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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 $CD4^+$ T cells. We further found that PE_PGRS protein-mediated activation of DCs involves participation of ERK1/2, p38 MAPK, and NF-κB signaling pathways. Priming of human DCs with IFN-γ 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.**

Mycobacterium 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). Therefore, 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-1β, 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.

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-κB 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-γ 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-κB 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–β-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′-CGCTCGAGAGCTGATTACCGACACCGTGT-3′ (reverse) for PE_PGRS 17; 5′-CGGGATCCATGTCATTTGTGATCGTGGCG-3′ (forward) and 5′-CCCAAGCTTTCATGGGATCAGGCTGGGCAG-3′ (reverse) for PE_PGRS 11. The amplified PCR product was ligated 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-β-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 μg/ml streptomycin, IL-4 (500 IU/10^6 cells), and GM-CSF (1000 IU/10^6 cells). Immature 7-d-old DCs (0.5×10^6 /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

$CD4^+$ 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 1×10^5 responder allogeneic $CD4^+$ 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 μCi of [^{3}H]thymidine. Radioactive incorporation was measured by standard liquid scintillation counting. The proliferation of cells was measured as cpm (mean ± 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 μM), SB203580 (1 μM), SP600125 (10 μM), Bay 11-0782 (20 μM), and TPCK (20 μM). 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-α–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× 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 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, 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 × *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 × *g* for 20 min at 4˚C.

Transient transfections and immunoflourescence

HEK-293 cells were transiently transfected with TLR2 or TLR2 dominant-negative constructs using ESCORT III (Sigma-Aldrich). For immunoflourescence 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 μg/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× 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 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, 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× 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 ± 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 μg 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. 1*A*, 1*B*). 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. 2*A–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 $CD4^+$ T cell proliferation in vitro

Ag-activated and matured DCs can trigger activation and proliferation of $CD4^+$ 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 $CD4^+$ T cells in an allogeneic MLR. As shown in Fig. 3*A*, DCs matured by PE_PGRS 17 and PE_PGRS 11 Ags significantly stimulated $CD4^+$ T cell proliferation as analyzed by $[^3H]$ thymidine incorporation. In concordance with these data, augmented secretion of IL-5 (Supplemental Fig. 3*A*), IFN-γ (Supplemental Fig. 3*B*), as well as TNF-α (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. 3*B* and 3*C*, both PE_PGRS 17 and PE_PGRS 11 augmented IFN-γ and IL-5 secretion from T cells of patients with pulmonary tuberculosis as

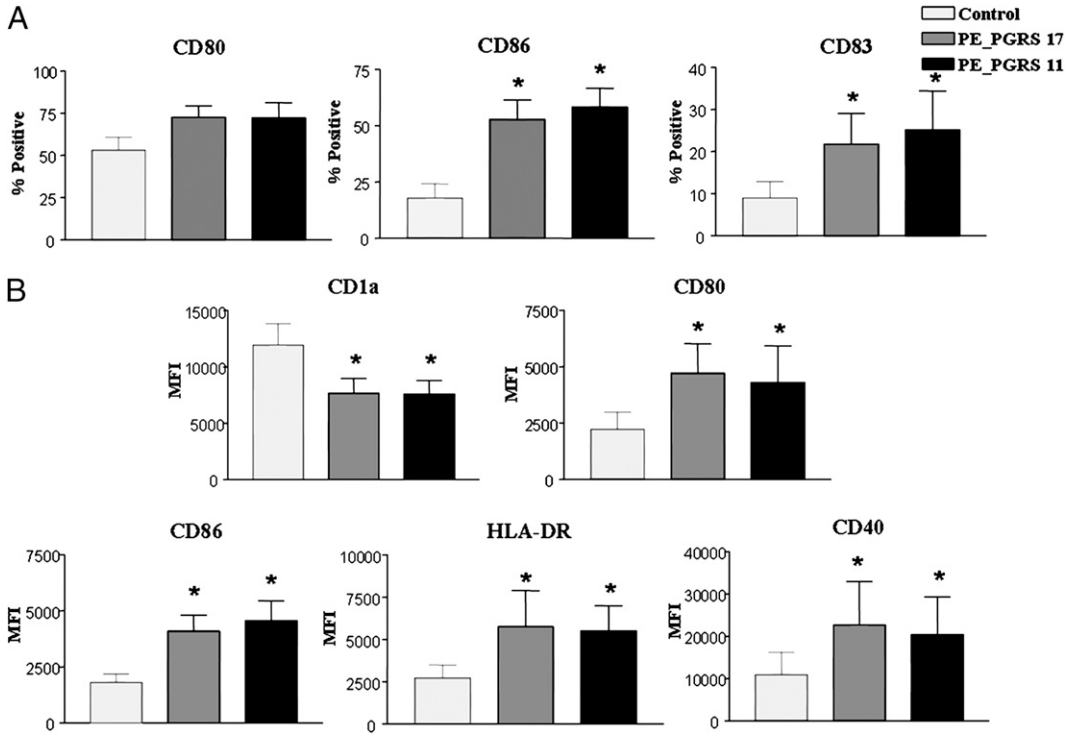

FIGURE 1. PE_PGRS 17 and PE_PGRS 11 induce maturation of human DCs. *A* and *B*, Seven-day-old immature DCs (0.5×10^6 cells/ml) were cultured with GM-CSF and IL-4 alone (Control) or GM-CSF, IL-4, and 5 μg/ml PE_PGRS 17 or PE_PGRS 11 for 48 h and analyzed for the expression of surface markers. The percentage of cells expressing the indicated markers is shown in *A*, whereas mean fluorescence intensities (MFI) are shown in *B*. Data are presented as mean ± 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-γ/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. 4*A*). Furthermore, to establish physical interaction of PE_PGRS 17 or PE_PGRS 11 with TLR2, vector or TLR2-transfected HEK-293

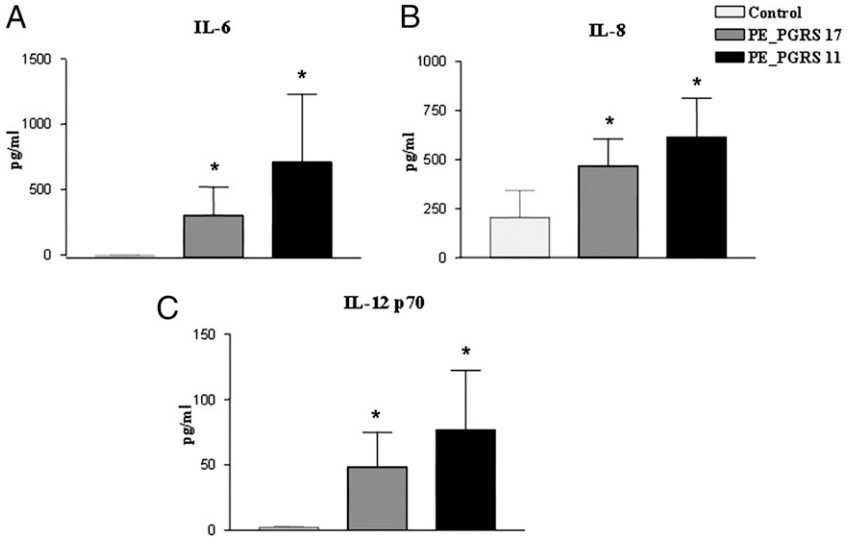

FIGURE 2. PE_PGRS 17 or PE_PGRS 11 triggers DCs to secrete high amounts of proinflammatory cytokines. DCs (0.5×10^6 cells/ml) were cultured with GM-CSF and IL-4 alone (Control) or GM-CSF, IL-4, and 5 μg/ml PE_PGRS 17 or PE_PGRS 11 for 48 h, and secretion of IL-6 (*A*), IL-8 (*B*), and IL-12 (*C*) in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

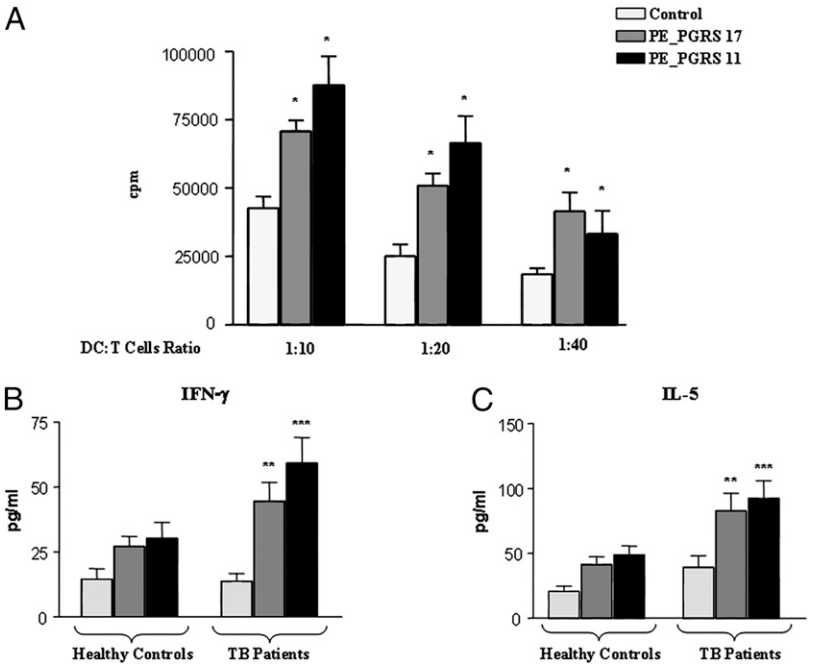

FIGURE 3. PE_PGRS 17 or PE_PGRS 11 triggers strong T cell response. *A*, DCs were cultured with GM-CSF and IL-4 alone (Control) or GM-CSF, IL-4, and 5 μg/ml of PE_PGRS 17 or PE_PGRS 11 for 48 h. Following extensive washings, DCs were cocultured with allogenic $CD4^+$ T cells at various DC:T cell ratios. The T cell proliferation was quantified by $[^3H]$thymidine incorporation, and values were expressed as cpm (mean ± 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 μg/ml PE_PGRS17 or PE_PGRS 11, and cell-free supernatants collected on day 4 were tested for concentrations of secreted IFN-γ (*B*) and IL-5 (*C*). Data are presented as mean ± 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 pulldown 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

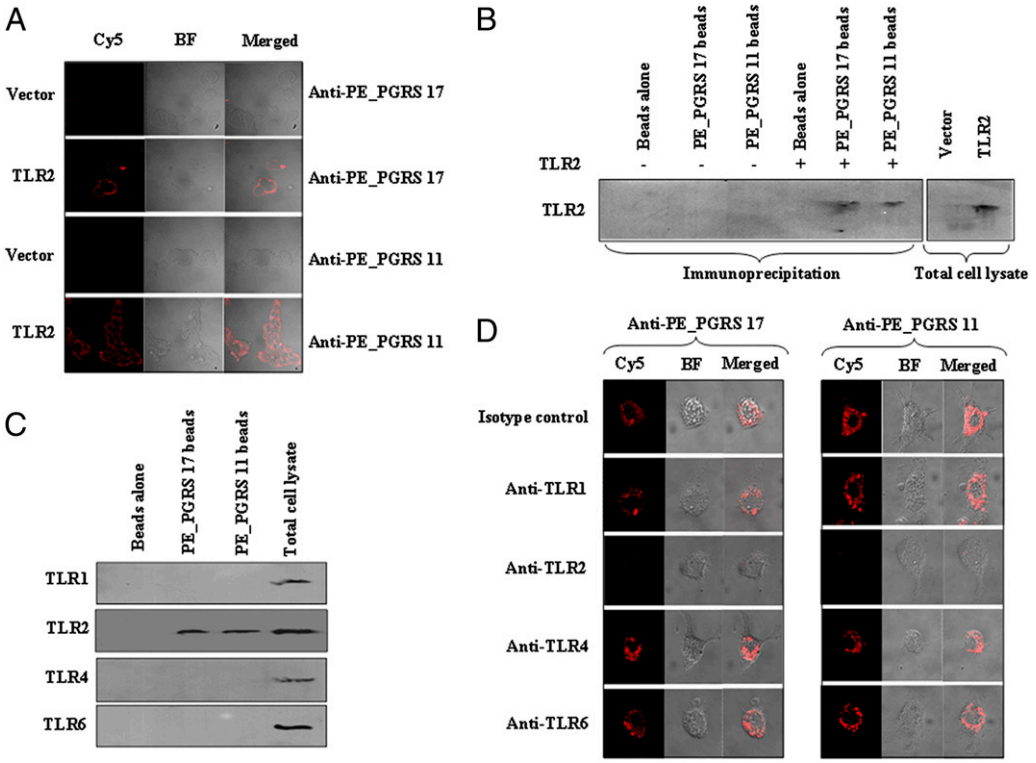

FIGURE 4. PE_PGRS 17 and PE_PGRS 11 proteins interact with TLR2. *A*, TLR2 or vector-transfected HEK-293 cells were treated with PE_PGRS 17 or PE_PGRS 11. The cells were further stained with Ab specific to PE_PGRS 17 or PE_PGRS 11 followed by Cy5-labeled secondary Ab. The immunofluorescent staining of 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.

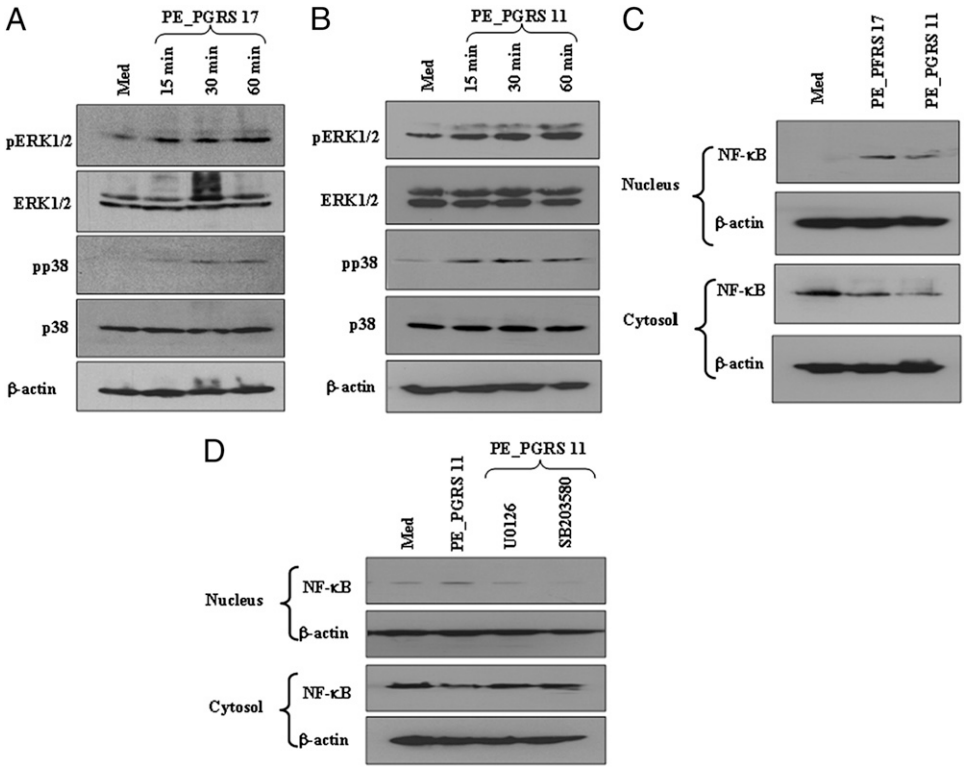

FIGURE 5. Maturation of DCs triggered by PE_PGRS 17 and PE_PGRS 11 involves ERK1/2 and p38 MAPK-dependent activation of NF-κB. DCs were treated with PE_PGRS 17 (*A*) or PE_PGRS 11 (*B*) for indicated time points, and phosphorylation of ERK1/2 (pERK1/2) and p38 MAPK (pp38) was analyzed by immunoblotting. *C*, PE_PGRS 17 and PE_PGRS 11 trigger nuclear translocation of p65 NF-κB. *D*, DCs were pretreated with ERK1/2 inhibitor (U0126, 10 μM) and p38 inhibitor (SB203580, 20 μM) followed by treatment with PE_PGRS 11, and nuclear translocation of p65 NF-κB was analyzed. Data are representative of three independent experiments. Med, medium.

validated by pulldown assay using whole cell extracts from DCs that endogenously express various TLRs. As demonstrated in Fig. 4*C*, 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

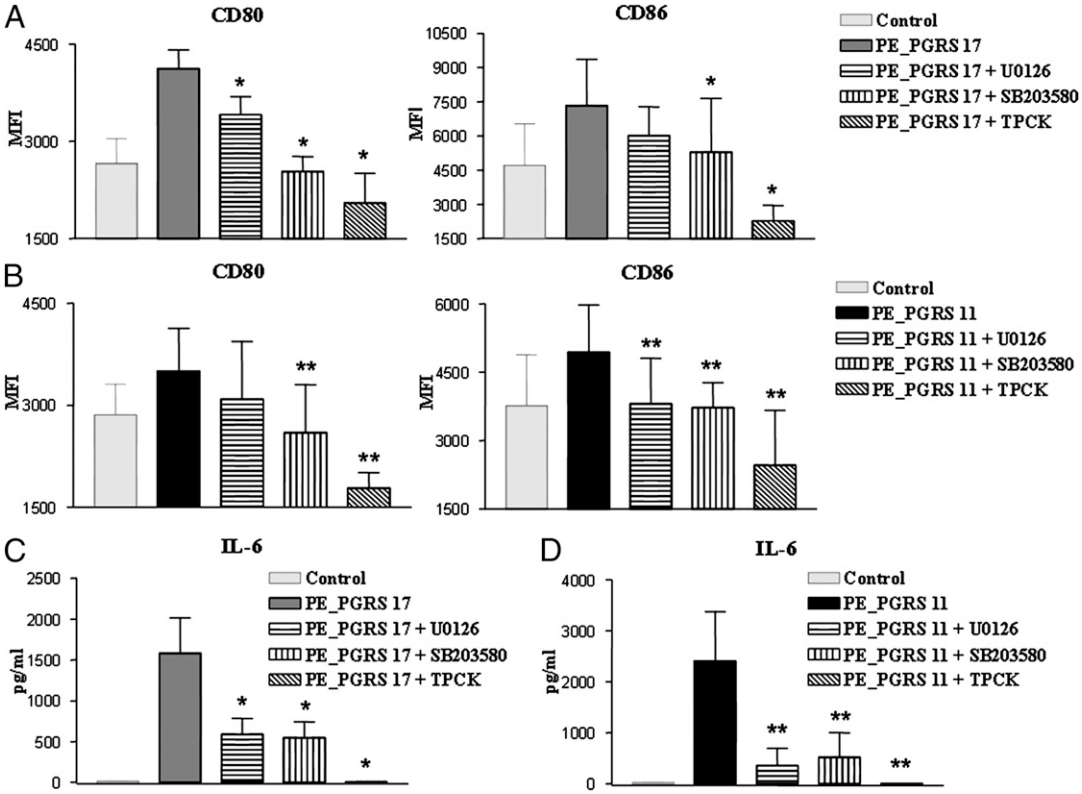

FIGURE 6. Involvement of ERK1/2, p38, and NF-κB pathways in PE_PGRS 17 or PE_PGRS 11-induced maturation of DCs. DCs were treated with pharmacological inhibitors of ERK1/2 (U0126, 10 μM), p38 (SB203580, 20 μM), NF-κB (TPCK, 20 μM), or DMSO (vehicle control) for 1 h prior to treatment with PE_PGRS 17 (*A*) 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 ± 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. 4*D*). Furthermore, the TLR2 dominant-negative construct significantly reduced PE_PGRS 17 or PE_PGRS 11 Ag-triggered NF-κB 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-κB 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-κB 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. 5*A*, 5*B*) and significant translocation of p65 NF-κB from the cytosol to the nucleus within 60 min of stimulation (Fig. 5*C*) 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-κB (Fig. 5*D* 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-κB pathway.

Furthermore, pharmacological inhibitors U0126 (ERK1/2), SB203580 (p38), TPCK (NF-κB) (59, 60), or Bay 11-0782 (NF-κB) (61) abrogated PE_PGRS 17- or PE_PGRS 11-induced expression of costimulatory molecules CD80, CD86, and CD40 (Fig. 6*A*, 6*B* and data not shown) and secretion of inflammatory cytokines (shown with IL-6 as an example) from DCs (Fig. 6*C*, 6*D*). Together, our results thus suggest that NF-κB and MAPK pathways represent important signaling partnership links in PE_PGRS 17- or PE_PGRS 11-triggered maturation and activation of human DCs.

IFN-γ potentiates PE_PGRS 17- or PE_PGRS 11-induced maturation of DCs

Among proinflammatory cytokines, IFN-γ 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-γ–deficient mice exhibit susceptibility to mycobacterial infection (62). It was suggested that the lack of protective immunity in IFN-γ–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-γ 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-γ for 6 h followed by treatment with PE_PGRS 17 or PE_PGRS 11 for 42 h. As represented in Fig. 7*A*, pretreatment with IFN-γ 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-γ priming augmented PE_PGRS 17 or PE_PGRS 11 Ag-mediated secretion of both IL-6 and IL-8 by DCs (Fig. 7*B*).

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

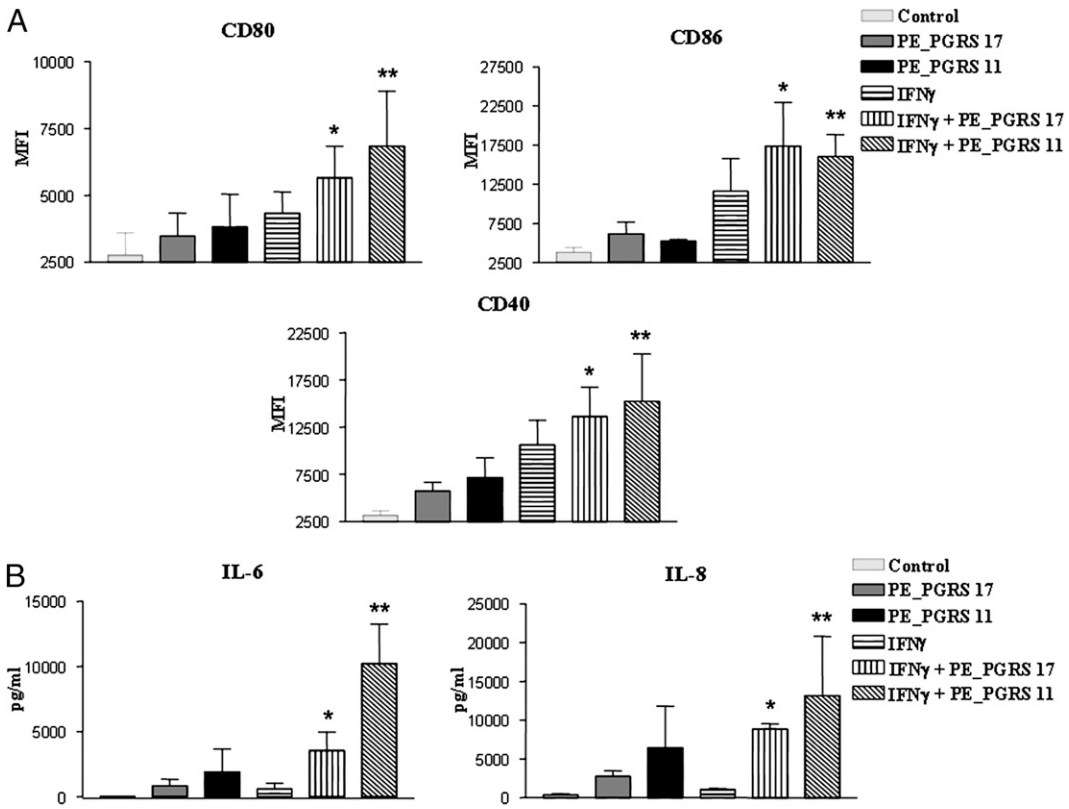

FIGURE 7. IFN-γ treatment augments PE_PGRS 17 and PE_PGRS 11-induced DC maturation. DCs were treated either with PE_PGRS 17 or PE_PGRS 11 alone or with IFN-γ for 48 h, and expression of maturation markers CD80, CD86, and CD40 (*A*) and secretion of IL-6 and IL-8 (*B*) was analyzed. Data are presented as mean ± 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-γ 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 $CD4^+$ 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-κB 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-κB pathways abrogated PE_PGRS Ag-induced maturation of DCs or secretion of inflammatory cytokines. These results implicate signaling integration of TLR2, MAPKs, and NF-κB 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-γ, by PBMCs is shown to be severely compromised in patients with tuberculosis (69). In this regard, augmentation by IFN-γ 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-γ not only enhances the maturation of DCs but also the secretion of proinflammatory cytokines. This observation implicates a crucial role of IFN-γ 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.

References

1. World Health Organization. 2007. Report: Global Tuberculosis Control—Surveillance, Planning, Financing. WHO/HTM/TB/2007.376. World Health Organization, Geneva, Switzerland.
2. Brandt, L., J. Feino Cunha, A. Weinreich Olsen, B. Chilima, P. Hirsch, R. Appelberg, and P. Andersen. 2002. Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect. Immun.* 70: 672–678.
3. Andersen, P., and T. M. Doherty. 2005. The success and failure of BCG - implications for a novel tuberculosis vaccine. *Nat. Rev. Microbiol.* 3: 656–662.
4. Walker, L., and D. B. Lowrie. 1981. Killing of *Mycobacterium microti* by immunologically activated macrophages. *Nature* 293: 69–71.
5. Flesch, I., and S. H. Kaufmann. 1987. Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. *J. Immunol.* 138: 4408–4413.
6. Flesch, I. E., and S. H. Kaufmann. 1990. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. *Infect. Immun.* 58: 2675–2677.
7. Ding, A. H., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141: 2407–2412.
8. Feng, C. G., A. G. Bean, H. Hooi, H. Briscoe, and W. J. Britton. 1999. Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with *Mycobacterium tuberculosis*. *Infect. Immun.* 67: 3242–3247.

9. Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J. Immunol.* 162: 5407–5416.
10. Goren, M. B., P. D'Arcy Hart, M. R. Young, and J. A. Armstrong. 1976. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA* 73: 2510–2514.
11. Chan, J., X. D. Fan, S. W. Hunter, P. J. Brennan, and B. R. Bloom. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infect. Immun.* 59: 1755–1761.
12. Chan, J., T. Fujiwara, P. Brennan, M. McNeil, S. J. Turco, J. C. Sibille, M. Snapper, P. Aisen, and B. R. Bloom. 1989. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc. Natl. Acad. Sci. USA* 86: 2453–2457.
13. Pathak, S. K., A. Bhattacharyya, S. Pathak, C. Basak, D. Mandal, M. Kundu, and J. Basu. 2004. Toll-like receptor 2 and mitogen- and stress-activated kinase 1 are effectors of *Mycobacterium avium*-induced cyclooxygenase-2 expression in macrophages. *J. Biol. Chem.* 279: 55127–55136.
14. Beatty, W. L., and D. G. Russell. 2000. Identification of mycobacterial surface proteins released into subcellular compartments of infected macrophages. *Infect. Immun.* 68: 6997–7002.
15. Andersen, P. 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. *Infect. Immun.* 62: 2536–2544.
16. Roberts, A. D., M. G. Sonnenberg, D. J. Ordway, S. K. Furney, P. J. Brennan, J. T. Belisle, and I. M. Orme. 1995. Characteristics of protective immunity engendered by vaccination of mice with purified culture filtrate protein antigens of *Mycobacterium tuberculosis. Immunology* 85: 502–508.
17. Roche, P. W., J. A. Triccas, D. T. Avery, T. Fifis, H. Billman-Jacobe, and W. J. Britton. 1994. Differential T cell responses to mycobacteria-secreted proteins distinguish vaccination with bacille Calmette-Guérin from infection with *Mycobacterium tuberculosis. J. Infect. Dis.* 170: 1326–1330.
18. Münz, C., R. M. Steinman, and S. Fujii. 2005. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J. Exp. Med.* 202: 203–207.
19. Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat. Rev. Immunol.* 6: 476–483.
20. Reis e Sousa, C. 2001. Dendritic cells as sensors of infection. *Immunity* 14: 495–498.
21. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2: 151–161.
22. Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat. Rev. Immunol.* 7: 543–555.
23. Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, et al. 1998. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* 188: 2163–2173.
24. Henderson, R. A., S. C. Watkins, and J. L. Flynn. 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis. J. Immunol.* 159: 635–643.
25. Giacomini, E., E. Iona, L. Ferroni, M. Miettinen, L. Fattorini, G. Orefici, I. Julkunen, and E. M. Coccia. 2001. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J. Immunol.* 166: 7033–7041.
26. Jang, S., S. Uematsu, S. Akira, and P. Salgame. 2004. IL-6 and IL-10 induction from dendritic cells in response to *Mycobacterium tuberculosis* is predominantly dependent on TLR2-mediated recognition. *J. Immunol.* 173: 3392–3397.
27. Bodnar, K. A., N. V. Serbina, and J. L. Flynn. 2001. Fate of *Mycobacterium tuberculosis* within murine dendritic cells. *Infect. Immun.* 69: 800–809.
28. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393: 537–544.
29. Brennan, M. J., and G. Delogu. 2002. The PE multigene family: a 'molecular mantra' for mycobacteria. *Trends Microbiol.* 10: 246–249.
30. Brennan, M. J., G. Delogu, Y. Chen, S. Bardarov, J. Kriakov, M. Alavi, and W. R. Jacobs, Jr. 2001. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. *Infect. Immun.* 69: 7326–7333.
31. Dheenadhayalan, V., G. Delogu, and M. J. Brennan. 2006. Expression of the PE_PGRS 33 protein in *Mycobacterium smegmatis* triggers necrosis in macrophages and enhanced mycobacterial survival. *Microbes Infect.* 8: 262–272.
32. Singh, K. K., X. Zhang, A. S. Patibandla, P. Chien, Jr., and S. Laal. 2001. Antigens of *Mycobacterium tuberculosis* expressed during preclinical tuberculosis: serological immunodominance of proteins with repetitive amino acid sequences. *Infect. Immun.* 69: 4185–4191.
33. Narayana, Y., B. Joshi, V. M. Katoch, K. C. Mishra, and K. N. Balaji. 2007. Differential B-cell responses are induced by *Mycobacterium tuberculosis* PE antigens Rv1169c, Rv0978c, and Rv1818c. *Clin. Vaccine Immunol.* 14: 1334–1341.
34. Singh, K. K., Y. Dong, S. A. Patibandla, D. N. McMurray, V. K. Arora, and S. Laal. 2005. Immunogenicity of the *Mycobacterium tuberculosis* PPE55 (Rv3347c) protein during incipient and clinical tuberculosis. *Infect. Immun.* 73: 5004–5014.
35. Choudhary, R. K., S. Mukhopadhyay, P. Chakhaiyar, N. Sharma, K. J. Murthy, V. M. Katoch, and S. E. Hasnain. 2003. PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. *Infect. Immun.* 71: 6338–6343.
36. Le Moigne, V., G. Robreau, C. Borot, J. L. Guesdon, and W. Mahana. 2005. Expression, immunochemical characterization and localization of the *Mycobacterium tuberculosis* protein p27. *Tuberculosis (Edinb.)* 85: 213–219.
37. Beatty, W. L., H. J. Ullrich, and D. G. Russell. 2001. Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. *Eur. J. Cell Biol.* 80: 31–40.
38. Koh, K. W., N. Lehming, and G. T. Seah. 2009. Degradation-resistant protein domains limit host cell processing and immune detection of mycobacteria. *Mol. Immunol.* 46: 1312–1318.
39. Schnappinger, D., S. Ehrt, M. I. Voskuil, Y. Liu, J. A. Mangan, I. M. Monahan, G. Dolganov, B. Efron, P. D. Butcher, C. Nathan, and G. K. Schoolnik. 2003. Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages: Insights into the Phagosomal Environment. *J. Exp. Med.* 198: 693–704.
40. Talaat, A. M., R. Lyons, S. T. Howard, and S. A. Johnston. 2004. The temporal expression profile of *Mycobacterium tuberculosis* infection in mice. *Proc. Natl. Acad. Sci. USA* 101: 4602–4607.
41. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7: 179–190.
42. Pecora, N. D., A. J. Gehring, D. H. Canaday, W. H. Boom, and C. V. Harding. 2006. *Mycobacterium tuberculosis* LprA is a lipoprotein agonist of TLR2 that regulates innate immunity and APC function. *J. Immunol.* 177: 422–429.
43. Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis. J. Immunol.* 163: 3920–3927.
44. Almeida, P. E., A. R. Silva, C. M. Maya-Monteiro, D. Töröcsik, H. D'Avila, B. Dezsö, K. G. Magalhães, H. C. Castro-Faria-Neto, L. Nagy, and P. T. Bozza. 2009. *Mycobacterium bovis* bacillus Calmette-Guérin infection induces TLR2-dependent peroxisome proliferator-activated receptor gamma expression and activation: functions in inflammation, lipid metabolism, and pathogenesis. *J. Immunol.* 183: 1337–1345.
45. Kincaid, E. Z., A. J. Wolf, L. Desvignes, S. Mahapatra, D. C. Crick, P. J. Brennan, M. S. Pavelka, Jr., and J. D. Ernst. 2007. Codominance of TLR2-dependent and TLR2-independent modulation of MHC class II in *Mycobacterium tuberculosis* infection in vivo. *J. Immunol.* 179: 3187–3195.
46. Nigou, J., T. Vasselon, A. Ray, P. Constant, M. Gilleron, G. S. Besra, I. Sutcliffe, G. Tiraby, and G. Puzo. 2008. Mannan chain length controls lipoglycans signaling via and binding to TLR2. *J. Immunol.* 180: 6696–6702.
47. Ferwerda, G., B. J. Kullberg, D. J. de Jong, S. E. Girardin, D. M. Langenberg, R. van Crevel, T. H. Ottenhoff, J. W. Van der Meer, and M. G. Netea. 2007. *Mycobacterium paratuberculosis* is recognized by Toll-like receptors and NOD2. *J. Leukoc. Biol.* 82: 1011–1018.
48. Ryffel, B., C. Fremond, M. Jacobs, S. Parida, T. Botha, B. Schnyder, and V. Quesniaux. 2005. Innate immunity to mycobacterial infection in mice: critical role for toll-like receptors. *Tuberculosis (Edinb.)* 85: 395–405.
49. Tapping, R. I., and P. S. Tobias. 2003. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *J. Endotoxin Res.* 9: 264–268.
50. Farhat, K., S. Riekenberg, H. Heine, J. Debarry, R. Lang, J. Mages, U. Buwitt-Beckmann, K. Röschmann, G. Jung, K. H. Wiesmüller, and A. J. Ulmer. 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J. Leukoc. Biol.* 83: 692–701.
51. Nakao, Y., K. Funami, S. Kikkawa, M. Taniguchi, M. Nishiguchi, Y. Fukumori, T. Seya, and M. Matsumoto. 2005. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. *J. Immunol.* 174: 1566–1573.
52. Nair, S., P. A. Ramaswamy, S. Ghosh, D. C. Joshi, N. Pathak, I. Siddiqui, P. Sharma, S. E. Hasnain, S. C. Mande, and S. Mukhopadhyay. 2009. The PPE18 of *Mycobacterium tuberculosis* interacts with TLR2 and activates IL-10 induction in macrophage. *J. Immunol.* 183: 6269–6281.
53. Schorey, J. S., and A. M. Cooper. 2003. Macrophage signalling upon mycobacterial infection: the MAP kinases lead the way. *Cell. Microbiol.* 5: 133–142.
54. Boislève, F., S. Kerdine-Römer, and M. Pallardy. 2005. Implication of the MAPK pathways in the maturation of human dendritic cells induced by nickel and TNF-alpha. *Toxicology* 206: 233–244.
55. Nakahara, T., Y. Moroi, H. Uchi, and M. Furue. 2006. Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. *J. Dermatol. Sci.* 42: 1–11.
56. Dowling, D., C. M. Hamilton, and S. M. O'Neill. 2008. A comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with LPS compared with a panel of TLR ligands. *Cytokine* 41: 254–262.
57. Pathak, S. K., S. Basu, K. K. Basu, A. Banerjee, S. Pathak, A. Bhattacharyya, T. Kaisho, M. Kundu, and J. Basu. 2007. Direct extracellular interaction between the early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* and TLR2 inhibits TLR signaling in macrophages. *Nat. Immunol.* 8: 610–618.
58. Binder, R. J. 2009. CD40-independent engagement of mammalian hsp70 by antigen-presenting cells. *J. Immunol.* 182: 6844–6850.
59. Rescigno, M., M. Martino, C. L. Sutherland, M. R. Gold, and P. Ricciardi-Castagnoli. 1998. Dendritic cell survival and maturation are regulated by different signaling pathways. *J. Exp. Med.* 188: 2175–2180.
60. Valentinis, B., A. Bianchi, D. Zhou, A. Cipponi, F. Catalanotti, V. Russo, and C. Traversari. 2005. Direct effects of polymyxin B on human dendritic cells maturation. The role of IkappaB-alpha/NF-kappaB and ERK1/2 pathways and adhesion. *J. Biol. Chem.* 280: 14264–14271.
61. Bansal, K., N. Kapoor, Y. Narayana, G. Puzo, M. Gilleron, and K. N. Balaji. 2009. PIM2 Induced COX-2 and MMP-9 expression in macrophages requires PI3K and Notch1 signaling. *PLoS One* 4: e4911.
62. Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* 178: 2243–2247.

63. Tundup, S., N. Pathak, M. Ramanadham, S. Mukhopadhyay, K. J. Murthy, N. Z. Ehtesham, and S. E. Hasnain. 2008. The co-operonic PE25/PPE41 protein complex of *Mycobacterium tuberculosis* elicits increased humoral and cell mediated immune response. *PLoS One* 3: e3586.
64. Rhoades, E., F. Hsu, J. B. Torrelles, J. Turk, D. Chatterjee, and D. G. Russell. 2003. Identification and macrophage-activating activity of glycolipids released from intracellular *Mycobacterium bovis* BCG. *Mol. Microbiol.* 48: 875–888.
65. Balaji, K. N., G. Goyal, Y. Narayana, M. Srinivas, R. Chaturvedi, and S. Mohammad. 2007. Apoptosis triggered by Rv1818c, a PE family gene from *Mycobacterium tuberculosis* is regulated by mitochondrial intermediates in T cells. *Microbes Infect.* 9: 271–281.
66. Jain, S. K., M. Paul-Satyaseela, G. Lamichhane, K. S. Kim, and W. R. Bishai. 2006. *Mycobacterium tuberculosis* invasion and traversal across an in vitro human blood-brain barrier as a pathogenic mechanism for central nervous system tuberculosis. *J. Infect. Dis.* 193: 1287–1295.
67. Srivastava, V., A. Jain, B. S. Srivastava, and R. Srivastava. 2008. Selection of genes of *Mycobacterium tuberculosis* upregulated during residence in lungs of infected mice. *Tuberculosis (Edinb.)* 88: 171–177.
68. Latchumanan, V. K., B. Singh, P. Sharma, and K. Natarajan. 2002. *Mycobacterium tuberculosis* antigens induce the differentiation of dendritic cells from bone marrow. *J. Immunol.* 169: 6856–6864.
69. Vilcek, J., A. Klion, D. Henriksen-DeStefano, A. Zemtsov, D. M. Davidson, M. Davidson, A. E. Friedman-Kien, and J. Le. 1986. Defective gamma-interferon production in peripheral blood leukocytes of patients with acute tuberculosis. *J. Clin. Immunol.* 6: 146–151.