protein complex in inducing necrosis, but not apoptosis, in macrophages. We propose that these protein complexes of *M. tuberculosis*, secreted by similar/unique transport system (Type VII), have an important role in *M. tuberculosis* virulence and disease reactivation.

1. Introduction

Cell death either due to apoptosis or necrosis is very common during infection with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis (TB). Apoptosis of antigen presenting cells (APCs) is known to be an innate mechanism of the host to fight infections. *M. tuberculosis* induces apoptosis in APCs through TNF-α, toll like receptors (TLRs), Fas or by altering the expression of Bax/Bcl-xL *via* an oxygen dependent pathway [1–4]. Virulent strains of *M. tuberculosis* have been known to develop mechanisms that resist host apoptotic cell death [5–8], however they are capable of inducing necrotic cell death, which helps in bacterial multiplication and dissemination [9–12]. On the other hand non-virulent strains like *Mycobacterium bovis* BCG and *Mycobacterium smegmatis* fail to induce necrosis of the host cells [10,11], suggesting that the factors released by the virulent strains interfere with the host cell death machinery for their release and subsequent infection of the neighboring (fresh) cells [8]. Although necrotic cell death during *M. tuberculosis* infection is a common observation, very little is known about the mechanisms. A unique property of $H_{37}Rv$ strain in preventing apoptotic envelope formation leading to necrotic cell death of macrophages has been recently reported [13]. Even though it is clear that host-pathogen interactions at the interface of cell death are a key to *M. tuberculosis* dissemination and virulence, the underlying mechanism(s) and the *M. tuberculosis* factors involved in the processes are very poorly understood.

RD1 locus, present in the genome of only the virulent mycobacterial strains, is believed to encode virulent factors ESAT-6 and CFP-10 complex secreted *via* a secretory locus called *esx-1* [14]. ESX-1 locus also contains a pair of genes encoding PE/PPE proteins. PE/PPE proteins are coded by a family of genes that include PE, PPE and PGRS (or PE/PPE/PGRS) and these represent 10% of the coding capacity of the mycobacterial genome Although the exact roles of PE/PPE proteins in virulence and regulation of their expression in mycobacteria are not completely understood, it is believed that these have important function in mycobacterial virulence and pathogenesis [15–17]. The findings that PE/PPE genes are duplicated and expanded in the genomes of pathogenic mycobacteria during the course of evolution lends further support to their role in virulence [18]. We earlier reported that the PE/PPE genes in the genome of *M. tuberculosis* are organized in operons with the members of the same family as well as those belonging to ESAT-6 like family [19]. Proteins belonging to these families are secreted by pathogenic mycobacteria using specialized (Type VII) secretion systems. For example, ESAT-6 protein of RD-1 locus and PE25/PPE41 protein

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complex are secreted by pathogenic mycobacteria *via* Esx-1 and Esx-5 loci, respectively [20–22]. This supports the observation that the PE/PPE proteins and the ESAT-6 family of genes are co-evolved and co-expanded in the genome of pathogenic mycobacteria [18,19]. This is also evident from the observations that the pathogenic mycobacteria harbor multiple *esx* secretory system containing genes encoding both PE/PPE and ESAT-6 family of proteins [15,18,20]. Functionally, the *esx-5* locus induces bacterial dissemination by cell lysis during mycobacterial infection, suggesting that the proteins secreted through this locus play an important role in virulence [22]. We previously reported that the PPE41 and PE25 proteins interact with each other and form complex in a fashion similar to ESAT-6 and CFP-10 [19]. To form soluble complex, PE25 and PPE41 interact at the translational or translocation levels since PE25 or PPE41, when expressed individually in *Escherichia coli* cells, remain insoluble as opposed to when co-expressed as an operon [19]. That this is indeed the case is evident from recent observation that co-expression of PE25 and PPE41, as well as the C-terminal of PE25 is indispensable for the secretion of PPE41 protein in pathogenic mycobacteria [20]. We also reported that the PE25/PPE41 complex elicited stronger immune responses in TB patients as well as in a mouse model, compared to individually expressed PE25 or PPE41 proteins [23,24], thereby suggesting that PE25/PPE41 complex is recognized by the host immune system. The *esx* secretory systems, including *esx-1* and *esx-5*, are also involved in secretion of other PE/PPE and ESAT-6 proteins, which potentially form a part of the virulence component factors that help in reactivation and dissemination of the pathogenic mycobacteria. Based on the evidences that the PE25/PPE41 complex and ESAT-6 family of proteins are exported out of mycobacteria (*via* special secretory loci, *esx-1* and *esx-5*) and induce necrosis or host cell lysis, but not apoptosis, we hypothesize that the genes belonging to PE/PPE and ESAT-6 family facilitate bacterial dissemination, multiplication and infection of fresh/neighboring cells leading to increased virulence, pathogenicity and disease reactivation.

2. Materials and methods

2.1. Protein expression and purification

The purification of the recombinant protein was carried out as described previously [19]. Briefly, *E. coli* BL-21 cells were transformed with pETDuet vector carrying both the *pe25* and *ppe41* genes for the expression of recombinant proteins. Soluble fraction of the co-expressed protein was loaded onto a cobalt affinity column and washed with 1X PBS containing 25 mM Imidazole. The bound proteins were eluted with 200 mM imidazole in 1X PBS and dialyzed against 1X PBS. To obtain the protein complex, the co-purified proteins were loaded onto Superose 6 column and the fractions corresponding to the sharp (~70 kDa) peak containing both PE25 and PPE41 proteins were collected, as described [19]. The concentration of the recombinant proteins was measured using BCA protein assay kit (Pierce). The purified recombinant protein was treated with Polymyxin B sepharose and the endotoxin level was measured with LAL (Limulus Amebocyte Lysates) method, which was <10 pg/ml.

2.2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

RAW264.7 macrophages were seeded and stimulated with different concentrations of the recombinant protein in a final volume of 250 µl of RPMI per well. The cell culture was incubated at 37 °C under 5% CO_2 and 70% relative humidity. Cell cytotoxicity was tested by MTT assay by adding 10 µl of 5 mg/ml MTT. Cells were then incubated at 37 °C for 3–4 h and then 100 µl of solubilization buffer (20% SDS, 50% N-Methyl Formamide) was added and absorbance was measured at 570 nm.

2.3. Enzyme linked immunosorbent assay (ELISA)

The levels of cytokines present in the culture supernatants were quantified by commercially available two-site sandwich enzyme-linked immunosorbent assay kit (BD OptEIA™ set Mouse, IL-12, IL-10 and TNF-α) according to the manufacturer's instructions. Briefly, 96 well plates were coated with coating antibody, incubated at 4 °C overnight, blocked with 2% BSA (blocking buffer) and incubated for 2 h at 37 °C. The culture supernatant was then added followed by detection antibody for 1 h at RT. The plates were then incubated with HRP conjugated secondary antibody for 1 h at 37 °C and the HRP activity was determined by using the substrate, o-phenylenediamine tetra-hydrochloride and H_2O_2. The plates were washed at every step of the incubation with wash buffer (1XPBS-Tween-20) for 5–7 times. The reaction was terminated
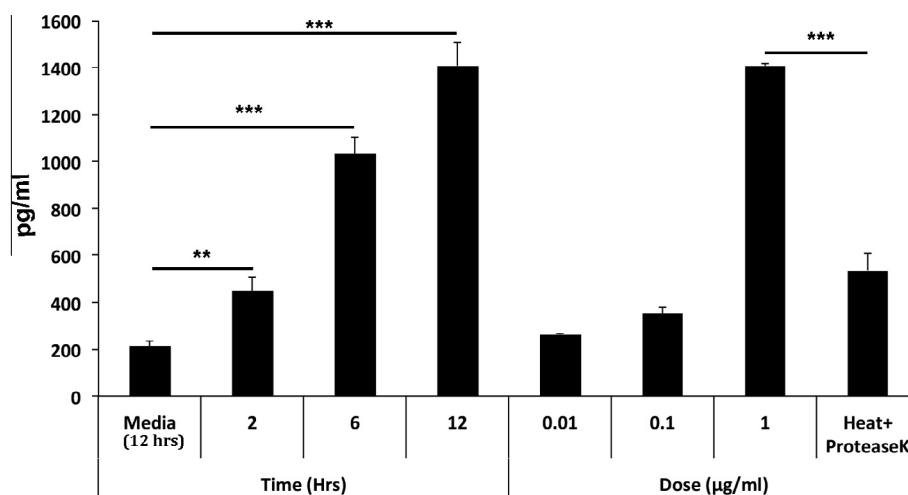

Fig. 1. The PE25/PPE41 protein complex induces TNF-α secretion in macrophages. RAW264.7 macrophages were stimulated with 1 µg/ml of PE25/PPE41 protein complex and TNF-α levels were measured at 2, 6 and 12 h, as indicated. In a parallel experiment, macrophages were incubated for 12 h with varying concentrations (0.01, 0.1 and 1 µg/ml) of the PE/PPE protein complex. The PE25/PPE41 complex was treated with protease cocktail followed by inactivation by autoclaving to serve as a control (Heat+Protease K). Supernatants were collected and ESLISA was performed to measure TNF-α level as described in materials and methods. Statistical significance was determined using student's *t* test (**$p < 0.01$; ***$p < 0.001$).
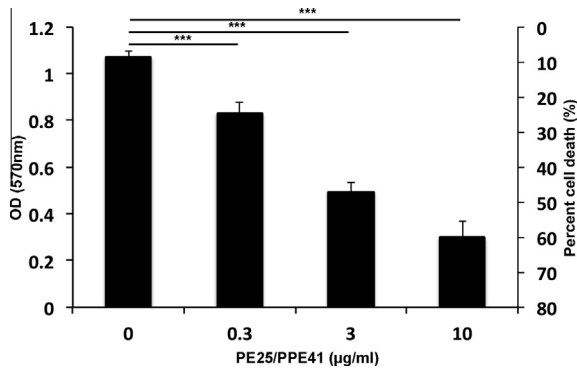
Fig. 2. The PE25/PPE41 protein complex induces cell death. MTT assay was carried out (as described in Section 2) to score for cell death in RAW264.7 macrophages stimulated with different concentration of PE25/PPE41 protein complex, as indicated. Cell death was determined by MTT assay (described in Section 2). Data plotted for O.D value as well as percentage cell death. Statistical significance was determined using student's t test (***$p < 0.001$).

using 2 N H_2SO_4 and the absorbance was measured at 492 nm in an ELISA reader.

2.4. TUNEL assay

Apoptosis was assayed using TUNEL assay kit (Promega), according to the manufacturer's instructions, in a 4 well chamber slide (BD, Bioscience). In brief, cells were washed with PBS, fixed with 3% paraformaldehyde and incubated for 30 min at RT. Cells were permeabilized with 0.1% Triton X-100 and incubated with reaction mixture containing fluorescein labeled dTTP and rdTTP transferase and allowed for nick end labeling for one hour at 37 °C. Reaction was stopped with 2X SSC. The slides were then rinsed with PBS and analyzed using confocal microscope.

2.5. Flow cytometry

Cell death assay was performed using Annexin V and propidium iodide (PI) staining kit (BD) according to the manufacturer's instructions. In brief, 0.2×10^6 cells were stimulated with different

concentrations of recombinant protein and incubated for different intervals of time. Cells were then harvested and washed in PBS twice and resuspended in 1X Annexin V binding buffer. Cells were stained with Annexin V and PI dyes, incubated for 15 min at RT and processed for flow cytometric analysis (BD, Vantage SE).

2.6. LDH assay

LDH release assay was carried out with the culture supernatant of RAW 264.7 macrophages stimulated with different concentration of recombinant protein and LPS (5 µg/ml). The reaction was carried out in a buffer containing 80 mM Tris–HCl (pH 7.2), 200 mM NaCl, 1.6 mM pyruvate, 0.2 mM NADH and 50 µl of culture supernatant. Optical density at 340 nm was recorded for 30 min using spectrophotometer. LDH activity was measured by calculating the rate of oxidation of NADH per unit time per mg of protein.

2.7. Statistical analyses

The experiments were carried out in triplicate and repeated 4 or more independent times. Statistical significance was determined using student's t test.

3. Results

3.1. The PE25/PPE41 complex drives TNF-α production in macrophages

We used the polymyxin B treated recombinant PE25/PPE41 complex protein to stimulate *in vitro* cultured RAW264.7 macrophages and scored for inflammatory cytokines TNF-α, IL-10 and IL-12. Strong induction of TNF-α, indicative of pro-inflammatory response, could be seen when RAW264.7 macrophages were stimulated with the PE25/PPE41 protein complex. The up regulation of TNF-α by recombinant PE25/PPE41 complex was observed in a dose and time dependent manner (Fig. 1). The production of TNF-α was observed as early as 2 h of incubation (~400 pg/ml). Recombinant PE or PPE protein when expressed individually could also induce TNF-α expression (data not shown). Control experiments with heat or protease treated protein failed to stimulate
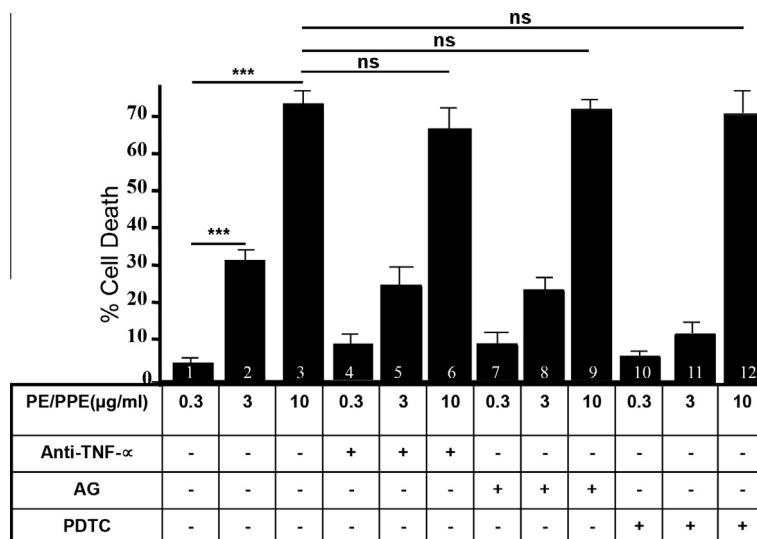

Fig. 3. Cell death is independent of TNF-α, NO or (NFκ-B) mediated signaling. MTT assay was carried out with RAW264.7 cells, either incubated with anti-TNF-α antibody (bars 4–6), amino guanidine (AG, 100 µg/ml) (bars 7–9), 10 µg/ml of PDTC (bars 10–12) or none (bars 1–3) prior to stimulation with 0.3 µg/ml (lanes 1, 4, 7 and 10), 3 µg/ml (bars 2, 5, 8 and 11) and 10 µg/ml (lanes 3, 6, 9 and 12) of PE25/PPE41 protein complex. Data plotted for percent cell death by MTT assay (as described in materials and methods). Statistical significance was determined using student's t test (***$p < 0.001$; ns = not significant).
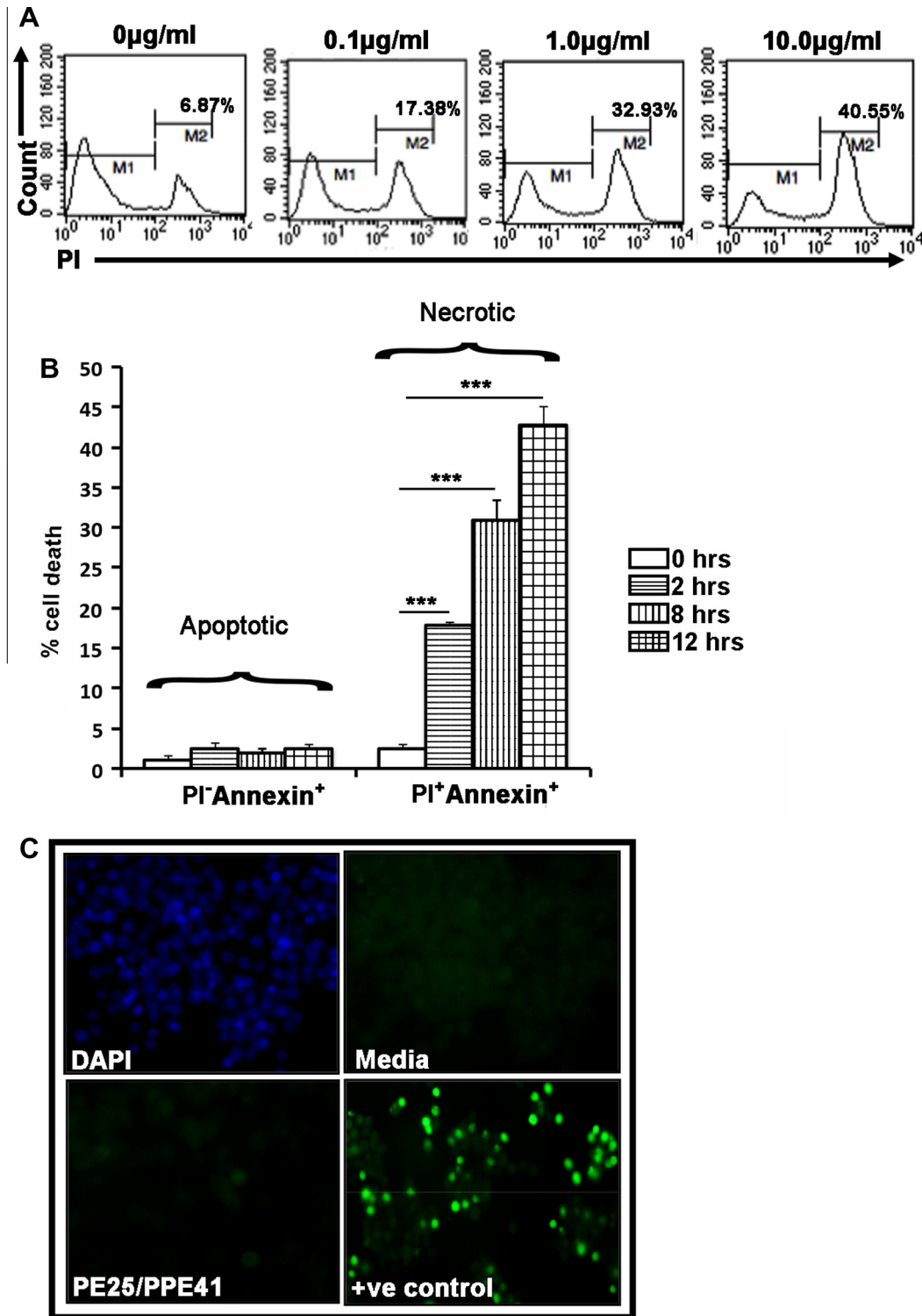
Fig. 4. The PE25/PPE41 complex induces necrosis, but not apoptosis. Necrosis of macrophages stimulated with the recombinant protein was monitored by propidium iodide (PI) exclusion assay. (A) RAW264.7 macrophages were stimulated with different concentrations of the PE25/PPE41 protein complex, as indicated. The peaks in the M2 region represent the uptake of PI dye by the necrotic cells when stimulated with increased concentration of the recombinant PE25/PPE41 protein complex. The number mentioned in M2 region of each of the graph represents the percentage of cells undergoing necrosis in response to the stimulation with PE25/PPE41 protein complex in a dose dependent manner. Data represent results of 3 independent experiments. (B) RAW264.7 macrophages were stimulated with 10 µg/ml of the PE25/PPE41 protein complex for 0, 2, 8 and 12 h, as indicated. Staining with PI/Annexin-V was carried out, cells acquired and data analyzed on flow cytometer (BD Vantage, SE). Necrotic (PI$^+$Annexin V$^+$) or apoptotic (PI$^-$Annexin V$^+$) populations were gated and compared between cells stimulated for 0, 2, 8 and 12 h. Statistical significance was determined using student's t test (***p < 0.001). (C) TUNEL assay with macrophages stimulated with 10 µg/ml of recombinant PE25/PPE41 protein complex show absence of TUNEL staining in the presence of the PE25/PPE41 complex. Positive control (cells treated with DNase A) shows staining with fluorescein tagged nucleotides at the nicked ends of nucleotides. DAPI staining shows that the cells were alive.

TNF-α secretion (Fig. 1), clearly demonstrating that the increase in TNF-α secretion is not due to endotoxin contamination but is due to the PE/PPE complex. The PE/PPE complex failed to drive production of IL-12 and IL-10 after incubation with the macrophages even up to 48 h (data not shown). These results demonstrate that the PE25/PPE41 protein complex is able to mount a strong pro-inflammatory response in the form of TNF-α production.

3.2. PE25/PPE41 complex induced cell death is not due to TNF-α, NO or NFκB mediated signaling

In-vitro cultured murine macrophage RAW264.7 cells were treated with recombinant PE25/PPE41 protein complex and cell death was measured using MTT assay. It could be seen that PE25/PPE41 complex induces cell death in a concentration dependent manner, causing approximately 26%, 42% and 60% cell death at a concentration of 0.3, 3 and 10 μg/ml, respectively after 12 h of incubation (Fig. 2).

A member of the PE/PPE family – PE_PGRS33 protein – has been reported to induce TNF-α secretion in RAW264.7 macrophages leading to apoptotic cell death [25]. Therefore, experiments were designed to investigate the role of TNF-α, nitric oxide (NO) or nuclear factor kappa B (NFκ-B) mediated signaling in PE25/PPE41 protein complex mediated cell death. MTT assay was carried out after pre-incubation of macrophage cells with anti-TNF-α antibody, amino guanidine (AG, inhibitor of nitric oxide synthesis), or PDTC (inhibitor of nuclear factor kappa B), followed by incubation with varying concentrations (0.3–10 μg/ml) of the PE/PPE complex. Expectedly, while PE/PPE protein complex caused cell death as a direct function of protein concentration, cell death remains unchanged in the absence of TNFα, NO or NFκB (Fig. 3). These results suggest that cell death induced by the PE/PPE protein complex is independent of NFκB, NO or TNF-α mediated signaling.

3.3. The PE25/PPE41 complex induces necrosis, but not apoptosis of mouse macrophages

In order to differentiate necrotic cell death from apoptosis, propidium iodide (PI)/Annexin V staining was carried out using macrophages stimulated with PE25/PPE41 complex (Fig. 4A and B). Propidium iodide easily passes through the ruptured membrane of dead cells and stains nucleic acids, but live cells or cells in early apoptotic phase are impermeable to PI dye. Unlike PI, Annexin V binds to phosphatidylserine with high affinity, which is externalized on the surface of apoptosed or dead cells, thus recognizing cells undergoing apoptosis even at early stage of cell death. Using flow cytometry staining, it could be seen that while PI uptake by the macrophages was a direct function of increasing concentration of the PE/PPE complex (Fig. 4A), the necrotic population of macrophages (PI+/AnnexinV+) increased at 2, 8 and 12 h of incubation, respectively (Fig. 4B). Absence of apoptotic population (PI−/AnnexinV+) suggests that PE/PPE protein complex induces necrosis of macrophages but not apoptosis (Fig. 4B). These results clearly suggest that the PE25/PPE41 protein complex induces necrosis in macrophages in a time dependent manner. Apoptotic cell death was also ruled out by TUNNEL staining. Macrophages incubated with PE25/PPE41 complex at a concentration as high as 10 μg/ml were negative for TUNEL staining (Fig. 4C). Necrotic cell death was further validated by lactate dehydrogenase (LDH) release assay. LDH assay involves measurement of NADH oxidation to NAD by LDH (released in to the medium from ruptured cells) in presence of pyruvic acid. RAW264.7 macrophages were stimulated with PE25/PPE41 at two different concentrations of PE25/PPE41 protein complex and LDH released was measured in the culture supernatant. It could be seen that the PE25/PPE41 protein complex induced release of LDH into the supernatant of RAW264.7 macrophages in a
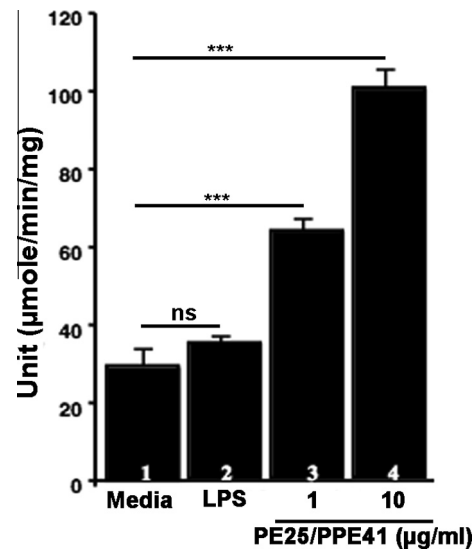

Fig. 5. The PE25/PPE41 protein complex induces necrosis. Lactate dehydrogenase (LDH) assay was carried out with the supernatant of cells incubated with 1 or 10 μg/ml of the PE25/PPE41 protein complex or stimulated with LPS or left unstimulated. The PE25/PPE41 protein complex stimulated release of LDH. The oxidation of NADH by LDH was measured in unit/mg/protein. Statistical significance was determined using student's *t* test (***$p < 0.001$).

concentration dependent manner (Fig. 5). Very low oxidation of NADH was seen in the supernatant of cells left unstimulated (Fig. 5) or stimulated with concentration as high as 5 μg/ml of LPS (Fig. 5). Taken together these results demonstrate that the PE25/PPE41 protein complex induces necrosis, but not apoptosis, in RAW 264.7 macrophage cells.

4. Discussion

Macrophages are the cellular host of mycobacterial infection and constitute the first line of defense without which the host is unable to provide complete protection [26]. M. tuberculosis employs various strategies to counteract macrophage protective responses [27]. Induction of necrotic cell death in macrophages is one such strategy that helps in bacterial dissemination and nutrients uptake inside the granulomas, while preventing apoptotic cell death, that acts as an innate defense against *M. tuberculosis* infection [28]. It is unclear how *M. tuberculosis* induces host cell necrosis and regulates its growth and multiplication especially during the latent phase of the growth. Here we report that a PE/PPE complex induces macrophage cell death by necrosis. Based on the current findings and previous reports we suggest that one of the important virulent features of the secretory PE/PPE proteins is to induce necrotic cell death of macrophages that helps the bacterium to survive and multiply in the host.

Necrotic cell death commonly observed in mycobacterial lesion and granulomas is driven by bacterial factors but not due to secretion of TNF-α during the infection [29,30]. Production of TNF-α in response to the PE25/PPE41 complex is suggestive of a host response elicited against the protein since it has been reported that TNF-α provides protection against *M. tuberculosis* infection perhaps through the induction of host cell apoptosis [28,31,32]. While various pathways are known that mediate apoptotic cells death [1–4], not much is known about the mechanisms driving necrotic cell death during mycobacterial infection. Our observations that necrotic cell death, in response to PE25/PPE41 complex, is independent of TNF-α, nitric oxide or NF-κB signaling suggests that PE25/PPE41 utilizes an unknown mechanism(s) to drive macrophage cell death that may ultimately contribute to virulence. Secretory
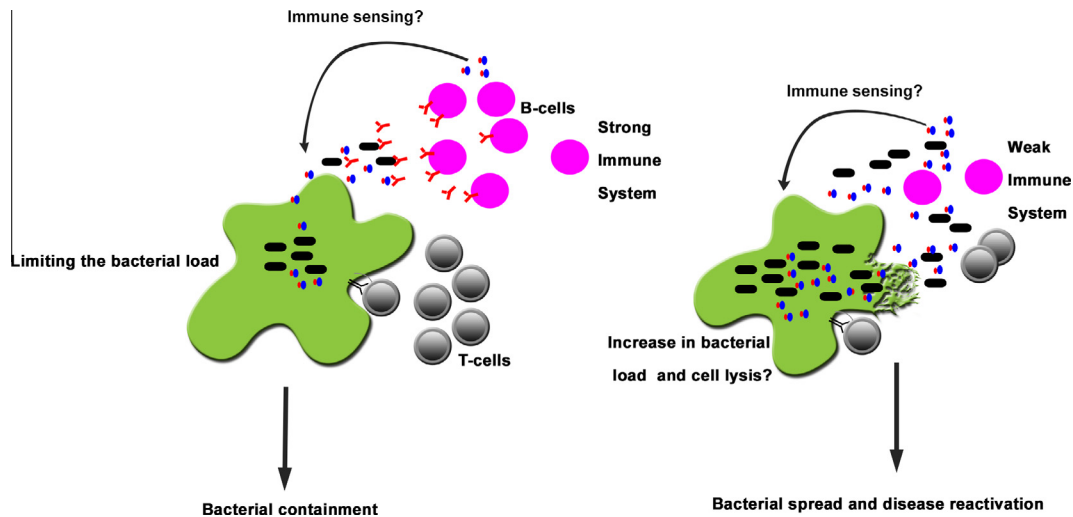
Fig. 6. Is the PE25/PPE41 complex involved in quorum sensing of the immune system? A schematic representation of the likely role of the secreted PE25/PPE41 complex on the host immune system. The secretion of the PPE41 or PE25/PPE41 complex is mediated by Type VII secretion system. The PPE41 or the PE25/PPE41 protein complex is secreted outside by the bacterium, which could be recognized by host immune system. But the proteins may help the bacterium by sensing the strength of the host immune system. Weak immune system may induce these proteins to regulate their own expression or signal for multiplication of the bacteria to spread outside the cells by inducing direct lysis of macrophages via necrosis, which in turn helps the bacterium to multiply and spread infection in the surrounding cells resulting into disease reactivation.

apparatus, *esx-5*, has been known to be involved in caspase independent, cathepsin-B dependent cell death of macrophages during mycobacterial infection. Since PPE41 protein secretion is mediated by *esx-5* apparatus, the role of cathepsin B in induction of cell death in response to PE25/PPE41 protein complex cannot be ruled out [21]. This ability of PE25/PPE41 protein complex to induce necrosis, supports the observation that necrotic cell death during mycobacterial infection is not driven by host response but by bacterial factor(s).

The association of PE/PPE and ESAT-6 gene families with virulence is evident from the observations that the duplication and expansion of the PE/PPE and ESAT-6 genes during the course of evolution occurred only in pathogenic mycobacteria [18,19]. These genes are very uniquely organized in the genome with *pe* and *ppe* genes in operon with another member of *pe/ppe* or *esat-6* family of genes [19] and evidently both ESAT-6 and PE/PPE family of proteins are secreted by Type VII/ESX secretory apparatus [15]. This also suggests that PE/PPE and ESAT-6 family of genes may have played important role in adaptation and host specificity, as these were earlier shown to be differentially expressed in different species of pathogenic mycobacteria [33–35]. Although exact function of PE/PPE proteins remain unknown, reports suggest that these are surface localized or secreted outside by mycobacteria and may have direct role in virulence and pathogenesis [16,22,36–43]. One of the important virulence mechanisms of pathogenic mycobacteria appears to be their ability to induce necrosis of host immune cells, as not only PE25/PPE41 complex, ESAT-6 protein has also been shown to induce necrosis of immune cells *via* an unknown mechanism [44]. The fact that the PE/PPE proteins mount both B- and T-cell immune responses [23,24,45] provokes us to also suggest a novel role for these protein families, present exclusively in the genus Mycobacterium, in 'immune quorum sensing' (Fig. 6) wherein these proteins are secreted to sense the immune status of the host after which through the possible involvement of cell surface constituents that influence interactions with other cells [38], convey a signal to stay dormant or undergo necrotic cell death. Based on our present and earlier findings, and existing evidences, we believe that the co-evolved and co-expanded PE/PPE and ESAT-6 family of proteins are secreted out, using sophisticated bacterial Type VII secretory systems, interact with host immune cells (Fig. 6), and facilitate mycobacterial dissemination and multiplication, which may ultimately lead to disease reactivation.

Acknowledgments

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