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Review Article

Immunomodulatory Role of Mycobacterial PE/PPE Family of Proteins

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Mycobacterium tuberculosis (M.*tb*) is an adept pathogen possessing unique strategies to exploit and corrupt the very core defense systems designed to curb its own survival and multiplication. Comparative analysis of mycobacterial genomes indicate that the pathogenic mycobacterium have acquired a multitude of genes which play important roles in modulating protective host-cell signaling that are triggered in response to an infection. Therefore, defining the "set of genes" expressed by intracellular pathogens as well as understanding modulations of host-signaling pathways during infection is extremely crucial to devise successful clinical intervention against the development of disease. The unique PE/PPE proteins, which constitute about 10% of coding capacity of M.*tb* genome, owing to their high abundance and expansion in pathogenic mycobacterial species warrants a better understanding of their role in mycobacterial pathogenesis. The PE/PPE families have been thought to play important roles in generating antigenic variation and immune evasion. Although a comprehensive understanding of their role is yet to be fully understood, emerging evidence supports a role of PE/PPE proteins at multiple levels of infectious process. This review delineates the importance of these proteins in the modulation of macrophage immune effector responses, as well as importance of these proteins in clinical manifestations of TB.

Key Words : Mycobacterium; PE/PPE Proteins; TLR; Immunomodulation

Introduction

More than 100 years after Koch's discovery, tuberculosis (TB) still remains a major threat tohuman existence. TB is one of the potential causes of mortality around the world, killing about two million people annually. Approximately, one-third of the world's population is asymptomatically infected with M.*tb*, the main causative agent of this disease. An exceedingly resistant cell wall, opportunistic switching over to latency and an efficient adaption to the hostile environment of phagocytic cells makes M.*tb*a very successful pathogen (Andersen and Doherty, 2005). The emergence of multiple drug-resistant (MDR) and extremely drug resistant (XDR) strains severely compromise traditional drug-based intervention. Except Bedaquiline which has been recently approved as part of combination therapy to treat adults with MDR pulmonary TB by FDA, very few novel/effective anti-mycobacterial drugs or vaccines are available, hence there is an escalating need for the development of new therapeutic agents to combat TB(Snider and La Montagne, 1994).

Mycobacterium has evolved highly sophisticated mechanisms like blocking phagosomal maturation, preventing apoptosis, limiting the activation of mitogen-activated protein kinase (MAPK) pathways in macrophages and suppressing the antibacterial immune response enabling it to invade, replicate and colonize the host tissues successfully (Fig. 1). One of the important mycobacterial survival strategies for intracellular survival is exploitation of host-cell signaling pathways (Koul *et al.*, 2004). Mycobacterial survival in the context of the host environment exemplifies a complex interplay between mycobacterial survival strategies and the consequent host responses to

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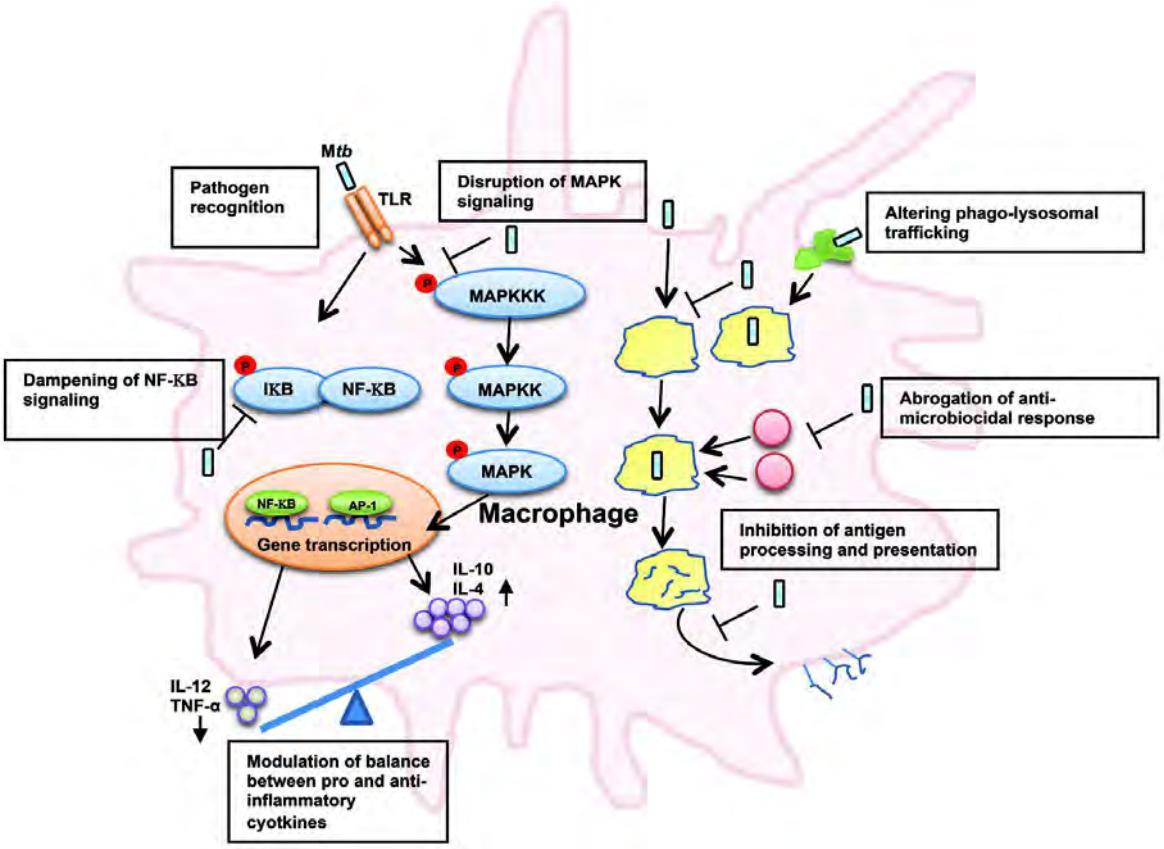

Fig. 1: An overview of the mechanisms employed by the M.*tb* for subverting macrophage signaling and effector functions. Macrophages are both sentinels and the first line of defense against M.*tb* and bacilli, which have acquired numerous strategies to perturb the macrophage signaling to favor its survival in macrophages as illustrated in figure. (AP-1, Activator protein; NF-κB, Nuclear factor kappa B, IκB, Inhibitor of NF-κB; MAPK, Mitogen activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; TLRs, Toll like receptors; ROI, Reactive oxygen intermediates; RNI, Reactive nitrogen intermediates)

infection. The host responses to infection begin with the identification of a foreign invader through the use of innate immune surveillance systems, which alert the body to an infection and induce a suite of inflammatory cytokines that ultimately shape the adaptive arm of immune response. Our understanding of the mechanisms of interaction between mycobacteria and host cells, and of consequent changes that are induced by mycobacteria in the host signaling machinery, is still incomplete.

Comparative analysis of mycobacterial genomes indicate that the pathogenic mycobacterium have acquired a multitude of genes which play important roles to modulate the host-cell signaling that are triggered in response to an infection. The interaction between the pathogen and the host is actually a dynamic confrontation where the microbe's strategy of survival by expressing virulence factors etc. challenges formidable defenses of the host immune system. Therefore, defining the "set of genes" expressed by intracellular pathogens as well as understanding modulations of host-signaling pathways during infection is extremely crucial to devise successful infection that will prevent the development of disease.

With the completion of genomic sequence of M.*tb*H37Rv, there have been extensive attempts to identify the mycobacterial components vital for establishment of infection (Cole, 1999; Cole *et al.*, 1998). The PE/PPE family of proteins that constitute 10% of the total coding capacity of the mycobacterial genome, on account of its unique presence in pathogenic mycobacterium merit a better understanding of its role in overall pathogenesis

associated with tuberculosis disease (Cole, 1999). This review provides an overview of the possible role of PE/PPE proteins in immunity against tuberculosis with emphasis on their immune-modulatory action and potential involvement in mycobacterial subversion of the host immune response.

PE/PPE Genomics

The names PE and PPE are derived from the motifs Pro-Glu (PE) (positions 8, 9) and Pro-Pro-Glu (PPE) (positions 8-10) found in most cases near the N-terminus conserved domains of 110 and 180 amino acid residues respectively. There are about 100 members of the PE family, whereas the PPE protein family comprises 70 members (Tekaia *et al*., 1999). The proteins can be classified into subgroups consisting of members with highly variable length and sequence features (Fig. 2). The PE_PGRS (polymorphic GC-rich sequence) proteins contain multiple tandem repeats of Gly-Gly-Ala (or a variant thereof), while the PPE_MPTR (major polymorphic tandem repeat) subgroup possess polypeptides that are rich in repeats with the signature Asn-X-Gly-X-Gly-Asn-X-Gly. PE-PGRS and PPE_MPTR genes represent some of the most variable regions of the M.*tb* chromosome and consequently speculated to represent a major source of antigenic variations which has been reviewed by Sampson (Sampson, 2011). The *pe* and *ppe* genes are mostly arranged in a unique regulon with the *pe* genes located always upstream to the ppe genes and scattered throughout the genome (Tundup *et al*., 2006). A total of 40 such *pe/ppe* gene operons exist and 22 of these exclusively contain *pe/ppe* gene pairs. Some *pe* and *ppe* genes are found to be associated with *esx* regions that encode the Type VII or ESX secretion systems. The mycobacterium genomes encode up to five of these transport systems. These clusters are conserved among various pathogenic mycobacteria (Gey van Pittius *et al*., 2006). *pe* and *ppe* genes have not been identified yet in any non-mycobacterial species, not even in closely related bacteria such as *Nocardiafarcinica* (Ishikawa *et al*., 2004). Surprisingly, although *ppe* genes are widely present in pathogenic mycobacteria, such as *M. avium*and *M. marinum*, the non-pathogenic species *M. smegmatis* conspicuously lacks these genes (Abdallah *et al*., 2009). The *M. smegmatis* genome, which is more than 50% larger than that of M.*tb*, contains only 4 putative *ppe* genes. Apparently, there is a strong evolutionary selection for PPE proteins in the pathogenic mycobacteria (Gey van Pittius *et al*., 2006). The most archaic members of these gene families were probably inserted during the first duplication of the ESX system (ESX-1) and were subsequently co-duplicated with the *esx* gene

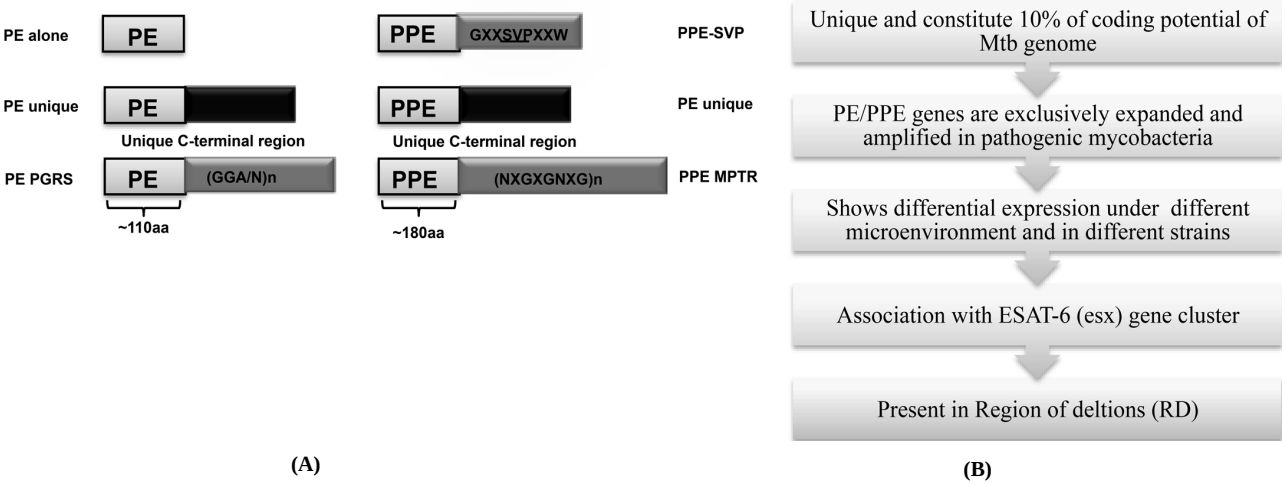

Fig. 2: Gene structure and distinct characteristics of PE/PPE family of proteins of M.*tb*. Schematic representation of the gene structure of the members of *pe/ppe* gene family, showing conserved N-terminal domains, motif positions and differences between different sub-families found in the two families respectively (A). A diagrammatic representation depicting the unique characteristics of PE/PPE family of proteins based on various studies (B)

clusters, until their recent expansion. ESX-1 and ESX-5 have been shown to play major roles in the secretion of PE/PPE proteins and in the virulence of different pathogenic mycobacteria, such as M.*tb* and *M. marinum* (Abdallah *et al*., 2008; Abdallah *et al*., 2009). The core machinery of the ESX-1 pathway is encoded in a genomic locus known as the region of difference 1 (RD1), which is absent in the attenuated BCG vaccine strain (Gordon *et al*., 1999; Guinn *et al*., 2004). ESX-1 is involved in the secretion of the potent T-cell Ags ESAT-6 (EsxA) and CFP-10 (EsxB), which are known to modulate TLR2 signaling and cytokine inhibition (Gao *et al*., 2004; Pathak *et al*., 2007; Pym *et al*., 2003; Skjot *et al*., 2000; Stanley *et al*., 2003). Recently, the ESX-1/RD1virulence locus has been shown to promote mycobacterial infection for local expansion and systemic dissemination (Davis and Ramakrishnan, 2009). In addition to the potential role of ESX-1, a study by Abdallah *et al.* has shown that ESX-5 locus is both necessary and sufficient to produce functional secretion machinery for PE and PPE proteins in *M. marinum* (Abdallah *et al*., 2008). However, secretion of PE/PPE protein by ESX-5is not generalized phenomenon as some of PE/PPE proteins carry functional N-terminal signal peptide cleavage sites, facilitating their secretion/localization via other mechanisms such as the *Sec*-dependent export pathway (Malen *et al*., 2007). This is emphasized by several studies showing cell-wall localization of PE/PPE proteins in *M. smegmatis*, which lacks the ESX-5 region (Cascioferro *et al*., 2007; Chaturvedi *et al*., 2010; Delogu *et al*., 2004; Sampson *et al*., 2001). Some PE/PPEs may be actively secreted or passively shed from the bacterium into the host cell milieu, and could even be released from host cells via exosomes (Balaji *et al*., 2007). Thus, they are ideally positioned to interact with the host immune system and have important consequences on the outcome of infection (Sampson, 2011). Structure-based predictions based on crystal structure of PE25/PPE41 protein pair showed considerable resemblance to the CFP-10/ESAT6 complex (Strong *et al*., 2006). Based on the close genomic, evolutionary and functional links between *pe/ppe* genes and the *esx* gene clusters, PE/PPE proteins might potentially be important virulence factors.

Differential Expression of *pe/ppe* Genes

There are ample evidences available to support differential expression of members of the PE/PPE family between different strains of M.*tb* (Flores and Espitia, 2003) as well as under different environmental and experimental conditions (Voskuil *et al*., 2004). A study of differential expression of *pe/ppe* genes between M.*tb* and *M. bovis* reveal that these genes are highly upregulated in M.*tb* as compared to *M. bovis*, suggesting that *pe/ppe* genes might be important in determining host preference and specificity during evolution (Rehren *et al*., 2007). Furthermore, divergent regulation of *pe/ppe* genes in genotypically diverse clinical isolates and within individual strains has been also reported (Delogu *et al*., 2006; Gao *et al*., 2005; Rindi *et al*., 2007). Differential regulation of *pe* and *ppe* genes was observed under 15 different conditions, hinting at diverse functions of these proteins under different stages of disease (Dheenadhayalan *et al*., 2006b). Many PE/PPE proteins are up-regulated during stress condition and upon macrophage infection in host tissue, suggesting a role in bacterial survival and intracellular survival respectively during mycobacterial infection (Betts *et al*., 2002; Haan *et al*., 2003; Voskuil *et al*., 2004). Expression levels of a subset of *pe/ppe* genes are altered in regulatory and other mutants of M.*tb* (Goldstone *et al*., 2009; Vallecillo and Espitia, 2009; Walters *et al*., 2006). Using an *in vivo* expression technology approach, which identified macrophage-specific gene expression, Srivastava *et al*. (2007) found one of the *pe/ppe* genes, rv3097c (PE_PGRS63), to be the most highly expressed gene as early as 24 h post infection (Srivastava *et al*., 2007). The promoters of several other *pe/ppe* genes (rv0977, rv1361c, rv1840c) were up-regulated in THP-1 macrophages (Dubnau *et al*., 2002). In a transposon site hybridization (TraSH) assay, a microarray-based technique, mutations in three genes belonging to the PE/PPE family (rv1807, rv3872, rv3873) had growth-attenuating effects (Sassetti and Rubin, 2003). Failure of mutations in other *pe/ppe* genes to produce a phenotype can probably be attributed to functional redundancy of these genes. Recently, the genes for PE13 and PPE18 have been shown to be part of the rv0485 regulon,

which encodes a putative transcription regulatory protein. Mutation studies indicate that Rv0485 is required for M.*tb* virulence (Goldstone *et al*., 2009). Some of the PE/PPE family proteins like Rv1168c, Rv2430c display stronger immuno reactivity against the sera obtained from patients with clinically active TB and could distinguish TB patients from *M. bovis* BCG-vaccinated healthy controls, indicating that probably these proteins are highly expressed during active pathogenesis of M.*tb* (Choudhary *et al*., 2003; Khan *et al*., 2008). Interestingly, analysis of M.*tb* proteome in the lungs of guinea-pigs revealed that PE/PPE proteins are the third most abundant category and showed the most consistent expression during infection. The PE-PGRS53/54, and PE-PGRS56/57, and PPE38 proteins were found to be amongst the ten most dominant proteins at 90 days post infection (Kruh *et al*., 2010). These findings are suggestive of diverse functional roles played by PE/PPE family of proteins at different stages of disease.

Role of Pattern Recognition Receptors in Recognition of M.*tb*

The activation of both innate and the acquired immune response to M.*tb* infection depends on a large degree on recognition of M.*tb* as a pathogen by the pattern-recognition receptors (PRRs) such as *Toll*-like receptors (TLRs) (Qureshi and Medzhitov, 2003). All known TLRs in mammals are type-I integral membrane glycoproteins containing an extracellular domain with leucine-rich repeats (LRRs) responsible for ligand recognition and a cytoplasmic *Toll/ Interleukin-I* receptor homology (TIR) domain required for initiating signaling cascades (Lee and Kim, 2007). Detection of microbial ligands by TLRs leads to the initiation of inflammatory cytokines (Aderem and Ulevitch, 2000; Janeway and Medzhitov, 2002). M.*tb* is known to activate at least two different families of pattern recognition receptors: TLR and the Nods (Ferwerda *et al*., 2005). TLRs initiate signal transduction by the binding of adaptor protein MyD88 (myeloid differentiation protein 88) to the TIR domain of TLRs, followed by the recruitment of IL-1 receptor associated kinases (IRAK), TNF receptor associated factor (TRAF) 6, TGFâ activated protein kinase 1 (TAK1), mitogen-activated protein (MAP) kinase, (leading to the activation of nuclear factor kappa B (NF-κB) and the induction of cytokines and chemokines, which play critical role in triggering the adaptive immunity against pathogens (Quesniaux *et al*., 2004). Remarkably TLRs recognize diverse range of microbial ligands, which are structurally and biochemically distinct, by just utilizing a conserved set of LRR motifs. TLRs may recognize such varied molecules possibly by mechanisms involving diversification of LRR motifs, cooperative interactions between different TLRs, and or the use of non-TLR coreceptors or other accessory molecules (West *et al*., 2006). The *in vitro* studies indicate that M.*tb* is recognized by several TLRs, including TLR2/ 1/6, TLR9, and possibly also TLR4 (Heldwein and Fenton, 2002; Quesniaux *et al*., 2004). A central role for TLR2 in M.*tb* recognition and protection has been prominently documented (Brightbill *et al*., 1999; Feng *et al*., 2003; Means *et al*., 1999; Underhill *et al*., 1999). Numerous studies *in vitro* and *in vivo* have shown that whole mycobacteria or mycobacterial components act as agonists for TLRs (Bafica *et al*., 2005; Pai *et al*., 2004; Quesniaux *et al*., 2004; Yoshimura *et al*., 1999).

TLR2-mediated immune activation of macrophages, plays an important role in early defense against invading M.*tb* through production of various cytokines, reactive oxygen, direct bactericidal effect or by the induction of apoptosis (Heldwein and Fenton, 2002). Classically, TLR2 is recognized as a principal inducer of the pro-inflammatory signal, like TNF-α, during infection with M.*tb* (Underhill *et al*., 1999). TLR2 in association with TLR1 and TLR6, mediates responses to mycobacterial lipoproteins, lipomannan, arabinose-capped lipoarabinomannan (ara-LAM) and phosphatidyl-*myo*-inositol mannoside (PIM) (Quesniaux *et al*., 2004). TLR activation by mycobacterial products not only leads to the synthesis of pro-inflammatory cytokines including TNF-α and IL-12, but also to the production of nitric oxide, a metabolite with potent anti-mycobacterial activity in mice (Brightbill *et al*., 1999; MacMicking *et al*., 1997; Pecora *et al*., 2006; Underhill *et al*., 1999). The mycobacterial cell wall contains a number of pro-inflammatory TLR2 ligands, including lipoproteins,

glycolipids, mycolylarabinogalactan–peptidoglycan complex (the cell wall core structure), lipids, and LAM (Berrington and Hawn, 2007). Various mycobacterial glycolipids and lipoproteins (Table 1) and proteins (Table 2) have been individually demonstrated to be involved in activating TLR-dependent signaling cascades and innate immune responses.

Interestingly, though TLR2 have been implicated in recognition of majority of M.*tb* ligands and known to induce pro-inflammatory responses, TLR2 signaling has been also implicated in modulation of phagocyte function. Studies have shown that engagement of TLR2 with M.*tb* ligands induces inhibition of macrophage MHC class II antigen presentation and also blocks macrophage responsiveness to IFN-γ (Fulton *et al*., 2004; Gehring *et al*., 2004; Noss *et al*., 2001; Pai *et al*., 2004; Wang *et al*., 2001). Surprisingly, in TLR2 deficient mice, though the ability to induce Th1 immune response is not compromised, an exaggerated immunopathology in response to M.*tb* is exhibited (Bhatt and Salgame, 2007). Thus, the M.*tb* is thought to hijack the TLR2-induced signaling to modulate the effector-APC functions of the macrophages or DCs for its own benefit. Ligation of TLR2 by various *M.tb* components inhibits antigen presentation, pro-inflammatory responses and induces apoptosis, thus compromising innate immune responses to mycobacterial infection (Fortune *et al*., 2004; Palma *et al*., 2009). Recently, *M. marinum* PPE38 protein

Table 1: TLR ligands (lipids) of mycobacterium and their physiological role

Mycobacterial TLR Ligands	Species	Pattern Recognition receptors	Modulation of immune response	References
		Glycolipids		
Ara LAM (lipoarabinomannan)	*M. smegmatis*	TLR2	Secretion of pro-inflammatory cytokines like TNF-α, IL-8 or IL-12	(Heldwein and Fenton, 2002; Vignal *et al*., 2003; Wieland *et al*., 2004)
PILAMs (phosphoinositide LAM)	*M. smegmatis*	TLR2		(Heldwein and Fenton, 2002)
PIM_2 (Dimannoside)	*M. bovis*	TLR2		(Jones *et al*., 2001)
PIM_6 (hexamannoside)	*M. bovis*	TLR2		(Gilleron *et al*., 2003)
ManLAM (mannosyl)	*M. tuberculosis, M. bovis, M. kansasii*	Mannose receptor, DC-SIGN	Stimulate IL-10 induction & inhibits the production of IL-12 & TNF-α	(Quesniaux *et al*., 2004)
		Lipoprotein		
LprG	*M. tuberculosis, M. bovis*	TLR2, TLR1, CD14	Inhibits class II MHC (MHC-II) expression and Ag processing. Short term exposure leads to TNF-α secretion in macrophages	(Drage *et al*., 2009; Gehring AJ, 2003)
Acylated LprA	*M. tuberculosis, M. bovis*	TLR2,TLR1 and CD36		(Drage *et al*., 2009; Pecora *et al*., 2006)
LpqH (19-kDa protein)	*M. tuberculosis, M. bovis*	TLR2, CD14	Inhibits class II MHC (MHC-II) expression and Ag processing. Short term exposure leads to TNF-α secretion in macrophages	(Aliprantis *et al*., 1999; Brightbill *et al*., 1999; Noss *et al*., 2001)
38-kDa glycolipoprotein (PhoS1)	*M. tuberculosis*	TLR2 and TLR4	TNF-α and IL-6 expression.	(Jung *et al*., 2006)

Table 2: TLR ligands (Proteins) of Mycobacterium and their physiological role

Mycobacterial TLR Ligands	Species	Pattern Recognition receptors	Modulation of immune response	References
		Proteins		
PE_PGRS33 (Rv1818c)	*M. tuberculosis*	TLR2	Induction of apoptosis through induction of TNF-α	(Basu *et al.*, 2007)
ESAT-6	*M. tuberculosis*	TLR2	Inhibition of pro-inflammatory responses	(Pathak *et al*., 2007)
HSP65 (Heat Shock Protein)	*M. tuberculosis*	TLR2		(Bulut *et al.*, 2005)
HSP70	*M. tuberculosis*	TLR2	Pro-inflammatory responses	(Bulut *et al.*, 2005)
HSP60	*M. tuberculosis*	TLR2, TLR4	Stimulates IL-10 via TLR2 signaling	(Khan *et al.*, 2008a)
Soluble tuberculosis factor (STF)	*M. tuberculosis*	TLR2	Induction of Pro-inflammatory responses	(Means *et al*., 1999)

was shown to trigger extensive inflammatory responses in macrophages and arrest MHC class I processing and presentationvia its interaction with TLR2 (Wang *et al*., 2013)**.**

Diverse Functional Role of PE/PPE Proteins

Various studies suggest a role of PE/PPE proteins in subverting critical innate immune pathways by interfering with various host components (Sampson, 2011). The most widely supported theory suggests the involvement of these proteins in generation of antigenic variation due to highly polymorphic nature of their C-terminal domains (Banu *et al*., 2002; Delogu and Brennan, 2001). Their roles in modulation of macrophage function have also been predicted (Banu *et al*., 2002; Brennan and Delogu, 2002; Cole *et al*., 1998; Delogu and Brennan, 2001). Two *M. marinum* orthologues of the PE_PGRS subfamily have been shown to be essential for replication in macrophages as well as persistence in granulomas (Ramakrishnan *et al*., 2000). A DNA vaccine construct based on the PE_PGRS region of protein Rv1818c (PE_PGRS33) was able to ablate the cellular immune response by influencing the antigen processing and presentation (Delogu and Brennan, 2001). A follow-up study by Dheenadhayalan and coworkers further strengthen the hypothesis that expression of complete PE_PGRS33 protein in the non-pathogenic fast-growing *M. smegmatis*, offers better survival advantage in infected macrophages cultures and mice, rather than a parental strain or a strain expressing only the PE domain of the protein (Dheenadhayalan *et al*., 2006a). The PE domain of PE_PGRS33 was found to be necessary for sub-cellular localization, while the PGRS domain is shown to affect the bacterial shape and colony morphology (Delogu *et al*., 2004). Further, a transposon insertion mutation in the PE_PGRS33 of a *M. bovis* BCG strain showed reduced survival in macrophages and dispersed growth in liquid media (Brennan *et al*., 2001). PE_PGRS33 has also been shown to elicit TNF-α production in macrophages in a TLR2-dependent manner and deletions within the PGRS domain (simulating those occurring in clinical strains) was shown to attenuate the TNF-α-inducing ability of PE_PGRS33 (Basu *et al*., 2007). Several other studies have confirmed the role of PE/PPE proteins in facilitating *in vivo* survival of M.*tb* (Mehta *et al*., 2006; Rengarajan *et al*., 2005; Singh *et al*., 2008).

Other diverse potential functions of the members of these families have been also reported. For example, Rodriguez and colleagues have found that the *ppe* gene Rv2123 (PPE37) is up-regulated

under low iron conditions, leading to a speculation that this gene may encode a siderophore involved in iron uptake (Rodriguez *et al*., 1999; Rodriguez *et al*., 2002). Another member of the PE_PGRS family, Rv1759c (*wag22*) was shown to possess fibronectin binding capacity, indicating an important role in host pathogen interactions (Abou-Zeid *et al*., 1991; Espitia *et al*., 1999). An *M. avium* PPE protein (Rv1787/ PPE25 orthologue), expressed only in macrophages, was shown to influence macrophage vacuole acidification, phagosome-lysosome fusion and replication in macrophage and found to be associated with virulence in mice (Li *et al*., 2005). Recently,some of the PE/PPE family members have also been implicated in the modulation of vacuole acidification (Jha *et al*., 2010; Li *et al*., 2005; Stewart *et al*., 2005). A high-throughput analysis of a *M. bovis* BCG tranposon mutant library identified four *pe_pgrs* mutants (pe_pgrs5, 28, 44, 59) and three *ppe_mptr* mutants (ppe_mptr10, 16, 21) that were enriched in acidified phagosomes. A more recent study showed that an M.*tb* ppe54 transposon mutant was impaired in its ability to arrest phagosome maturation and trafficked rapidly into acidified compartments (Brodin *et al*., 2010). Additional data supporting the notion that members of the PPE gene family may be involved in disease pathogenesisis a study that shows that a transposon mutant of the *ppe* gene Rv3018c (PPE46) had attenuated growth in macrophages (Camacho *et al*., 1999). Sassetti *et al*., further confirmed role of two PPE's Rv1807/PPE31 and Rv3873/PPE68 and one PE Rv3872/PE35 for mycobacterial growth *in vivo* during infection of mice (Sassetti and Rubin, 2003). Additionally, Jain and coworkers identified three PE_PGRS genes (Rv0977/ PE_PGRS16, Rv0978c/PE_PGRS17 and Rv0980c/ PE_PGRS18) and two PPE genes (Rv1801/PPE29 and Rv3021c/PPE47) that were up-regulated by at least 8-fold in human brain microvascular endothelial-cell-associated *M. tuberculosis* and showed that at least Rv0980c and Rv1801 are potentially required for endothelial-cell invasion and/ or intracellular survival of the bacilli (Jain *et al*., 2006). Interestingly, Talaat *et al*. identified the same *PE_PGRS* genes, i.e. *Rv0977, Rv0978c* and *Rv0980c* to form part of a so-called *in vivo*-expressed genomic island that was highly expressed only *in vivo* and not *in vitro* (Talaat *et al*., 2004). While enzymatic activity is not predicted to be a general feature of the PE/PPE proteins, enzymatic functions have been assigned to 3 PE proteins: PE_PGRS11 has been reported to be a functional phosphoglyceratemutase (Chaturvedi *et al*., 2010; Mishra *et al*., 2008), both PE_PGRS63 (LipY (Mishra *et al*., 2008)) and PE11 (LipX(Deb *et al*., 2006), have shown to demonstrate lipase activity. The latter two proteins may therefore play a role in energy provision during infection. Available data therefore indicates that selected PE/PPE family members play important roles in subverting innate immune responses, and together with other bacterial mediators, may assist the pathogen in establishing itself within the host. Once this has occurred, PE/ PPEs may then contribute to processes, which allow the pathogen to persist within host tissues. The observation that PE_PGRS proteins bear some resemblance to the Epstein-Barr virus nuclear antigen (EBVNA) gave rise to speculation that, akin to the EBVNA, the PE_PGRS proteins may inhibit antigen processing (Cole *et al*., 1998). This has subsequently been demonstrated for PE_PGRS33 (Brennan and Delogu, 2002) and PE_PGRS17 (Koh *et al*., 2009a), supporting the idea that these proteins may assist the immune evasion by limiting antigen presentation, thereby preventing recognition and killing the mycobacteria-infected host cells. Recently PPE2 protein, which was shown to display strong and specific immune-reactivity against sera from active tuberculosis, was also found be involved in down-regulation of NO production in activated macrophages (Bhat *et al*., 2013).

All these studies provide clues that the PE/PPE proteins may play distinct, but complementary roles as the infection progresses, acting in concert to facilitate the adaptation to hostile host environment. Members of these families may be critical mediators of host responses, which ultimately determine the outcome of infection. However, detailed molecular mechanisms by which the PE/PPE proteins can favour bacterial survival inside the host are not known. Thus, identification and characterization of the PE/PPE proteins that may play role in *M. tuberculosis* virulence are extremely important for understanding the mechanism of pathogenesis in tuberculosis.

Hijacking of TLR2 and Th1-Th2 Differentiation by Mycobacterial PE/PPE Proteins

Innate immunity relies on the ability of immune cells to recognize microbial products that distinguish classes of pathogens through germ-line encoded pattern recognition receptors (PRR). The recognition of microbial products alerts the immune system to mount an immediate response to counter the infection. Another important function of innate immune response is the activation and coordination of an adaptive immune response, which allows long-lasting specific immunity (Hoffmann *et al*., 2006; Janeway and Medzhitov, 2002). Upon recognition of microbial products, information regarding the nature of invading microorganism is translated to instruct proper differentiation of T-helper cells, thus ensuring elicitation of appropriate immune response to fight a particular pathogen. The ability to acquire protective immunity depends on the activation of correct type of response, and several diseases are known to be caused by an alteration of the Th1/Th2 balance, tuberculosis being one of them (Re and Strominger, 2004). A unique characteristic of mycobacterium is that they can cause chronic infection during which Th1/Th2 /T-cell balance is a critical determinant of persistence in the host (Mukhopadhyay, 2007).

Efficient control of microbial infection not only requires immune activation upon pathogen invasion, but also demands generation of appropriate immune response. Successful elimination of M.*tb* bacteria depends mainly on the interactions between infected macrophages and antigen-specific T-cells (Kaufmann and Flesch, 1988). Immunity to *M.tb* depends on robust Th1 cell-mediated response while susceptibility is associated with Th2 response (Rook *et al*., 2001). Among the several factors that regulate naïve T cell polarization and development into Th1or Th2 cell, the cytokines produced by activated macrophages and dendritic cells (DC) have the most influential role (Netea *et al*., 2005). The generation of host protective Th1 cells depends on IL-12 secreted by macrophage; on the contrary IL-10 from macrophages inhibits Th1 cells and promotes M.*tb* infection (Giacomini *et al*., 2001; Higgins *et al*., 2009). Although excessive production of Th1 response occurs during M.*tb* infection, higher production of Th1 cytokines does not in itself assure protection against TB. In contrast, a weak type 2 response evoked by M.*tb* has been shown to be sufficient to undermine the efficacy of Th1 mediated immunity (Rook *et al*., 2005). In fact M.*tb* has been shown to promote an environment characterized by Th2 cytokines like IL-10 and IL-4 during infection with expression of Th2 cytokines (Almeida *et al*., 2009; Rook, 2007). Indeed, this appears true in studies showing that anti-PPD T cell response in infected patients is biased towards the Th2 type and explains the occurrence of specific IgE antibody in human tuberculosis (Wilsher *et al*., 1999). Therefore, it seems that failure to resolve infection in susceptible individuals is a consequence of M.*tb* strategy to skew Th1 response to Th2, by expression of Th2 cytokines. Anti-mycobacterial protective immunity is dominantly mediated by the Th1 T cell response and since macrophage induced IL-12 strongly regulate Th1 response while IL-10 production from macrophages not only perturb the Th1 response but also bias the immune response towards the non-protective Th2 type, mycobacterial strategy at every step is likely to perturb the macrophage IL-12/IL-10 balance to subsequently establish a Th2 response. This hypothesis is supported by the observation that, virulent clinical strains of M.*tb* are proficient at stimulating high levels of IL-10 in the immunosuppressive etiology of M.*tb* (Manca *et al*., 2005). Similarly, IL-10 transgenic mice are shown to reactivate latent tuberculosis infection (Turner *et al*., 2002). Besides, genome-wide expression profiling of host cells responding to mycobacterial infection divulge that M.*tb* is a poor inducer of the expression of IL-12 p40 (Talaat *et al*., 2004). Therefore, the regulation of a decision of protection versus survival against tuberculosis is very much dependent on the production of IL-12 and IL-10 from macrophages. Understanding the regulation of IL-12 and IL-10 balance in macrophages during mycobacterial infection thus holds significance in comprehending mechanisms operating during mycobacterial pathogenesis.

M.*tb* alters its gene expression radically during the course of infection and these antigens might affect

the nature of immune response drastically in a chronic infection. Therefore, it can be assumed that PPE proteins which show differential expression under different environmental stimuli may be involved in corrupting protective immune responses by various mechanisms, one of them being the induction of Th2 cytokines. With this perspective, we characterized the role of PPE18 (Rv1196) a representative PPE family of protein to differentially modulate macrophage cytokine profiles. Previous studies revealed that PPE18 is a non-secretory protein expressed during infection, exclusively present in species belonging to M.*tb* complex (Dillon *et al*., 1999)and genetically linked to ESAT6 gene cluster (Gey van Pittius *et al*., 2006). PPE18 showed higher expression in M.*tb* as compared to BCG (Rehren *et al*., 2007) and when administered as a DNA vaccine in mice, afforded protection against challenges with aerosolized M.*tb* (Skeiky *et al*., 2004). These observations, prompted us to consider PPE18 to be potential virulence factor, involved in pathogenesis of M.*tb*. We found that PPE18 is surface-exposed, interacts with TLR2 receptor on macrophages and regulates cytokine secretion in macrophages to induce IL-10 with very little activation of pro-inflammatory cytokines (Nair *et al*., 2009). Induction of IL-10 was found to be dependent on TLR2 mediated activation of p38 MAPK. PPE18 was found to inhibit IL-12p40 in macrophages in response to PPD and LPS; skew the anti-PPD T-cell response towards Th2- type with increase in IL-5 and decrease in IFN-γ production in a T-cell activation assay. PPE18 protein was found to target p38MAPK-SOCS3-Rel signaling to down-regulate the IL-12/TNF pro-inflammatory responses (Nair *et al*., 2011). Further studies using *ppe18* genetic knock out M.*tb* strain revealed that lack of PPE18 leads to attenuation of M.*tb* in mice with reduced infection burden in lung, liver and spleen and better survival rates compared to mice infected with the wild-type M.*tb* strain (Bhat *et al*., 2012a). The anti-inflammatory effects of PPE18 through TLR2 are in contrast to the induction of pro-inflammatory cytokines such as TNF-α by the known mycobacterial TLR2 ligands (Basu *et al*., 2007; Jo, 2008; Underhill *et al*., 1999). Therefore, to get an insight into the mechanisms underlining this apparent contradiction, molecular basis of PPE18 interaction with TLR2 was investigated. The *in silico* docking analyses and mutation experiments indicated that PPE18 specifically interacts with LRR 11~15 domain of the TLR2 and this site of interaction was different from that of a synthetic lipopeptide Pam_3CSK_4 known to activate ERK 1/2and TNF-α induction or from PPE16 that induces pro-inflammatory signaling (Bhat *et al*., 2012b). The issues why and how TLR2 recognize a diverse array of microbial ligands and induce distinct cytokine signaling in macrophages are indeed enigmatic. It is hence fascinating to envision the mechanisms that guide the variegation of immunity in response to different ligands of TLR2. The inferences from this study provides a novel perspective to the notion that binding of different ligands to different yet overlapping sites on the ecto-domain of TLR2 may have impact on the assembly of the intracellular multi-protein signaling complex, which in turn translates into differential cytokine signaling. Plausibly, recognition of different pathogen associated molecular patterns (PAMPs) by TLR2 through specific LRR domains may underlie the functional specificity of TLR2 in production of unique signaling which provide PAMP tailored immune response. It appears that downstream TLR2 signaling cascades could be either pro- or anti-inflammatory depending interaction of the specific ligand with TLR2 (Re and Strominger, 2004). However, the mechanisms that dictate whether a TLR2-triggered cascade will swing towards pro-inflammatory or anti-inflammatory response is not well understood. One of the determining factors could be whether TLR2 ligands of mycobacteria trigger a downstream p38 MAPK or ERK 1/2 activation, since activation of ERK 1/2 mostly leads to the induction of TNF-α, whereas, activation of p38 MAPK is found to be essential for IL-10 induction (Reiling *et al*., 2002). As we have shown with PPE18, downstream TLR2 signaling cascades could be either pro- or anti-inflammatory depending interaction of the specific ligand with TLR2 (Nair *et al*., 2009). Recently, we have shown that M.*tb* heat shock protein 60 (Mtbhsp60, Cpn60.1, and Rv3417c) interacts with both TLR2 and TLR4 receptors, but its interaction with TLR2 leads to clathrin-dependent endocytosis

resulting in an increased production of interleukin (IL)-10 and activated p38 MAPK. However, induction of pro-inflammatory cytokines such as TNF-α by Mtbhsp60 required its sequestration to the membrane either through TLR2 or TLR4, resulting in activation of ERK1/2 signaling cascades (Parveen *et al*., 2013). Similarly PPE32, PE5-PE4 and PE15-PPE20 and PE35 and PPE68 were also shown to manipulate the host cytokine, via alterationof the MAPK signaling pathways (Tiwari *et al*., 2014; Tiwari *et al*., 2012). Thus, the capacity for distinct M.*tb* agonists to dictate these MAPK signaling cascades downstream of TLR2 could provide an additional means for the bacilli to modulate macrophage-effector responses.

Akin to our study on PPE18, recent reports now show a similar role of other PE/PPE proteins as well as ESAT-6 in subverting the normal macrophage innate immune response, by inhibiting the macrophage inflammatory responses by TLR2 signaling (Deng *et al*., 2014; Pathak *et al*., 2007; Tiwari *et al*., 2014; Tiwari *et al*., 2012), surmising that *pe/ppe* genes being in close proximity with *esat-6* like genes constitute the pathogens effort during evolution to cluster together and then induce effectors important for virulence and pathogenesis of *M. tuberculosis*. Because of their importance in detection and containment of mycobacterium, pathogenic mycobacterium may target TLR2 signaling as a mode of immune evasion. The evolutionary pressure for the expansion of the PE/PPE family in pathogenic mycobacterium may be to suppress TLR2-mediated innate immunity.

Humoral and Cell-mediated Immune Response Against PE/PPE Proteins

It is being increasingly appreciated that humoral immune responses accompanied by cellular immune response is important for controlling the *M.tb* infection. Mycobacteria-specific antibodies have been shown to influence mycobacterial dissemination and modulate potentially detrimental inflammatory tissue responses (Bosio *et al*., 2000; Maglione *et al*., 2007). PE/PPE family members on account of being either secreted or surface exposed are known targets of humoral responses. Several PE/PPE proteins have been shown to be potent B-cell antigens and serve as sero-diagnostics markers (Sampson, 2011). Mostly, the members of PE_PGRS and PPE_MPTR families possessing highly repetitive domains are found to elicit antibody responses (Chakhaiyar *et al*., 2004; Delogu and Brennan, 2001; Koh *et al*., 2009b). Delogu and Brennan were the first to dissect immunogenic domains of PE/PPE proteins. They found that in case of PE_PGRS33, the N-terminal part of protein, induced cell-mediated immunity in an animal model. In contrast, the C-terminal PGRS domain was not able to induce any cellular response, although it was found to be a good B-cell antigen and was recognized by sera from *M. tuberculosis* challenged mice (Delogu and Brennan, 2001). Concomitant to these results, other studies have shown that glycine and asparagine repeat domains are preferentially recognized in comparison to the PE-only versions (Koh *et al*., 2009b).

Cell-mediated immunity conferred by CD4+T cells is critical for controlling *M.tb* infection (Bhatt and Salgame, 2007). Hence, defining the repertoire of *M.tb* T cell antigen is central for vaccine development. Published traditional as well as latest proteome-wide antigen screens have reported the ability of PE/PPE proteins as major targets of human immune response (Kunnath-Velayudhan and Porcelli, 2013). Among the antigens identified by the peptide library screening for CD4+ T cells responses around 45% were from the PE/PPE family, though they constitute less than 5% of open reading frame of *M.tb* proteome. Identification of PE/PPE proteins as prominent antigenic targets of CD4+ T cells in these studies of the immune-proteome is consistent with other more targeted analysis showing that these proteins are recognized at multiple stages of infection and in different disease states in mycobacteria infected human and cattle (Vordermeier *et al*., 2012). Immuno-dominance of PE/PPE proteins is largely attributed to the presence of cross-reactive epitopes, which are shared by multiple homologous family members. This point is more appreciated in studies showing that both the level and frequency of T-cell response to PE/PPE proteins were greater to peptide pools, representing more conserved N-terminal

regions, than to the pools representing other regions of the proteins (Vordermeier *et al*., 2012).

T-cell expression cloning has been successfully exploited to identify T-cell immunogens in M.*tb* sensitized individuals (Dillon *et al*., 1999), like the identification of the potent T-cell antigen Mtb39a, which is encoded by Rv1196/ppe18. In subsequent work, this antigen has been evaluated as part of the poly-protein subunit vaccine candidate, Mtb72f (Leroux-Roels *et al*., 2010; Reed *et al*., 2009). Collectively, all these results indicate that PE/PPE proteins are worthy of further evaluation as potentially protective antigens for inclusion in new TB vaccine candidates.

Conclusions

The major challenge in rational design of novel drug targets to tuberculosis, is our insufficient understanding of the complex interplay between macrophages and M.*tb*. Understanding modulations in host-signaling pathways during infection with M.*tb* is therefore extremely important to increase our knowledge of pathogenic mechanisms that result in development of disease. Analysis of the M.*tb* genome sequence revealed presence of two unique gene families PE/PPE, found to be exclusively present and expanded in pathogenic mycobacterium. Though some clues exist attributing PE/PPE family of protein to immunogenicity and antigenic diversity, detailed characterization of physiological relevance during infection still remains elusive. Nevertheless, some interesting themes are emerging alluding to immune-modulatory potential of these proteins, important in establishing and maintenance of mycobacterial infection. Further, integrated studies on identifying the interaction partners and host pathways/processes targeted by PE/PPE family of proteins will provide crucial insights towards our understanding of mycobacterial survival inside the host and to uncover novel aspects of host cell biology. Ultimately, such insights might help us in designing new drugs to control TB.

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