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# PE\_PGRS Antigens of *Mycobacterium tuberculosis* Induce Maturation and Activation of Human Dendritic Cells

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*Mycobacterium tuberculosis*, the causative agent of pulmonary tuberculosis, infects one-third of the world's population. Activation of host immune responses for containment of mycobacterial infections involves participation of innate immune cells, such as dendritic cells (DCs). DCs are sentinels of the immune system and are important for eliciting both primary and secondary immune responses to pathogens. In this context, to understand the molecular pathogenesis of tuberculosis and host response to mycobacteria and to conceive prospective vaccine candidates, it is important to understand how cell wall Ags of *M. tuberculosis* and, in particular, the proline-glutamic acid polymorphic guanine-cytosine-rich sequence (PE\_PGRS) family of proteins modulate DC maturation and function. In this study, we demonstrate that two cell wall-associated/secretory PE\_PGRS proteins, PE\_PGRS 17 (Rv0978c) and PE\_PGRS 11 (Rv0754), recognize TLR2, induce maturation and activation of human DCs, and enhance the ability of DCs to stimulate CD4<sup>+</sup> T cells. We further found that PE\_PGRS protein-mediated activation of DCs involves participation of ERK1/2, p38 MAPK, and NF- $\kappa$ B signaling pathways. Priming of human DCs with IFN- $\gamma$  further augmented PE\_PGRS 17 or PE\_PGRS 11 Ag-induced DC maturation and secretion of key proinflammatory cytokines. Our results suggest that by activating DCs, PE\_PGRS proteins, important mycobacterial cell wall Ags, could potentially contribute in the initiation of innate immune responses during tuberculosis infection and hence regulate the clinical course of tuberculosis. *The Journal of Immunology*, 2010, 184: 3495–3504.

**M**ycobacterium tuberculosis, the causative agent of pulmonary tuberculosis, infects one-third of the world's population (1). The emergence of multidrug-resistant and extensively drug-resistant strains, alarmingly raising numbers of patients with coinfection of HIV and tuberculosis and variable efficacy of immunization with *Mycobacterium bovis* bacillus Calmette-Guérin, have stressed the urgency of developing novel therapeutic intervention strategies for tuberculosis (2, 3). There-

fore, identification and characterization of immunodominant mycobacterial Ags that are critical in eliciting protective immune responses is essential to understand host-pathogen interaction and to develop therapeutic strategies to combat tuberculosis.

Activation of inflammatory responses for containment of mycobacterial infections involves participation of innate as well as adaptive immunity (4–9). Despite robust host immune responses, *M. tuberculosis* exhibits remarkable ability to survive by interfering with functions of APCs, the cells critical for eliciting immune responses to pathogens (10–13). Although mycobacteria are suggested to persist in granulomas of infected hosts, many antigenic proteins are known to be secreted from the infected macrophages (14–17) that eventually act as key targets for APCs, such as dendritic cells (DCs), recruited to the focus of the infection. DCs are sentinels of the immune system and are important for eliciting both primary and secondary immune responses to pathogens (18–23). DCs express diverse cell surface markers, and phenotypic analysis broadly classifies the DCs into immature and mature stages. Immature DCs have the ability to capture and internalize Ags that involve surface expressed receptors, such as TLRs, lectin receptors, FcR, and complement receptors. Additionally, DCs express a number of cytokine receptors, an important feature in their ability to respond to inflammatory stimuli. In this perspective, immature DCs operate as immunological sensors to alert the immune system (19, 21). However, immature DCs are poor stimulators of diverse types of T cells, and it is necessary for them to undergo maturation and activation steps in order initiate robust innate immunity. In this context, the maturation process is defined by the series of phenotypic changes that enable DCs to initiate immunity as professional APCs. This complex process relies on several concomitant processes, including loss of endocytic/phagocytic receptors; upregulation of costimulatory and adhesion molecules, such as CD80, CD86, CD40, CD54, and CD58, the molecules involved in mediating clustering with and activation of T cells; and secretion of immunoregulatory cytokines and chemokines, such as IL-1 $\beta$ , IL-6,

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Abbreviations used in this paper: BF, bright field; DC, dendritic cell; Med, medium; Ni-NTA, Ni-nitrilotriacetic acid; NP-40, Nonidet P-40; PE, proline-glutamic acid; PE\_PGRS, proline-glutamic acid polymorphic guanine-cytosine-rich sequence; PGRS, polymorphic guanine-cytosine-rich sequences; PPE, proline-proline-glutamic acid.

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IL-10, IL-12, IL-23, TNF, CCL17, CCL19, and CCL22. Further, maturation process involves translocation of MHC class I/II compartments to the cell surface and eventual migration of mature DCs into regional/draining lymphoid tissue. This maturation and activation process renders DCs a high capability to activate Ag-specific T cells (22, 23).

Studies have suggested that infection of human DCs with *M. tuberculosis* results in their maturation reflected by increased surface expression of maturation markers as well as secretion of elevated levels of inflammatory cytokines (24–26). Further, infected DCs were shown to restrict the intracellular growth of *M. tuberculosis* (27). In this context, it is vital to understand molecular details on how cell wall Ags of *M. tuberculosis*, and in particular proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) gene family members of *M. tuberculosis*, modulate DC maturation and their function.

Ten percent of the coding capacity of *M. tuberculosis* genome is devoted to the PE and PPE gene family members, exemplified by the presence of PE and PPE motifs near the N-terminus of their gene products (28). Many members of the PE family exhibit multiple copies of polymorphic guanine-cytosine-rich sequences (PGRSs) at the C-terminal end, which are designated as the PE\_PGRS family of proteins (29). The uniqueness of the PE genes is further illustrated by the fact that these genes are restricted to mycobacteria (28, 29). Several PE genes are reported to be expressed upon infection of macrophages, and polymorphisms in PGRS region are suggested to play a critical role in antigenic diversity and to evade host immune recognition (30, 31). Many PE/PPE proteins are also known to induce a strong B cell response in humans (32–35). A number of PE\_PGRS proteins associate with the cell wall, and following surface exposure, these proteins traffic out of the mycobacterial phagosome into endocytic compartments. In addition, these PE\_PGRS proteins can gain access to the extracellular environment in the form of exocytosed vesicles (14, 30, 36, 37). However, little is known about the effect of PE\_PGRS proteins on the maturation and functions of human DCs and underlying signaling events.

In this study, we demonstrate that two cell wall-associated/secretory PE\_PGRS proteins, PE\_PGRS 17 (Rv0978c) and PE\_PGRS 11 (Rv0754), recognize TLR2 and induce maturation and activation of human DCs. We further found that PE\_PGRS protein-mediated activation of DCs involves activation of ERK1/2, p38 MAPK, and NF- $\kappa$ B pathways. Our results suggest that by acting on APCs, such as DCs, PE\_PGRS 17 and PE\_PGRS 11 proteins could potentially regulate immune responses to *M. tuberculosis* and the clinical course of tuberculosis.

## Materials and Methods

### *Abs and reagents*

Recombinant human IL-4, GM-CSF, and IFN- $\gamma$  were obtained from ImmunoTools (Friesoythe, Germany). FITC-conjugated mAbs to HLA-DR, CD80, and CD1a and PE-conjugated mAbs to CD86 and CD83 were from BD Biosciences (Le Pont-De-Claix Cedex, France), and PE-conjugated mAb to CD40 was from BD Biosciences. Anti-Thr202/Tyr204 phospho-ERK1/2, anti-ERK1/2, anti-Thr180/Tyr182 phospho-p38 MAPK, p38 MAPK, and anti-NF- $\kappa$ B p65 Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-TLR1, anti-TLR2, anti-TLR4, and anti-TLR6 were procured from Imgenex (San Diego, CA). Anti- $\beta$ -actin Ab (AC-15) was from Sigma-Aldrich (St. Louis, MO).

### *Expression and purification of PE\_PGRS 17 and PE\_PGRS 11*

The selected PE\_PGRS genes were PCR amplified from genomic DNA using gene-specific primers: 5'-GCGGATCCATGTCGTTTGTCAACGTGGC-3' (forward) and 5'-CGTCTCAGAGCTGATTACCGACACCGTGT-3' (reverse) for PE\_PGRS 17; 5'-CGGGATCCATGCATTTGTGATC-GTGGCG-3' (forward) and 5'-CCCAAGCTTTCATGGGATCAGGCTGGCAG-3' (reverse) for PE\_PGRS 11. The amplified PCR product was li-

gated into the pGEMT-Easy vector (Promega, Madison, WI), and the recombinant clones carrying the appropriate gene insert were confirmed by DNA sequencing. The gene inserts were subcloned into pRSET series vectors for protein expression and purification. *Escherichia coli* BL21 cells carrying recombinant plasmids were induced with isopropyl- $\beta$ -D-thiogalactopyranoside, and His-tagged recombinant proteins were purified with Ni-nitrilotriacetic acid (Ni-NTA) columns (Qiagen, Valencia, CA).

### *Generation of polyclonal Abs to PE\_PGRS 17 and PE\_PGRS 11*

The polyclonal Abs against PE\_PGRS 17 and PE\_PGRS 11 were generated in rabbits by s.c. injection of 1 mg purified proteins emulsified with equal volume of Freund's adjuvant (Sigma-Aldrich). The experiments were approved by the Institutional Ethics Committee for Animal Experimentation and Institutional Biosafety Committee, Indian Institute of Science, Bangalore. The first immunization was carried out with Freund's complete adjuvant followed by two booster immunizations with Freund's incomplete adjuvant at 21-d intervals. The Ab titers in the serum were determined by ELISA 2 wk postfinal immunization.

### *Generation and culture of human DCs*

PBMCs were isolated from buffy coats of healthy donors purchased from Hôpital Hôtel Dieu, Etablissement Français du Sang, Paris, France, upon ethical approval for the use of such materials. Monocytes from PBMCs were positively isolated using CD14 magnetic beads (Miltenyi Biotec, Paris, France). The purity of the monocytes was >98%. Monocytes were differentiated into immature DCs by culturing them for 7 d in RPMI 1640 containing 10% FCS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, IL-4 (500 IU/10<sup>6</sup> cells), and GM-CSF (1000 IU/10<sup>6</sup> cells). Immature 7-d-old DCs (0.5  $\times$  10<sup>6</sup> /ml) were cultured with cytokines alone or cytokines and individual PE\_PGRS protein for 48 h.

### *Patients with tuberculosis and healthy subjects*

The study population was comprised of tuberculosis bacilli-infected patients ( $n = 9$ ) or healthy controls ( $n = 4$ ) reporting to the National Institute of Mental Health and Neurosciences, Bangalore, India. The diagnosis of tuberculosis was established based on clinical and radiological data together with the identification of acid-fast bacilli in sputum. The healthy donors were negative for active tuberculosis disease. The samples from HIV-positive subjects were excluded from the study. The study subjects had given written consent, and the study was approved by the Institutional Bioethics Committee.

### *Analysis of the expression of surface molecules by flow cytometry*

Cell surface staining was performed with specifically labeled mAbs, and samples were proceeded for flow cytometry (LSR II, BD Biosciences). For each sample, 5000 events were recorded. Data were analyzed using BD FACSDiva software (BD Biosciences).

### *MLR*

CD4<sup>+</sup> T cells used in allogenic MLR were isolated from PBMCs of healthy donors using CD4 magnetic beads (Miltenyi Biotec). After 48 h of treatment with individual proteins, DCs were washed extensively and cocultured with  $1 \times 10^5$  responder allogeneic CD4<sup>+</sup> T cells at DC:T cell ratios of 1:10, 1:20, and 1:40. After 4 d, the cells were pulsed for 16–18 h with 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine. Radioactive incorporation was measured by standard liquid scintillation counting. The proliferation of cells was measured as cpm (mean  $\pm$  SEM of quadruplicate values) after subtracting values of responder T cell cultures alone.

### *Analysis of cytokines*

Cytokines were quantified in cell-free culture supernatants using BD CBA Human Inflammation kit and Human Th1/Th2 kits (BD Biosciences).

### *Treatment of DCs with pharmacological inhibitors of signaling pathways*

All the pharmacological inhibitors were obtained from Calbiochem (San Diego, CA). They were reconstituted in sterile, cell-culture grade DMSO (Sigma-Aldrich) and used at following concentrations after determining the viability of DCs in titration experiments using MTT assay: U0126 (10  $\mu$ M), SB203580 (1  $\mu$ M), SP600125 (10  $\mu$ M), Bay 11-0782 (20  $\mu$ M), and TPCK (20  $\mu$ M). DMSO at 0.1% concentration was used as the vehicle control. In experiments with inhibitors, the cells were treated with a given inhibitor for 60 min before treatment with individual proteins. Additionally, when a given inhibitor was tested, its efficacy in terms of inhibition of

phosphorylation of intended signaling molecule as well as a nonintended signaling molecule was also tested (for example, SB203580 inhibits TNF- $\alpha$ -triggered p38, but not ERK1/2 phosphorylation).

#### Immunoblotting analysis

Total cell lysates were prepared after washing the cells briefly with ice-cold PBS in 1 $\times$  radio immunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40 [NP-40], 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF). Equal amounts of proteins from each sample were subjected to SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in TBST buffer (0.02 M Tris-HCl [pH 7.5], 0.15 M NaCl and 0.1% Tween 20) containing 5% nonfat dried milk and probed with a primary Ab overnight at 4°C. After washing with TBST, membranes were incubated with secondary Ab linked to HRP (Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were then developed with an ECL detection system (PerkinElmer, Wellesley, MA) as per the manufacturer's instructions.

#### Nuclear and cytosolic subcellular fractionation

DCs were washed with ice-cold PBS and gently resuspended in ice-cold Buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). Postincubation on ice for 15 min, cell membranes were disrupted with 10% NP-40, and the nuclear pellets were recovered by centrifugation at 13,000  $\times$  g for 15 min at 4°C. The supernatants from this step were used as cytosolic extracts. Nuclear pellets were lysed with ice-cold Buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), and nuclear extracts were collected postcentrifugation at 13,000  $\times$  g for 20 min at 4°C.

#### Transient transfections and immunofluorescence

HEK-293 cells were transiently transfected with TLR2 or TLR2 dominant-negative constructs using ESCORT III (Sigma-Aldrich). For immunofluorescence studies, vector or TLR2 cDNA construct-transfected HEK-293 cells and DCs were seeded in a 35-mm dish on cover slips. On the day of experiment, HEK-293 cells were washed and treated with 2  $\mu$ g/ml purified PE\_PGRS 17 or PE\_PGRS 11 proteins for 1 h, whereas DCs were pretreated with either anti-TLR1, -TLR2, -TLR4, or -TLR6 blocking Abs or isotype control Abs followed by treatment with purified PE\_PGRS 17 or PE\_PGRS 11 proteins. Cells were fixed with cold methanol for 15 min and stained with primary Abs (anti-PE\_PGRS 17 or anti-PE\_PGRS 11) for 1 h, followed by incubation with secondary Ab (Cy5-conjugated anti-rabbit IgG) in the dark for 1 h at room temperature. Cover slips with cells were mounted on a slide with fluoromount G, immunofluorescent images were acquired by a Zeiss LSM 510 Meta confocal laser scanning microscope, and the images were analyzed for the integrated density of the fluorescence and for the area of the cells using Zeiss LSM image browser software (Zeiss, Oberkochen, Germany).

#### Immunoprecipitation assay

HEK-293 cells transfected with TLR2 or vector construct and DCs were washed briefly with ice-cold PBS and lysed in 1 $\times$  radio immunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1  $\mu$ g/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF). The cell lysate was incubated with PE\_PGRS 17 or PE\_PGRS 11 immobilized on Ni-NTA beads at 4°C overnight on a rotor. The beads were harvested, washed, and boiled in 5 $\times$  Laemmli buffer for 5 min. The proteins were separated on 12% SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membranes (Millipore). The membranes were further probed with anti-TLR1, -TLR2, -TLR4, or -TLR6 Abs as indicated.

#### Statistical analysis

Levels of significance for comparison between samples were determined by Student *t* test distribution. The data in the graphs are expressed as the mean  $\pm$  SEM. GraphPad Prism 3.0 software (GraphPad, San Diego, CA) was used for all the statistical analysis.

## Results

### PE\_PGRS 17 and PE\_PGRS 11 Ags induce maturation of human DCs

Several lines of evidence suggest that the members of PE\_PGRS family proteins PE\_PGRS 17 and PE\_PGRS 11 have a critical role in the pathogenesis of tuberculosis and in the host innate and adaptive immune responses to bacilli (33, 38–40). Because DCs are at the

crossroads of innate and adaptive immunity, we first analyzed the effect of PE\_PGRS 17 and PE\_PGRS 11 proteins on the maturation process of human DCs.

Immature DCs were cultured with PE\_PGRS 17 or PE\_PGRS 11 for 48 h, and expression of various surface markers on cells was analyzed by flow cytometry. We have used Ags at a concentration of 5  $\mu$ g for all of the experiments after carrying out titration analysis. As shown in Fig. 1, stimulation of DCs with PE\_PGRS 17 and PE\_PGRS 11 resulted in the significantly increased expression of costimulatory molecules CD80, CD86, and CD40, Ag-presenting molecule HLA-DR, and DC terminal maturation marker CD83, whereas the expression of DC differentiation marker CD1a was decreased (Fig. 1).

We confirmed that the stimulatory effects of PE\_PGRS 17 and PE\_PGRS 11 proteins on DCs were not due to endotoxin or LPS contamination in the protein preparations. For all of the experiments, we have used protein preparations that were passed through polymyxin B agarose column. Consequently, we could not detect endotoxins in protein preparations as analyzed by the E-Toxate kit (Sigma-Aldrich). Furthermore, a nonrelated mycobacterial lipase protein produced and processed by the same procedure failed to trigger expression of maturation markers on DCs (Supplemental Fig. 1A, 1B). Also, proteinase K treatment abrogated the ability of PE\_PGRS 17 or PE\_PGRS 11 to trigger maturation of DCs, indicating that intact protein in its native form was required for inducing the maturation of DCs (Supplemental Fig. 2).

### PE\_PGRS 17 and PE\_PGRS 11 induce the secretion of proinflammatory cytokines by DCs

DC-derived cytokines play a critical role in the polarization of T cells and in mediating inflammatory responses (18, 19). Therefore, we analyzed whether PE\_PGRS Ag-mediated maturation process of DCs is associated with the secretion of proinflammatory cytokines. In fact, both PE\_PGRS 17 and PE\_PGRS 11 significantly stimulated DCs to secrete high levels of IL-6, IL-8, and IL-12 (Fig. 2A–C). However, untreated DCs secreted negligible amounts of these cytokines. Interestingly, PE\_PGRS 17 or PE\_PGRS 11 failed to trigger secretion of immunoregulatory and suppressive cytokine IL-10 (data not shown).

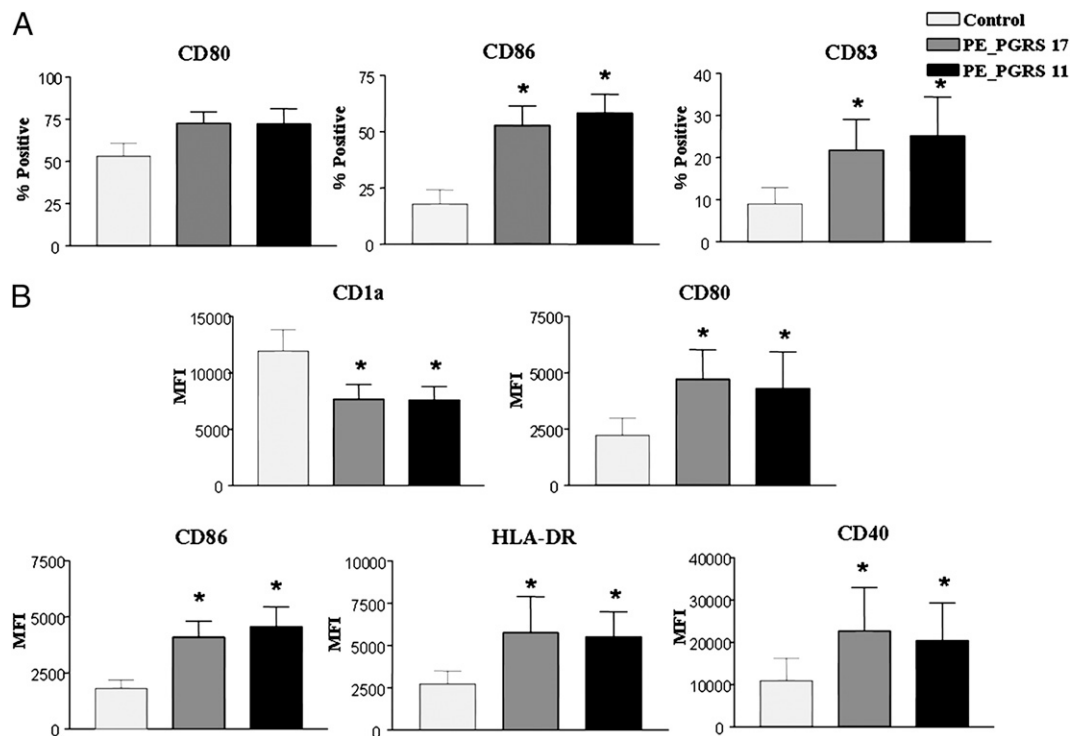
### PE\_PGRS 17 and PE\_PGRS 11 Ag-stimulated DCs induce CD4<sup>+</sup> T cell proliferation *in vitro*

Ag-activated and matured DCs can trigger activation and proliferation of CD4<sup>+</sup> T cells at very low stimulator to responder ratio (18–21). In this regard, we ascertained whether maturation of DCs by PE\_PGRS 17 and PE\_PGRS 11 Ags is reflected in their ability to stimulate CD4<sup>+</sup> T cells in an allogeneic MLR. As shown in Fig. 3A, DCs matured by PE\_PGRS 17 and PE\_PGRS 11 Ags significantly stimulated CD4<sup>+</sup> T cell proliferation as analyzed by [<sup>3</sup>H] thymidine incorporation. In concordance with these data, augmented secretion of IL-5 (Supplemental Fig. 3A), IFN- $\gamma$  (Supplemental Fig. 3B), as well as TNF- $\alpha$  (data not shown) by these DC-stimulated T cells implicate the potential of PE\_PGRS 17 and PE\_PGRS 11 Ags to stimulate potent T cell stimulation via DCs.

### PE\_PGRS 17 and PE\_PGRS 11 Ags induce strong T cell responses *ex vivo*

We have previously shown that PE\_PGRS 17 and PE\_PGRS 11 exhibit a very high antigenic index for the potential antigenic determinants (33 and data not shown). Therefore, we have hypothesized that patients with tuberculosis have a high frequency of T cells specific for these proteins. To examine this, we have analyzed the ability of PE\_PGRS 17 and PE\_PGRS 11 to trigger T cell cytokine secretion from PBMCs of patients with pulmonary tuberculosis. As demonstrated in Fig. 3B and 3C, both PE\_PGRS 17 and PE\_PGRS 11 augmented IFN- $\gamma$  and IL-5 secretion from T cells of patients with pulmonary tuberculosis as





**FIGURE 1.** PE\_PGRS 17 and PE\_PGRS 11 induce maturation of human DCs. *A* and *B*, Seven-day-old immature DCs ( $0.5 \times 10^6$  cells/ml) were cultured with GM-CSF and IL-4 alone (Control) or GM-CSF, IL-4, and 5  $\mu$ g/ml PE\_PGRS 17 or PE\_PGRS 11 for 48 h and analyzed for the expression of surface markers. The percentage of cells expressing the indicated markers is shown in *A*, whereas mean fluorescence intensities (MFI) are shown in *B*. Data are presented as mean  $\pm$  SEM from six independent donors. \* $p < 0.05$  versus Control.

compared with healthy subjects. Further, the ratios of cytokine responses were examined in healthy subjects and in patients with pulmonary tuberculosis to obtain a broader measure of the effect of PE\_PGRS 17 or PE\_PGRS 11 Ags on the balance of immunity. However, calculation of median ratios of PE\_PGRS 17 or PE\_PGRS 11 Ag-driven IFN- $\gamma$ /IL-5 indicated more balanced Th1/Th2 responses in both patients and healthy subjects (data not shown).

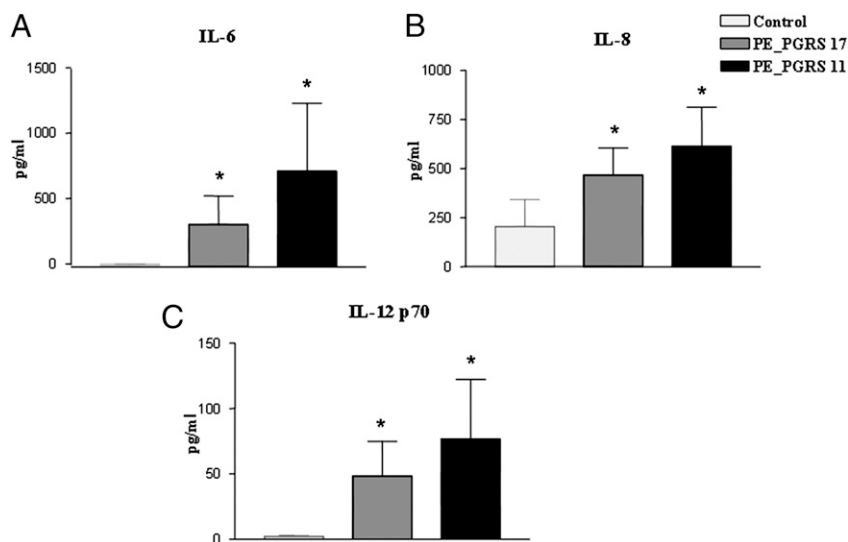
#### PE\_PGRS 17 and PE\_PGRS 11 proteins recognize TLR2

Innate immune cells express several pattern recognition receptors (41). Pattern recognition receptors, such as TLRs, recognize pathogen-associated molecular patterns on mycobacteria or mycobacterial cell wall components, leading to intracellular signaling events,

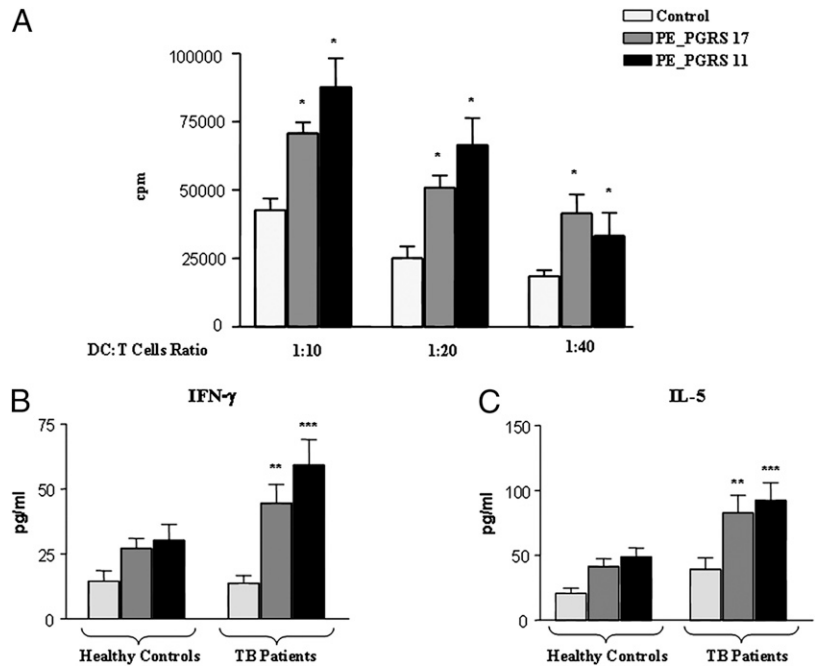
activation of innate cells, secretion of inflammatory cytokines, and initiation of immune responses to mycobacteria bacilli (42–45). Therefore, we aimed at identifying TLR that is recognized by PE\_PGRS 17 and PE\_PGRS 11 Ags.

In view of the critical role of TLR1, TLR2, TLR4, and TLR6 in mediating the recognition of mycobacterial Ags (42, 43, 46–52), we first analyzed the binding of PE\_PGRS 17 or PE\_PGRS 11 Ags to TLR2-transfected HEK-293 cells by confocal microscopy. Interestingly, we found that both PE\_PGRS 17 or PE\_PGRS 11 specifically recognize TLR2, but neither of these two Ags recognized control vector DNA-transfected HEK-293 cells (Fig. 4A). Furthermore, to establish physical interaction of PE\_PGRS 17 or PE\_PGRS 11 with TLR2, vector or TLR2-transfected HEK-293

**FIGURE 2.** PE\_PGRS 17 or PE\_PGRS 11 triggers DCs to secrete high amounts of proinflammatory cytokines. DCs ( $0.5 \times 10^6$  cells/ml) were cultured with GM-CSF and IL-4 alone (Control) or GM-CSF, IL-4, and 5  $\mu$ g/ml PE\_PGRS 17 or PE\_PGRS 11 for 48 h, and secretion of IL-6 (*A*), IL-8 (*B*), and IL-12 (*C*) in cell free culture supernatants was analyzed by cytokine bead array assay. Data are presented as mean  $\pm$  SEM from six independent donors. \* $p < 0.05$  versus Control.

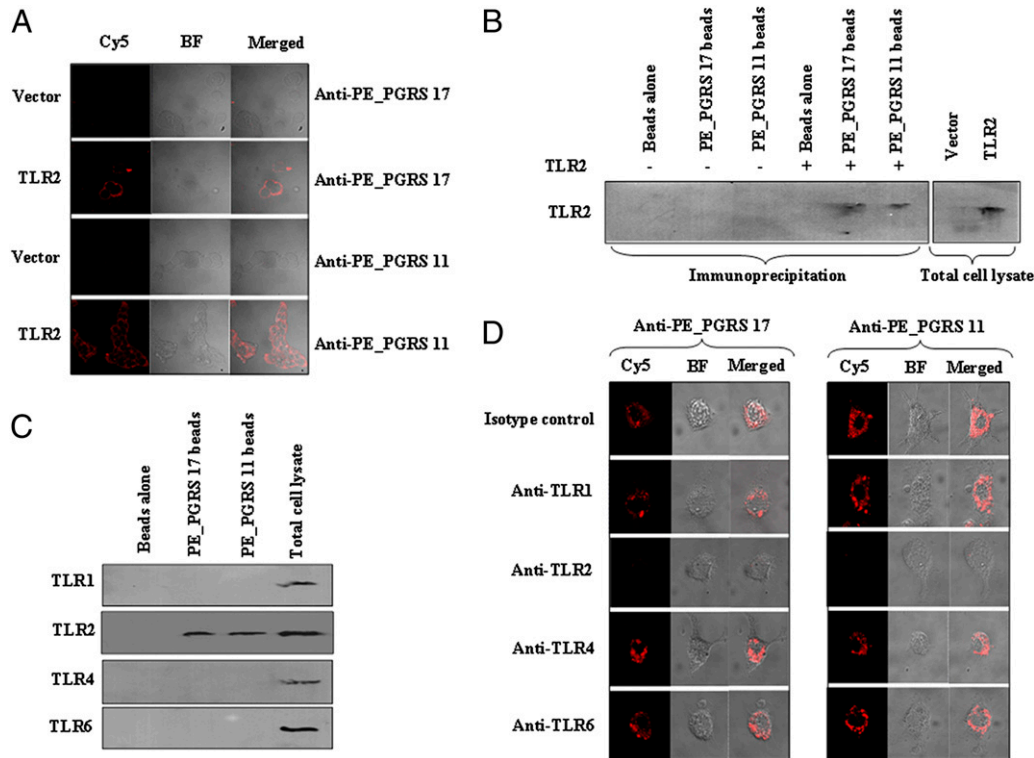


**FIGURE 3.** PE\_PGRS 17 or PE\_PGRS 11 triggers strong T cell response. *A*, DCs were cultured with GM-CSF and IL-4 alone (Control) or GM-CSF, IL-4, and 5  $\mu\text{g/ml}$  of PE\_PGRS 17 or PE\_PGRS 11 for 48 h. Following extensive washings, DCs were cocultured with allogenic CD4<sup>+</sup> T cells at various DC:T cell ratios. The T cell proliferation was quantified by [<sup>3</sup>H]thymidine incorporation, and values were expressed as cpm (mean  $\pm$  SEM of quadruplet values). The data are representative of one of three independent donors. *B* and *C*, PBMCs obtained from patients with tuberculosis ( $n = 9$ ) and healthy control subjects ( $n = 4$ ) were cultured with or without 5  $\mu\text{g/ml}$  PE\_PGRS17 or PE\_PGRS 11, and cell-free supernatants collected on day 4 were tested for concentrations of secreted IFN- $\gamma$  (*B*) and IL-5 (*C*). Data are presented as mean  $\pm$  SEM. \* $p < 0.05$  versus Control; \*\* $p < 0.05$  versus PE\_PGRS 17 (Healthy Controls); \*\*\* $p < 0.05$  versus PE\_PGRS 11 (Healthy Controls).

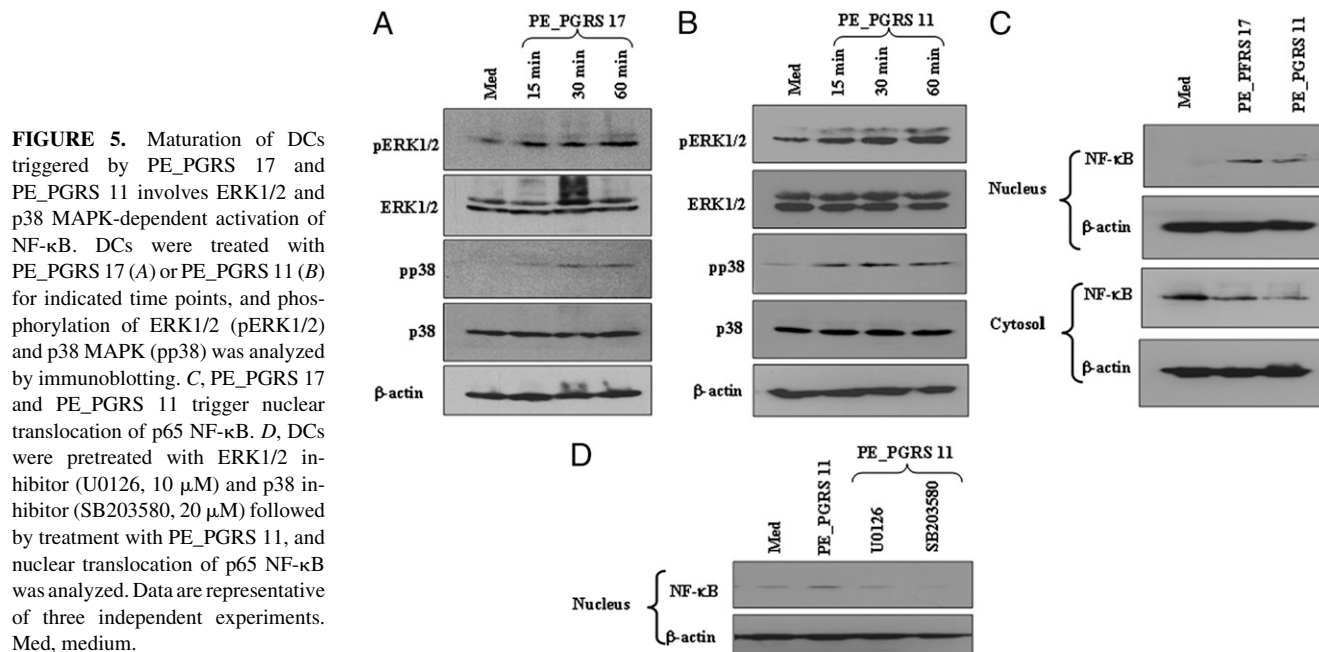


cell extracts were incubated with PE\_PGRS 17 or PE\_PGRS 11 proteins immobilized on Ni-NTA beads, and pull-down proteins were analyzed for TLR2 by immunoblotting. Data presented in Fig. 4*B* demonstrate that immobilized PE\_PGRS 17 or PE\_PGRS

11 could pull down TLR2, suggesting PE\_PGRS 17 or PE\_PGRS 11 Ags interact specifically in a significant manner with TLR2. In addition, specific interaction of PE\_PGRS 17 or PE\_PGRS 11 Ags with TLR2 as opposed to TLR4, TLR1, or TLR6 was further

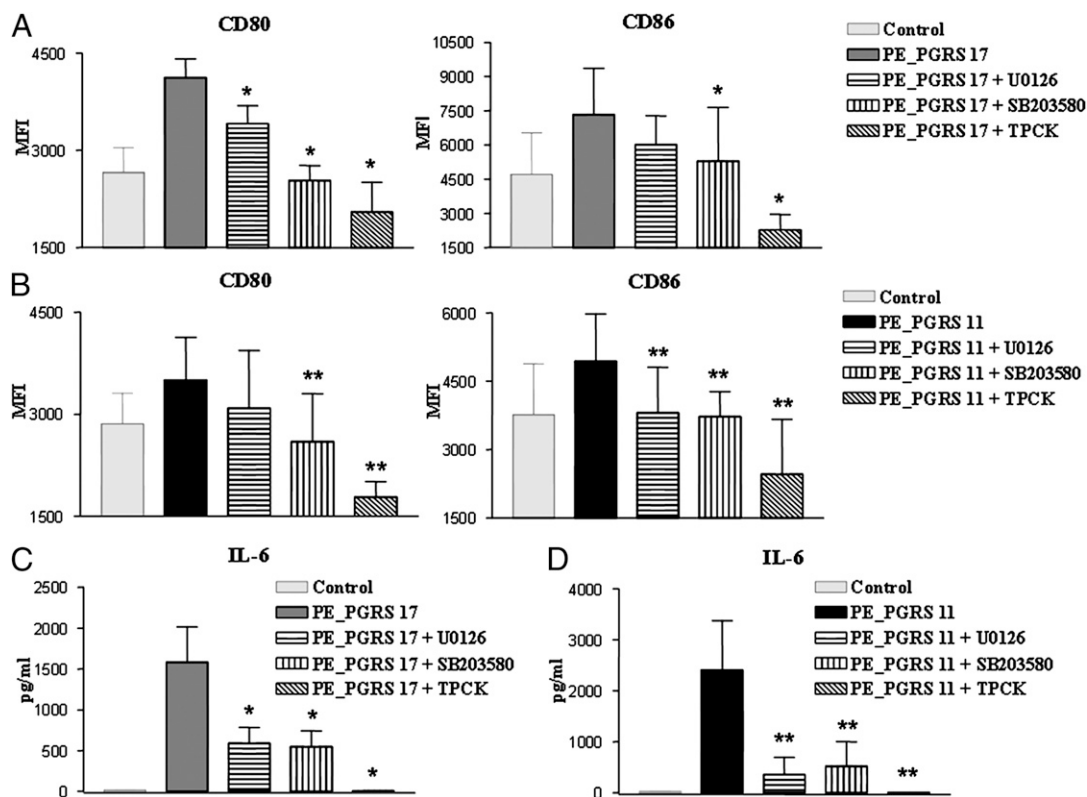


**FIGURE 4.** PE\_PGRS 17 and PE\_PGRS 11 proteins interact with TLR2. *A*, TLR2 or vector-transfected HEK-293 cells were treated with PE\_PGRS 17 or PE\_PGRS 11. The cells were further stained with Ab specific to PE\_PGRS 17 or PE\_PGRS 11 followed by Cy5-labeled secondary Ab. The immunofluorescent staining of cells was analyzed by confocal microscopy. *B*, Cell lysates from HEK-293 cells transfected with TLR2 or vector were incubated with PE\_PGRS 17 or PE\_PGRS 11 immobilized on Ni-NTA beads. The proteins bound on beads were extracted and analyzed for the presence of TLR2 by immunoblotting. *C*, Cell lysates from DCs were incubated with PE\_PGRS 17 or PE\_PGRS 11 immobilized on Ni-NTA beads, and bead-bound proteins were loaded on gel followed by immunoblotting for TLR1, TLR2, TLR4, and TLR6. *D*, DCs were pretreated with anti-TLR1, -TLR2, -TLR4, and -TLR6 blocking or isotypic Abs followed by incubation with PE\_PGRS 17 or PE\_PGRS11. Specificity of interaction of PE\_PGRS 17 or PE\_PGRS 11 was evaluated by confocal microscopic analysis. Data are representative of two independent experiments. BF, bright field.



validated by pulldown assay using whole cell extracts from DCs that endogenously express various TLRs. As demonstrated in Fig. 4C, PE\_PGRS 17 or PE\_PGRS 11 interacted specifically with

TLR2 as opposed to TLR1, TLR4, and TLR6. Further, to ascertain physical binding of PE\_PGRS 17 or PE\_PGRS 11 Ags specifically to TLR2 on DC surface, we blocked respective TLRs with



**FIGURE 6.** Involvement of ERK1/2, p38, and NF- $\kappa$ B pathways in PE\_PGRS 17 or PE\_PGRS 11-induced maturation of DCs. DCs were treated with pharmacological inhibitors of ERK1/2 (U0126, 10  $\mu$ M), p38 (SB203580, 20  $\mu$ M), NF- $\kappa$ B (TPCK, 20  $\mu$ M), or DMSO (vehicle control) for 1 h prior to treatment with PE\_PGRS 17 (A) or PE\_PGRS 11 (B) for 48 h. The expression of CD80 and CD86 was analyzed by flow cytometry. C and D, DCs were treated as in A or B, and the level of secreted IL-6 was analyzed. Data are presented as mean  $\pm$  SEM from three independent donors. \* $p$  < 0.05 versus PE\_PGRS 17; \*\* $p$  < 0.05 versus PE\_PGRS 11.

blocking Abs, and confocal microscopic studies clearly ascertain the specific interaction of PE\_PGRS 17 or PE\_PGRS 11 with TLR2 on DCs (Fig. 4D). Furthermore, the TLR2 dominant-negative construct significantly reduced PE\_PGRS 17 or PE\_PGRS 11 Ag-triggered NF- $\kappa$ B promoter activity, as well as expression of TLR2 target gene cyclooxygenase-2 at transcript as well as its protein levels (data not shown). Additionally, the ability of PE\_PGRS 17 or PE\_PGRS 11 to trigger the expression of TLR2 target gene cyclooxygenase-2 was not compromised in macrophages derived from TLR4-defective C3H/HeJ mice (data not shown). These results thus signify the role of TLR2 in PE\_PGRS 17- or PE\_PGRS 11-mediated signaling events.

#### Role of MAPK and NF- $\kappa$ B pathways in PE\_PGRS 17 or PE\_PGRS 11 Ag-induced maturation of DCs

Maturation of DCs induced by mycobacterial Ags often involves activation of NF- $\kappa$ B and MAPK pathways such as ERK1/2 and p38 MAPK (53–58). In this context, we found that both PE\_PGRS 17 or PE\_PGRS 11 Ags trigger the activation of MAPKs ERK1/2 and p38 (Fig. 5A, 5B) and significant translocation of p65 NF- $\kappa$ B from the cytosol to the nucleus within 60 min of stimulation (Fig. 5C) in human DCs. Interestingly, we found that U0126 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor) abolished PE\_PGRS 17- or PE\_PGRS 11-induced nuclear translocation of NF- $\kappa$ B (Fig. 5D and data not shown). These results thus indicate that PE\_PGRS 17 or PE\_PGRS 11 proteins trigger DC maturation via MAPK-dependent activation of the NF- $\kappa$ B pathway.

Furthermore, pharmacological inhibitors U0126 (ERK1/2), SB203580 (p38), TPCK (NF- $\kappa$ B) (59, 60), or Bay 11-0782 (NF- $\kappa$ B) (61) abrogated PE\_PGRS 17- or PE\_PGRS 11-induced expression of costimulatory molecules CD80, CD86, and CD40 (Fig. 6A, 6B and data not shown) and secretion of inflammatory

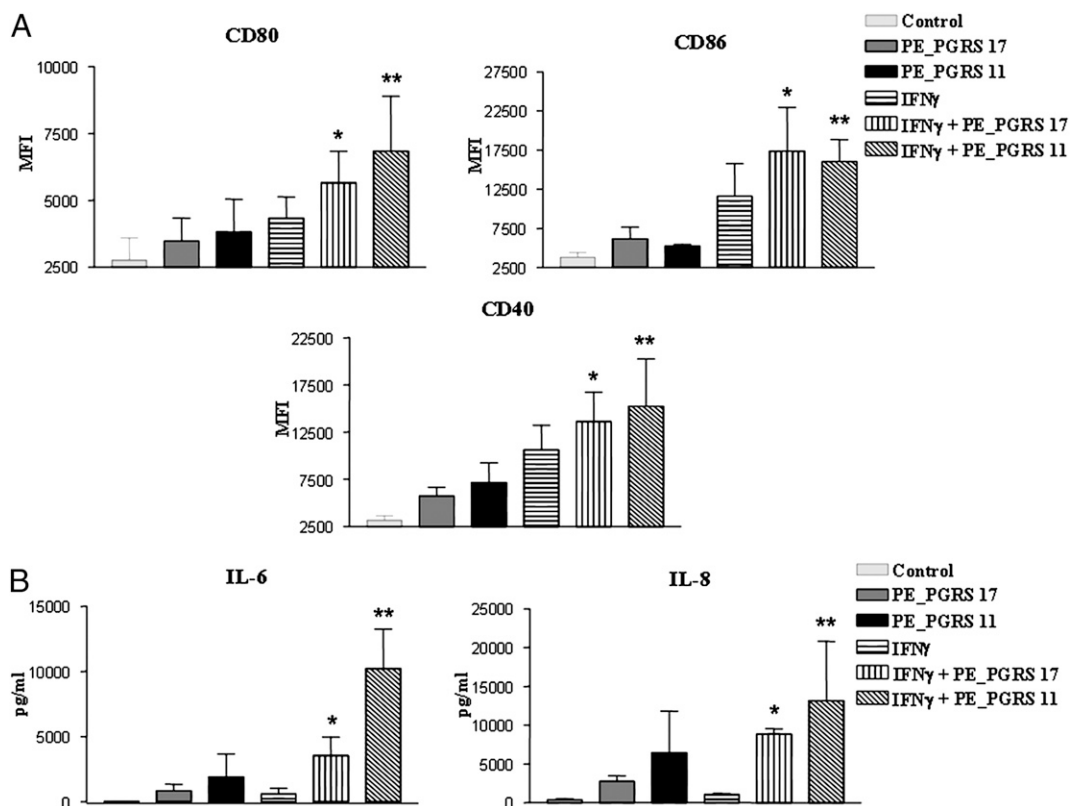
cytokines (shown with IL-6 as an example) from DCs (Fig. 6C, 6D). Together, our results thus suggest that NF- $\kappa$ B and MAPK pathways represent important signaling partnership links in PE\_PGRS 17- or PE\_PGRS 11-triggered maturation and activation of human DCs.

#### IFN- $\gamma$ potentiates PE\_PGRS 17- or PE\_PGRS 11-induced maturation of DCs

Among proinflammatory cytokines, IFN- $\gamma$  occupies an important place, as it regulates the diverse antimycobacterial cellular functions, such as activation of innate cells, Ag processing and presentation, and generation of effector CTLs. Indeed, IFN- $\gamma$ -deficient mice exhibit susceptibility to mycobacterial infection (62). It was suggested that the lack of protective immunity in IFN- $\gamma$ -deficient mice was primarily due to their inability to activate innate cells. In this perspective, we hypothesize that the priming of human DCs with IFN- $\gamma$  leads to augmentation of PE\_PGRS 17 or PE\_PGRS 11 Ag-induced DC maturation and secretion of inflammatory cytokines. Immature DCs were primed with 1000 IU/ml IFN- $\gamma$  for 6 h followed by treatment with PE\_PGRS 17 or PE\_PGRS 11 for 42 h. As represented in Fig. 7A, pretreatment with IFN- $\gamma$  led to enhancement of PE\_PGRS 17- or PE\_PGRS 11-induced maturation process of DCs as exemplified by the enhanced expression of costimulatory molecules CD80, CD86, and CD40. Additionally, IFN- $\gamma$  priming augmented PE\_PGRS 17 or PE\_PGRS 11 Ag-mediated secretion of both IL-6 and IL-8 by DCs (Fig. 7B).

#### Discussion

DCs are principal mediators of initiation as well as activation of host immune responses to tuberculosis infection. Activation of T cell-mediated immunity during infection is regulated by the signaling cascades that are initiated upon encounter of Ags by DCs and



**FIGURE 7.** IFN- $\gamma$  treatment augments PE\_PGRS 17 and PE\_PGRS 11-induced DC maturation. DCs were treated either with PE\_PGRS 17 or PE\_PGRS 11 alone or with IFN- $\gamma$  for 48 h, and expression of maturation markers CD80, CD86, and CD40 (A) and secretion of IL-6 and IL-8 (B) was analyzed. Data are presented as mean  $\pm$  SEM from three independent donors. \* $p$  < 0.05 versus PE\_PGRS 17; \*\* $p$  < 0.05 versus PE\_PGRS 11.



subsequent productive engagement of TCR on T cells in the context of costimulation by DCs (18, 19). Further, cytokines secreted from DCs, such as IL-6, IL-8, IL-12, or IL-10, direct the T cell polarization toward Th1, Th2, Th17, or regulatory T cells (19). Interestingly, mycobacterial Ags have been shown to possess the potential of regulating immune responses in humans and animal models (31, 26, 63). Although mycobacteria reside within phagolysosomes, cell wall Ags including lipoarabinomannan, phosphatidyl-myoinositol mannosides, and PE\_PGRS Ags are released and traffic out of the mycobacterial phagosome into endocytic compartments as well as gain access to the extracellular environment in the form of exocytosed vesicles (14, 61, 64, 65). In this perspective, deciphering the interaction of cell wall Ags of pathogenic mycobacteria with human DCs is important to understand the molecular pathogenesis of tuberculosis and host response to mycobacteria and to conceive prospective vaccine candidates.

As described previously, the PE\_PGRS subfamily of PE genes is enriched in genes with a high probability of being essential in *M. tuberculosis* survival in the host and hence can act as virulent factors (30, 31). Further, selective expression of specific PE\_PGRS proteins in vivo could address the various pathological attributes of *M. tuberculosis*. In this regard, we explored the modulation of human DC activation by PE\_PGRS Ags of *M. tuberculosis*.

PE\_PGRS 17 and PE\_PGRS 11 Ags display differential antigenic profiles and are associated with pathological conditions, as evident from DNA microarray expression data (39, 40, 66). In comparison with other PE\_PGRS Ags, the expression of PE\_PGRS 17 and PE\_PGRS 11 is selectively increased in *M. tuberculosis* bacilli upon infection of mouse macrophages (39), and PE\_PGRS 17 was shown to be upregulated, by at least 8-fold, in human brain microvascular endothelial cell-associated *M. tuberculosis* (66). In addition, PE\_PGRS 17 and PE\_PGRS 11 have been detected in lung tissues of infected mice (40, 67). We have previously shown that among all the proteins belonging to the PE family (including PE\_PGRS Ags), PE\_PGRS 17 and PE\_PGRS 11 exhibit a very high antigenic index for the potential antigenic determinants as mentioned (33 and data not shown). In addition, our previous studies have suggested that among studied PE Ags, PE\_PGRS 17 and PE\_PGRS 11 exhibit immunoreactivity in sera from adult patients with pulmonary tuberculosis and child patients with pulmonary or extrapulmonary infection. More importantly, the immunoreactivity elicited in patients' sera demonstrated differential and stronger reactivities of the humoral Abs to PE\_PGRS 17 and PE\_PGRS 11 compared with other PE or mycobacterial Ags. These observations suggested the possible differential serodiagnostic utility of PE\_PGRS 17 and PE\_PGRS 11 during different stages of tuberculosis infection (33 and data not shown). Further, PE\_PGRS 17 and PE\_PGRS 11 elicited stronger IFN- $\gamma$  and IL-5 cytokine secretion from T cells in patients with pulmonary tuberculosis compared with healthy subjects. In view of these observations, we proposed that PE\_PGRS 17 and PE\_PGRS 11 represent an ideal model to study mycobacteria-specific innate immune responses in humans.

We show that PE\_PGRS 17 and PE\_PGRS 11 proteins induce maturation of DCs characterized by upregulation of costimulatory and Ag-presenting molecules with a concomitant increased production of proinflammatory cytokines. Further, PE\_PGRS protein-matured DCs were functionally competent as they induced robust proliferation and secretion of cytokines from CD4<sup>+</sup> T cells, thus implicating the immunopotent nature of PE\_PGRS 17 and PE\_PGRS 11.

TLRs in general and TLR2 specifically play a pivotal role in the activation of inflammatory immune responses by mycobacteria (41–44). The activation threshold of TLR2 signaling in DCs during infection could act as a rate-limiting step that eventually controls the strength of innate or adaptive immune responses.

TLR2 signaling in DCs is suggested to involve the participation of MAPK and NF- $\kappa$ B signaling pathways (57, 61). In this context, PE\_PGRS 17 and PE\_PGRS 11 interact with cell surface TLR2, and disruption of the activation of MAPK and NF- $\kappa$ B pathways abrogated PE\_PGRS Ag-induced maturation of DCs or secretion of inflammatory cytokines. These results implicate signaling integration of TLR2, MAPKs, and NF- $\kappa$ B pathways during mycobacterial Ag-triggered DC maturation. In addition to PE\_PGRS Ags described in our study, a few other mycobacterial Ags, ESAT-6 and Ag85b, are reported to trigger murine DC maturation (68). However, our study is the first of its kind to report a novel activity associated with two selectively expressed PE\_PGRS proteins to which hitherto no functional annotation has been accredited as yet.

Many studies have clearly emphasized the observation of a lack of adequate immune response in patients with tuberculosis against many well-characterized Ags of tuberculosis bacilli. This lack of immune response is more prominent in newly diagnosed cases of pulmonary tuberculosis, wherein the host immune system is not sufficiently primed to elicit a strong immune response against most Ags of *M. tuberculosis* (33). The production of a key cytokine, IFN- $\gamma$ , by PBMCs is shown to be severely compromised in patients with tuberculosis (69). In this regard, augmentation by IFN- $\gamma$  of PE\_PGRS protein-mediated activation of DCs could act as a determining factor in the initiation of robust innate immunity to *Mycobacterium*. We show that in vitro priming of DCs with IFN- $\gamma$  not only enhances the maturation of DCs but also the secretion of proinflammatory cytokines. This observation implicates a crucial role of IFN- $\gamma$  in the pathogenesis of tuberculosis as well as in its utilization as an immunoadjuvant. Overall, our study assigns a novel role for PE\_PGRS Ags of *M. tuberculosis* in the activation of innate immunity and supports their possible utility in new therapeutic strategies to combat tuberculosis.

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## Disclosures

The authors have no financial conflicts of interest.

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