

Modulation of Gamma Interferon Receptor 1 by *Mycobacterium tuberculosis*: a Potential Immune Response Evasive Mechanism[∇]

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***Mycobacterium tuberculosis* inhibits gamma interferon (IFN- γ)-mediated antimycobacterial action by adopting diverse mechanisms. IFN- γ binds to its receptor, IFN- γ R, in order to initiate proper signaling. We have observed reduced surface expression levels of IFN- γ receptor 1 (IFN- γ R1) in untreated pulmonary tuberculosis patients compared to those in healthy individuals ($P < 0.01$). Following antitubercular therapy, the expression of IFN- γ R1 was restored in these patients. To delineate the mechanism by which *M. tuberculosis* modulates IFN- γ R1, in vitro experiments were designed, wherein the down modulation of IFN- γ R1 surface expression was observed for CD14⁺ cells in peripheral blood mononuclear cells (PBMCs) cocultured with live *M. tuberculosis* compared to that for uninfected cells ($P < 0.01$). No modulation of IFN- γ R1 expression was observed for CD14⁺ cells in PBMCs infected with *Mycobacterium smegmatis*. A time-dependent decrease in IFN- γ R1 mRNA expression was observed for PBMCs infected with *M. tuberculosis*. Similar down modulation of IFN- γ R1 protein and mRNA expression in phorbol myristate acetate-differentiated THP-1 cells (pdTHP-1) by *M. tuberculosis* was observed ($P < 0.01$). Using reporter gene analysis of 5' deletion constructs of the IFN- γ R1 gene (*IFNGR1*) promoter, the decrease in IFN- γ R1 mRNA in *M. tuberculosis*-infected pdTHP-1 cells was shown to be due to the decreased transcription of *IFNGR1*. By immunoblotting and electrophoretic mobility shift assays, the down regulation of stimulating protein 1 (Sp1) expression and its recruitment on the phorbol ester-responsive element of the *IFNGR1* promoter in *M. tuberculosis*-infected pdTHP-1 cells was observed. This down regulation of Sp1 in pdTHP-1 cells cocultured with *M. tuberculosis* may be responsible for the down regulation of IFN- γ R1 expression, thereby potentially altering its receptivity to IFN- γ .**

Mycobacterium tuberculosis is a facultative intracellular pathogen that resides and multiplies within human macrophages. Gamma interferon (IFN- γ), the predominant inducer of macrophage-mediated microbicidal functions (36), has been shown to be required for the prevention of progressive *M. tuberculosis* infection (13, 19). In tuberculosis patients, IFN- γ has been detected in CD4⁺ T cells and culture supernatants (8) as well as in infectious foci (7). Despite the availability of IFN- γ , the immune system is unable to eradicate the infection, indicating that *M. tuberculosis* selectively inhibits macrophage responsiveness to IFN- γ (56, 61). This reduced response results in the inefficient induction of IFN- γ -inducible genes, such as major histocompatibility complex class II and others (24, 61).

IFN- γ binds to its cell surface receptor, IFN- γ R, which consists of two heterodimeric subunits, IFN- γ R1 (α , ligand binding) and IFN- γ R2 (β , signaling subunit) (6). The IFN- γ R is expressed on lymphoid cells (such as monocytes/macrophages, T, B, and NK cells) and nonlymphoid cells (such as fibroblasts and endothelial cells) (62). Various infections, such as *Leishmania donovani* (43), *Trypanosoma cruzi* (23), and *Mycobacterium avium* (22), have been shown to down modulate IFN- γ R1 expression. In addition, individuals with mutations in IFN- γ R

genes have been shown to be susceptible to mycobacterial infections (reviewed in reference 17). This provides convincing evidence that the functional integrity of this receptor is crucial in the defense against mycobacterial infection in humans.

The aim of this study was to investigate the cellular mechanism by which *M. tuberculosis* modulates the expression of IFN- γ R1. Our results indicate that *M. tuberculosis* down regulates the expression of IFN- γ R1 in human cells, such as peripheral blood mononuclear cells (PBMCs), CD14⁺ cells, and phorbol myristate acetate (PMA)-differentiated THP-1 cells (pdTHP-1). Further, *M. tuberculosis* down regulates the transcription of *IFNGR1* by inhibiting the expression of stimulating protein 1 (Sp1) in pdTHP-1 cells. The binding and interaction of IFN- γ with its heterodimeric receptor mediate the signaling cascade, which in due course leads to the expression of IFN- γ -responsive genes (1). The inability of infected cells to respond to IFN- γ , owing to the down modulation of IFN- γ R1 expression, leads to the survival and persistence of *M. tuberculosis* in the infected host.

MATERIALS AND METHODS

Subjects. Informed consent obtained from a cohort of 13 pulmonary tuberculosis (PTB) patients of the outpatient department of LRS Hospital of Tuberculosis and Respiratory Diseases and 16 laboratory personnel of the Biotechnology Department, AIIMS, New Delhi, India, were included in the study. All patients were administered standard antitubercular therapy (ATT). Six of them were available for the assessment of the level of expression of IFN- γ R1 at various time points prior to and after therapy. Scrutinizing patient clinical histories, physical examinations, and laboratory investigations ruled out the occurrence of concom-

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itant intracellular infections in the individuals enrolled. The study was approved by the institutional ethical committee.

Reagents. The following reagents were procured—anti-human IFN- γ R1-phycoerythrin, anti-human leukocyte antigen DR alpha (HLA-DR α)-fluorescein isothiocyanate (FITC), and anti-CD14-FITC monoclonal antibodies (Abs); recombinant human IFN- γ (Pharmingen); anti-Sp1 Ab (eBioscience); reverse transcription-PCR (RT-PCR) reagents, DNase and *Taq* DNA polymerase (MBI Fermentas); protease inhibitor (Roche Diagnostics); anti-mouse-horseradish peroxidase Ab (Bangalore Genei); Trizol, RPMI 1640, and fetal calf serum (FCS) (Invitrogen); pGL3 basic luciferase vector (Promega); DEAE-dextran and Ficoll-Hypaque (Sigma); and PMA, luciferase estimation kit, and SYBR Premix Ex *Taq* (Clontech).

Mycobacterium cultivation. *M. tuberculosis* H37Rv was grown in 7H9 supplemented with 10% albumin-dextrose complex and 0.1% Tween 80, with shaking at 200 rpm at 37°C until an optical density at 590 nm of 0.6 to 0.8 was reached. The culture was vortexed along with 3-mm glass beads and allowed to stand for 20 min. The single-cell suspension was retrieved from the top and used in infection experiments. Similarly, *Mycobacterium smegmatis* mc² was propagated in Luria-Bertani (LB) supplemented with 10% glycerol, and a single-cell suspension was prepared.

Isolation of human PBMCs and infection with mycobacteria. PBMCs were isolated from patients and healthy donors by Ficoll-Hypaque gradient centrifugation as described previously (8). Isolated PBMCs were washed with RPMI, resuspended in RPMI supplemented with 10% autologous serum, and maintained for 30 min at 37°C in 5% CO₂ before infection. PBMCs from healthy donors were cocultured with *M. tuberculosis*/M. *smegmatis* mc² (multiplicity of infection [MOI] = 1:1) for various time points as indicated. After infection, PBMCs were harvested and stained for IFN- γ R1 and CD14/processed for RT-PCR analysis. Uninfected PBMCs were included as controls.

THP-1 cell culture, M. tuberculosis infection, and IFN- γ stimulation. THP-1 cells (NCCS, Pune, India) were maintained in RPMI supplemented with 10% FCS and 100 U/ml penicillin G and differentiated by PMA (20 ng/1 \times 10⁶ cells/ml) for 24 h (30). pdTHP-1 cells were cocultured with live *M. tuberculosis* (MOI = 1:1)/whole-cell lysate of *M. tuberculosis* (WCL; TB research material, NIH). Cells were washed and incubated with IFN- γ (100 U/ml). After 24 h, cells were harvested and processed for flow cytometric/RT-PCR analysis/nuclear extract preparation along with controls.

Staining of PBMCs and THP-1 cells and flow cytometric analysis. PBMCs (infected and uninfected and/or for basal-level expression) suspended in staining buffer (phosphate-buffered saline with 1% FCS) were incubated for 45 min on wet ice with anti-IFN- γ R1 and anti-CD14 Abs to stain surface IFN- γ R1 and CD14. THP-1 cells were similarly stained for surface CD14, HLA-DR α , and IFN- γ R1 expression. For the staining of total IFN- γ R1 expression in THP-1 cells, cells were first permeabilized and then incubated with anti-IFN- γ R1 Ab. Stained cells were washed and fixed overnight in 1% paraformaldehyde. A total of 20,000 cells were acquired per sample for flow cytometric analysis (Becton Dickinson; courtesy of the NII, New Delhi, India). The data were analyzed using CellQuest software.

Real-time RT-PCR for IFN- γ R1 expression. Real-time RT-PCR for IFN- γ R1 expression was performed on cDNA samples as described previously (35). For the amplification of IFN- γ R1 and housekeeping (β -actin) genes, the following primers were used: IFN- γ R1-forward (5' CATCAGTCATACAGCCATTT 3'), IFN- γ R1-reverse (5' CTGGATTGTCTTCGGTATGCAT 3'), β -actin-forward (5' TTGTTACAGGAAGTCCCTTGCC 3'), and β -actin-reverse (5' ATG CTATCACCTCCCCTGTGTG 3'). Briefly 2 μ g of DNase-treated RNA was used to prepare cDNA. Duplicate reactions were set up using SYBR Premix Ex *Taq*, and 0.5 μ M of each pair of primers and samples (2.5 μ l of cDNA) was subjected to thermal cycling on a Bio-Rad MyIQ under the following conditions: 95°C for 30 s for stage 1 and 95°C for 15 s, 58°C for 20 s, and 72°C for 40 s for stage 2. Stage 2 was repeated for 35 cycles. Relative quantification of IFN- γ R1 for each sample used was performed using the standard curve method and was normalized to the level of the endogenous control, β -actin. This was done to account for variability in the (i) initial concentration and quality of total RNA and (ii) conversion efficiency of the reverse transcription reaction. For the quantification of IFN- γ R1 transcripts normalized to β -actin, standard curves were prepared for both IFN- γ R1 and β -actin. For each experimental sample, the amounts of both IFN- γ R1 and β -actin were determined from the standard curve. The normalized values have been presented as ratios of IFN- γ R1 to β -actin.

Semiquantitative RT-PCR of HLA-DR α expression. RNA isolated from THP-1 cells and PBMCs was treated with DNase, and 2 μ g was used to prepare cDNA. To the 25- μ l reaction mixture (0.2 mM each of dATP, dTTP, dCTP, and dGTP; 0.4 μ M of each pair of primers), 1 U of *Taq* DNA polymerase and 2.5 μ l of 10 \times buffer and cDNA were added. The reaction mixture was subjected to an

initial denaturation at 94°C for 5 min and 35 cycles each of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. The annealing temperature for *GAPDH* was 60°C, and amplification was for 30 cycles. *GAPDH* gene expression was used to normalize the cDNA used. PCR products were separated by agarose gel (2%) electrophoresis and visualized by ethidium bromide staining. The primer sequences and the expected sizes of the amplified products were as follows: 5' CGAGTCTATCTGAATCCTGACCA 3' (forward), 5' GTTCTGCTGCATTGCTTTTGCGCA 3' (reverse), and 693 bp for HLA-DR α and 5' TGAAGTCTGGAGTCAACGGAT 3' (forward), 5' CGGT GCCATGGAATTTGCCA 3' (reverse), and 167 bp for glyceraldehyde-3-phosphate dehydrogenase.

Plasmid construction. Luciferase reporters containing the 5' deletion constructs of the human *IFNGR1* promoter (GenBank accession number U19241) in a pGL3 basic luciferase vector were constructed as described previously (49). pGLR1A, pGLR1B, and pGLR1C have *IFNGR1* promoters from -840 to +1 bp, -128 to +1 bp, and -109 to +1 bp, respectively.

Transient transfection of THP-1 cells. The DEAE-dextran method was used to transfect THP-1 cells with modifications (65). A total of 1 \times 10⁷ cells were washed with TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 7 mM CaCl₂, and 5 mM MgCl₂) and resuspended in 1 ml of TBS containing 5 μ g of reporter plasmid DNA and 500 μ g DEAE-dextran. The cells were incubated for 30 min at room temperature, washed with RPMI, resuspended in 12 ml of RPMI with 10% FCS, and plated. The cells were allowed to stabilize for 6 h. Following this, cells were cocultured with live *M. tuberculosis* (MOI = 1:1) or WCL (500 μ g) for an additional time period of 36 h. PMA (20 ng/ml) was added 21 h posttransfection. All cultures were terminated at 42 h. Lysates were prepared and filtered (0.45 μ m) to remove *M. tuberculosis*. The luciferase activity was estimated, and the results are expressed as the relative intensity of light generated by luciferase (RLU) per microgram of protein of the lysate. The protein concentration was estimated by the Bradford method (9).

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA). Nuclear protein extracts were prepared from infected and uninfected cells (27). Extracts prepared were filtered (0.45 μ m) to remove *M. tuberculosis*. A synthetic double-stranded oligonucleotide containing the sequence of the phorbol ester-responsive element (PRE) of the *IFNGR1* promoter (5'-GGTCCCCTCCTGCCGA-3') was end labeled with [γ -³²P]ATP by using T4 polynucleotide kinase (49). Each 20- μ l reaction mixture contained 20,000 cpm of labeled PRE, 1.5 to 2.0 μ g of nuclear extracts, and 0.25 μ g of poly(dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 5 mM NaCl, 10 mM MgCl₂, 5 mM CaCl₂, 5% glycerol, and 1 mM EDTA). The mixture was incubated at 4°C for 30 min and electrophoresed on a 6% polyacrylamide gel (0.5 \times Tris-borate-EDTA; 100 V for 1 h). The DNA-protein complex was visualized by autoradiography. For competitive assays, a 100-fold excess of unlabeled double-stranded PRE and anti-Sp1 Ab was used. Densitometric analysis was performed using Labworks software (UVV, United Kingdom).

Immunoblot analysis of Sp1. Nuclear proteins (15 μ g) separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were electroblotted onto nitrocellulose membranes. The electroblots were blocked, probed with murine polyclonal anti-human Sp1 Ab, washed (phosphate-buffered saline-Tween 20), and incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G. The bound Ab was visualized by ECL (Amersham, IL). Densitometric analysis was performed as described above.

Statistical analysis. Nonparametric Mann-Whitney test, two-tailed, unpaired Student's *t* test, and one-way analysis of variance (ANOVA) with Tukey's test were used to analyze the data.

RESULTS

Decreased surface expression of IFN- γ R1 in PBMCs and CD14⁺ cells of PTB patients compared to that in healthy controls. Flow cytometric estimation of the basal surface level expression of IFN- γ R1 on PBMCs isolated from PTB patients and healthy individuals was performed. Acquired cells were gated for mononuclear cells, and the mean fluorescence intensity (MFI) of IFN- γ R1⁺ cells was calculated. The representative MFI profiles (histogram) seen in a patient and a healthy control are depicted in Fig. 1A. It was observed that the MFI for surface expression of IFN- γ R1 was lower in patient-derived PBMCs (3.9) than in PBMCs from the healthy control (9.10) (Fig. 1A). The cumulative MFI for the surface expression of

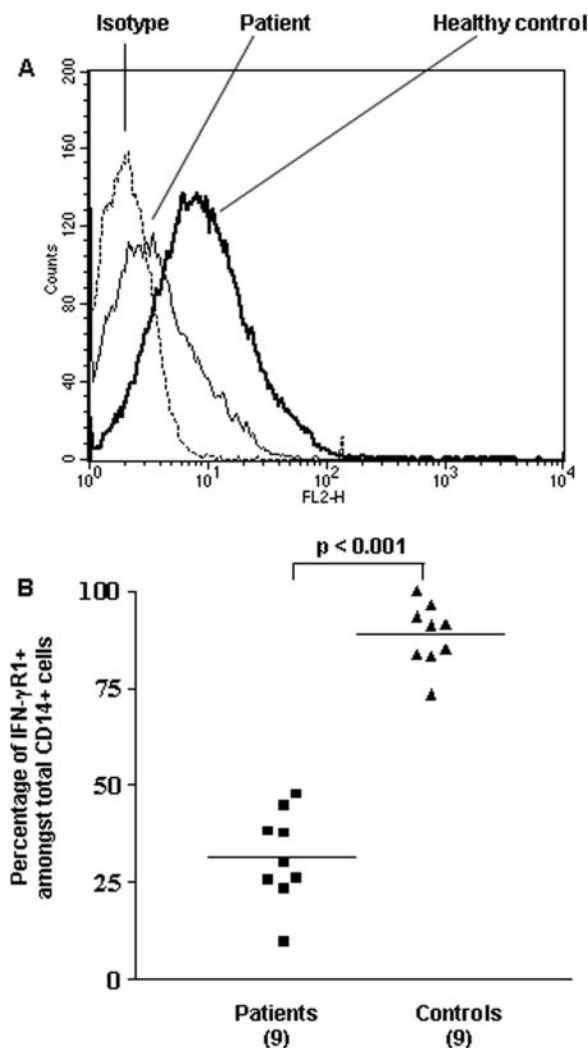


FIG. 1. Decreased surface expression of IFN- γ R1 on PBMCs and CD14⁺ cells of PTB patients. PBMCs were isolated from peripheral blood of patients and healthy controls and stained with phycoerythrin-conjugated anti-IFN- γ R1 Ab and FITC-conjugated anti-CD14 Ab. A total of 20,000 cells were analyzed by flow cytometry. (A) Representative histogram plot indicating IFN- γ R1⁺ cells in PBMCs of a patient and a healthy individual. (B) Histogram indicating a significant difference in the percentage of CD14⁺ IFN- γ R1⁺ cells in PBMCs from PTB patients ($n = 13$) and controls ($n = 10$). $P < 0.01$ by the Mann-Whitney U test. Bars represent the means.

IFN- γ R1 on PBMCs from patients ($n = 13$) was 4.6 ± 1.1 , compared to that of PBMCs from controls ($n = 9$; 8.9 ± 0.6) ($P < 0.01$). IFN- γ R1 surface expression on CD14⁺ cells was also estimated. It was observed that the mean percentage \pm standard deviation (SD) of CD14⁺ cells expressing IFN- γ R1 in patient-derived PBMCs ($n = 9$; 31.4 ± 11.9) was significantly lower than that of PBMCs from healthy controls ($n = 9$; 88.7 ± 8.1 ; $P < 0.001$) (Fig. 1B).

Recovery of IFN- γ R1 surface expression on PBMCs of PTB patients upon ATT. For this part of the study, a cohort of six PTB patients was assessed for the surface expression of IFN- γ R1 on PBMCs prior to the initiation of ATT and at 2 and 4 months, respectively. Prior to ATT, the MFI of surface expression of

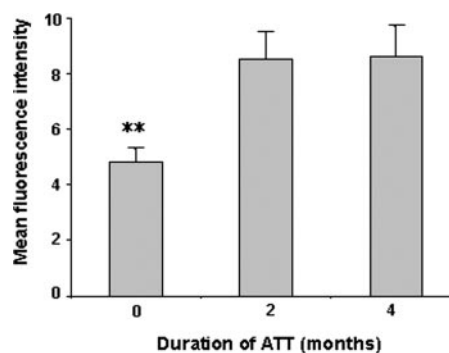


FIG. 2. Effect of ATT on surface expression of IFN- γ R1. PBMCs of PTB patients ($n = 6$) were isolated prior to and after ATT treatment for 2 and 4 months, respectively. The cells were stained for IFN- γ R1 and processed for flow cytometric analysis. A total of 20,000 cells were analyzed per sample. Each bar represents the mean percent \pm SD (error bars) (IFN- γ R1 expression at 0 versus 2 months of ATT). **, P was < 0.01 by nonparametric paired ANOVA.

IFN- γ R1 on PBMCs was 4.8 ± 0.5 (Fig. 2). After 2 months of ATT, a significant rise in the MFI of IFN- γ R1 surface expression was observed (8.5 ± 1.0 ; $P < 0.01$) in treated patients. The MFI of IFN- γ R1 surface expression in PBMCs remained at this level after 4 months of therapy (8.6 ± 1.2).

***M. tuberculosis* down modulates IFN- γ R1 surface expression on CD14⁺ cells.** To assess whether *M. tuberculosis* modulates the surface expression of IFN- γ R1 in vitro, PBMCs were cocultured with live *M. tuberculosis* for varying time periods (1 to 18 h) and the surface expression of IFN- γ R1 on CD14⁺ cells was monitored using flow cytometry. In all, PBMCs derived from three different individuals were investigated. A representative series of dot plots of PBMCs derived from a single individual exposed to *M. tuberculosis* and unexposed controls (2 and 8 h) and stained for IFN- γ R1 and CD14 is depicted in Fig. 3A. The percentage of CD14⁺ cells expressing IFN- γ R1 (Fig. 3A, upper right quadrant) among the total CD14⁺ population (upper and lower right quadrants) upon *M. tuberculosis* infection was 86.3% [$(4.4/5.1) \times 100$] and 20.0% [$(0.5/2.5) \times 100$] at 2 and 8 h, respectively. In uninfected PBMCs, the percentage of CD14⁺ cells expressing IFN- γ R1 among the total CD14⁺ population was 92.5% [$(6.2/6.7) \times 100$] and 76.6% [$(2.3/3.0) \times 100$] at 2 and 8 h, respectively. The down modulation of IFN- γ R1 surface expression was also observed for the CD14-positive population upon *M. tuberculosis* infection (31.3 and 25.5% at 2 and 8 h, respectively) relative to uninfected controls (35.7 and 38.6% at 2 and 8 h, respectively) (Fig. 3A).

The cumulative mean percentages \pm SD of IFN- γ R1-expressing cells among CD14⁺ cells in *M. tuberculosis*-infected and uninfected PBMCs are shown in Fig. 3B. After 8 and 18 h of exposure, ~ 3.4 - and 3.5 -fold decreases in the percentage of IFN- γ R1⁺ cells among CD14⁺ cells were observed for *M. tuberculosis*-infected PBMCs compared to that for uninfected cells ($P < 0.01$). Down modulation in the surface expression of IFN- γ R1 among CD14⁺ cells in uninfected PBMCs was observed for up to 4 h and subsequently remained unaltered.

***M. tuberculosis* down modulates IFN- γ R1 mRNA expression.** As flow cytometric analysis revealed that *M. tuberculosis* down modulates IFN- γ R1 surface protein expression, the mRNA

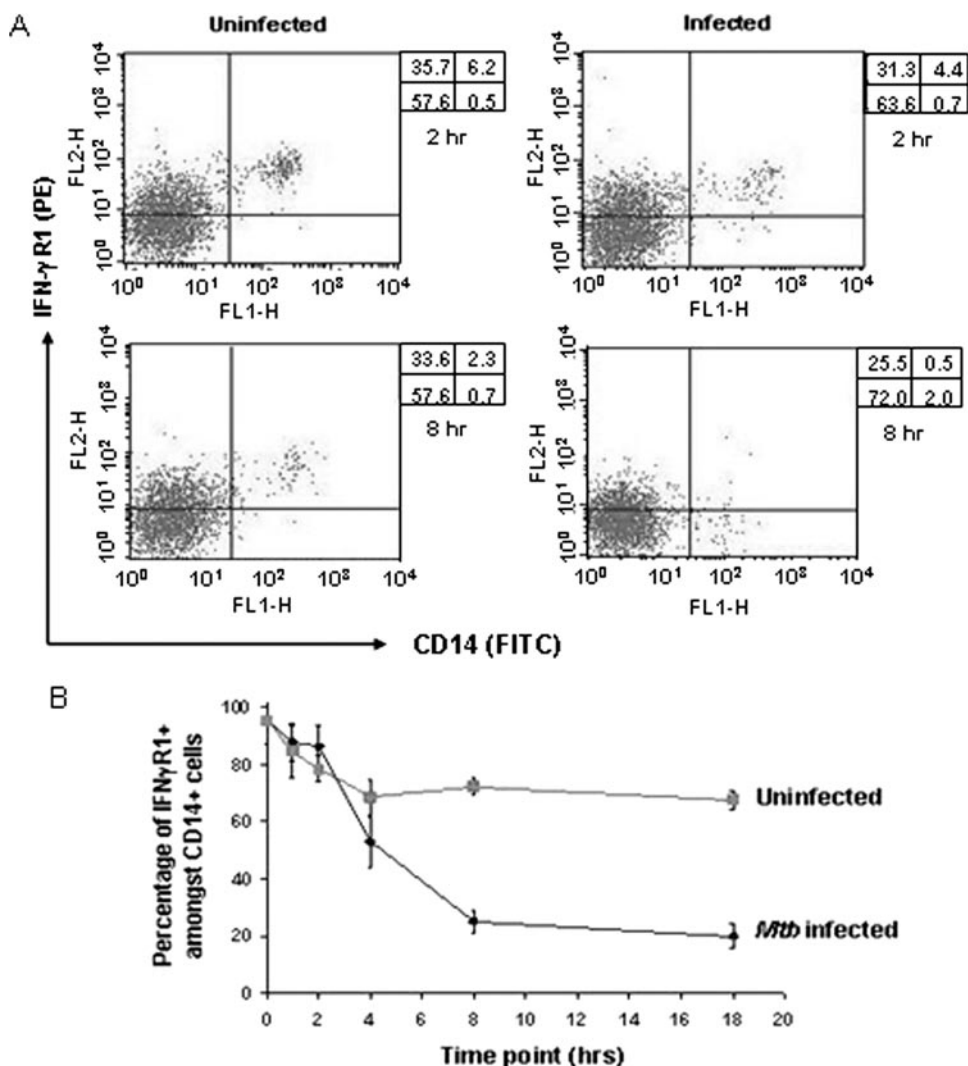


FIG. 3. *M. tuberculosis* down modulates surface expression of IFN- γ R1. PBMCs from a healthy donor were cocultured with live *M. tuberculosis* (MOI = 1:1) for 1, 2, 4, 8, and 18 h. After infection, cells were washed and stained for IFN- γ R1 and CD14. Uninfected time-matched PBMCs were used as controls. (A) Representative dot plots at 2 and 8 h for uninfected controls and infected PBMCs are shown. (B) Presented is a line diagram depicting cumulative data for the CD14⁺ IFN- γ R1⁺ cells amongst total CD14⁺ cells in PBMCs of three healthy donors for the time points investigated. Data are shown for *M. tuberculosis*-infected versus uninfected PBMCs at 8 and 18 h. $P < 0.01$ (two-tailed paired Student's *t* test). Error bars indicate standard deviations.

level of the IFN- γ R1 gene (*IFNGR1*) was assessed by quantitative real-time RT-PCR in PBMCs cocultured with live *M. tuberculosis* (Fig. 4). Time-dependent down modulation of IFNGR1 transcripts was observed for *M. tuberculosis*-infected PBMCs compared to uninfected controls, indicating that the down modulation in IFN- γ R1 surface expression by *M. tuberculosis* (Fig. 3) was due to the decreased expression of IFNGR1. *M. tuberculosis* infection significantly ($P < 0.01$) down regulates the IFN- γ R1 mRNA expression at 4 h after infection. This trend continued for up to 18 h postinfection. The reduction in IFN- γ R1 mRNA expression was three- to fivefold compared to that for uninfected controls ($P < 0.01$).

***M. smegmatis* does not modulate IFN- γ R1 surface expression on CD14⁺ cells.** In order to assess the specificity of the modulation of IFN- γ R1 by *M. tuberculosis*, a pathogenic mycobacterium, viz., *M. smegmatis* mc² (a nonpathogenic mycobacterium), was

used. As the generation time of *M. smegmatis* mc² is ~4 h, all experiments were terminated at 4 h. PBMCs were exposed to *M. smegmatis* mc² (MOI = 1:1; 0.5 to 4 h) and stained for IFN- γ R1 and CD14. The cumulative mean percentages \pm SD of IFN- γ R1-expressing cells among CD14⁺ cells in *M. smegmatis* mc²-infected and uninfected PBMCs have been shown in Fig. 5. At the time points investigated, no difference in the expression of IFN- γ R1 was observed between infected and uninfected PBMCs.

***M. tuberculosis* down modulates IFN- γ R1 expression in pdTHP-1 cells.** An increase in the expression of IFN- γ R1 in pdTHP-1 cells compared to that in undifferentiated cells has been reported (48). We confirmed the induction of IFN- γ R1 expression in pdTHP-1 cells compared to that in undifferentiated cells (Fig. 6A). Hence, pdTHP-1 cells were used as a model to determine the influence of *M. tuberculosis* on the modulation of IFN- γ R1 expression. Flow cytometric analysis

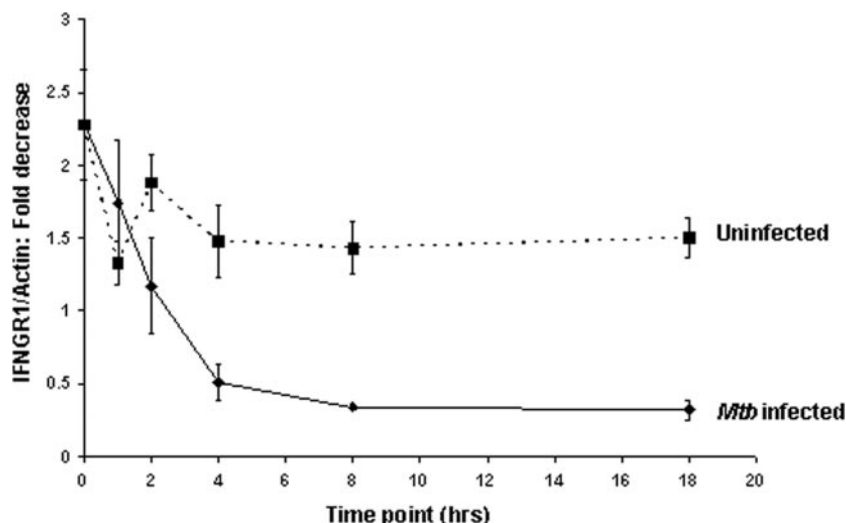


FIG. 4. *M. tuberculosis* (Mtb) down modulates IFN- γ R1 mRNA expression. PBMCs from a healthy donor were cocultured with live *M. tuberculosis* (MOI = 1:1) for varying time periods (1 to 18 h). Total RNA was isolated at indicated time points. mRNA was assayed by quantitative real-time RT-PCR for *IFNGR1* expression as described in Materials and Methods. All values were normalized to β -actin. Results are shown as decreases (n -fold) \pm SD (error bars) of normalized *IFNGR1* expression in infected and uninfected PBMCs.

demonstrated that *M. tuberculosis* infection of pdTHP-1 cells resulted in the down regulation of surface and total expression of IFN- γ R1 (Fig. 6A and B). *M. tuberculosis* infection inhibited both IFN- γ R1 surface and total (surface and intracellular) expression by \sim 50% compared with uninfected cells ($P < 0.01$) (Fig. 6B). To correlate the decrease in surface expression with the decrease in *IFNGR1* transcripts in *M. tuberculosis*-infected pdTHP-1 cells, as observed with PBMCs (Fig. 3 and 4), quantitative real-time RT-PCR was performed on RNA isolated from uninfected and infected pdTHP-1 cells. An increase in IFN- γ R1 mRNA transcripts was observed in pdTHP-1 cells compared to that in THP-1 cells ($P < 0.01$) (Fig. 6C). Significant reduction in *IFNGR1* mRNA was observed in *M. tuberculosis*-infected pdTHP-1 cells compared to that in uninfected

pdTHP-1 cells ($P < 0.01$) (Fig. 6C). *M. tuberculosis* infection resulted in a seven- to eightfold decrease in *IFNGR1* mRNA in pdTHP-1 cells.

***M. tuberculosis* infection of pdTHP-1 cells inhibits IFN- γ -mediated HLA-DR α expression.** Flow cytometric (Fig. 7A) and RT-PCR (Fig. 7B, lanes 3 and 4) analysis showed that IFN- γ -mediated HLA-DR α expression was detected in uninfected and *M. tuberculosis*-infected pdTHP-1 cells. However, HLA-DR α expression was significantly reduced (\sim 66%, $P < 0.01$) in infected cells compared to that in uninfected cells (Fig. 7A and B).

***M. tuberculosis* infection of pdTHP-1 cells inhibits IFNGR1 promoter activity.** PRE (Fig. 8A) is located in the -128 - to -109 -bp region of the *IFNGR1* promoter (49). 5' deletion constructs of the *IFNGR1* promoter were constructed in a pGL3 basic luciferase vector (Fig. 8B). To determine the effect of *M. tuberculosis* on the promoter activity of *IFNGR1*, the constructs were transfected into THP-1 cells and cocultured with live *M. tuberculosis* (MOI = 1:1; \sim 250 ng of protein)/WCL (500 μ g protein) and the levels of luciferase activity were determined (14). Figure 8B shows the structure of the deletion derivatives used in the study. The corresponding promoter activities are shown in Fig. 8C. A twofold inhibition of pGLR1B promoter activity by live *M. tuberculosis* was observed (Fig. 8C) ($P < 0.05$). Similar inhibition of pGLR1A and pGLR1B promoter activity was observed with WCL ($P < 0.05$), suggesting that *M. tuberculosis* mediated the inhibition of *IFNGR1* promoter activity.

***M. tuberculosis* inhibits expression of Sp1 and its recruitment on the PRE of the IFNGR1 promoter.** Binding of Sp1 to the PRE of the *IFNGR1* promoter has been shown to induce the up regulation of *IFNGR1* expression (49). Initially Sp1 expression in the nuclear extracts prepared from *M. tuberculosis*-infected pdTHP-1 cells was investigated (Fig. 9A, panel i). A densitometric plot of the Sp1 expression detected in various experimental conditions has been depicted in Fig. 9A, panel ii. PMA differen-

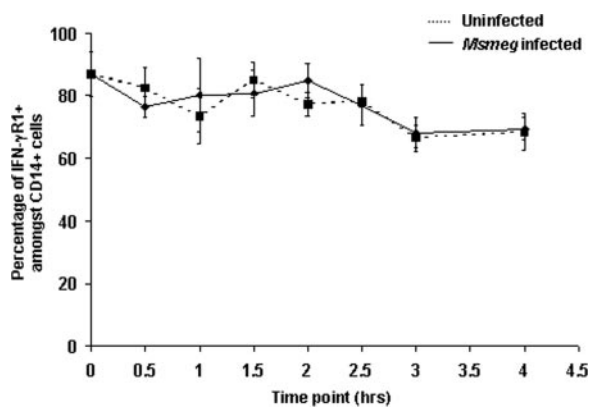


FIG. 5. *M. smegmatis* (Msmeg) does not modulate IFN- γ R1 surface expression. PBMCs from a healthy donor were cocultured with *M. smegmatis* (MOI = 1:1) for varying time periods (0.5 to 4 h). After infection, cells were washed and stained for IFN- γ R1 and CD14. Uninfected time-matched PBMCs were used as controls. Presented is a line diagram depicting cumulative data of the CD14⁺ IFN- γ R1⁺ cells amongst total CD14⁺ cells in PBMCs of three healthy donors for the time point investigated. Error bars indicate standard deviations.

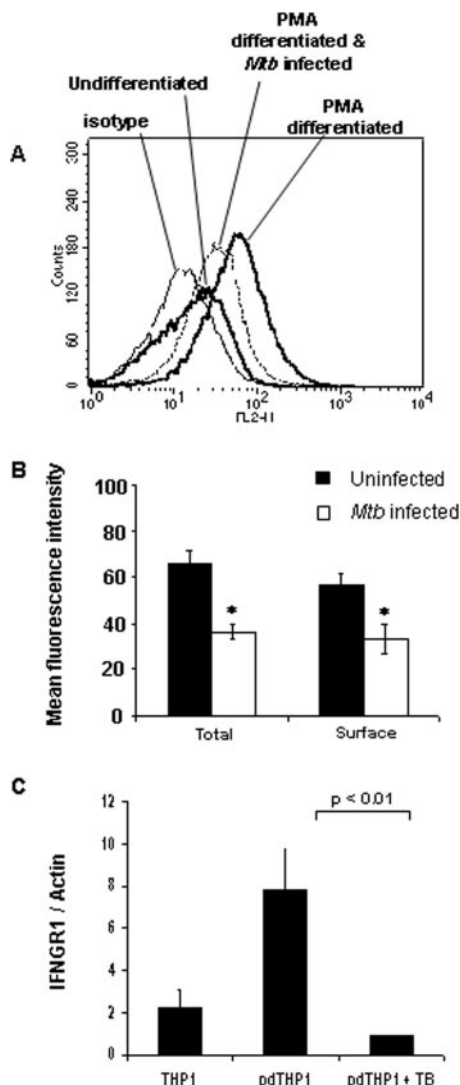


FIG. 6. *M. tuberculosis* (*Mtb*) down modulates expression of IFN- γ R1 in THP-1 cells. pdTHP-1 cells were infected with live *M. tuberculosis* (MOI = 1:1) or left uninfected for 24 h and harvested either for staining for total (surface and intracellular) and surface expression of IFN- γ R1 or for RNA isolation. For the total expression of IFN- γ R1 protein, cells were first permeabilized and then stained for IFN- γ R1. A total of 20,000 cells were acquired for analysis. Transcripts of IFNGR1 were quantified by real-time quantitative RT-PCR as described in Materials and Methods. (A) Histogram plot showing variation in the IFN- γ R1 surface expression in uninfected (MFI = 75.4) and *M. tuberculosis*-infected (MFI = 39.5) pdTHP-1 cells. (B) Histogram depicting the cumulative MFIs of total and surface expression of IFN- γ R1 in uninfected and *M. tuberculosis*-infected cells. *, $P < 0.01$. (C) Bar diagram indicating normalized transcript of IFNGR1 (IFNGR1/ β -actin) in THP-1 cells, pdTHP-1 cells, and *M. tuberculosis*-infected pdTHP-1 cells. The results of panel A are representative of eight independent experiments, which yielded comparable results. Error bars indicate standard deviations.

tiation increased the expression of Sp1 compared to that of undifferentiated THP-1 cells (Fig. 9A, lanes 1 and 4). The level of Sp1 expression in pdTHP-1 cells was significantly inhibited by live *M. tuberculosis* (Fig. 9A, lane 2) ($P < 0.01$) and WCL (lane 3) ($P < 0.01$) compared to that in uninfected pdTHP-1 cells (lane 4).

The interaction of nuclear extracts derived from infected and

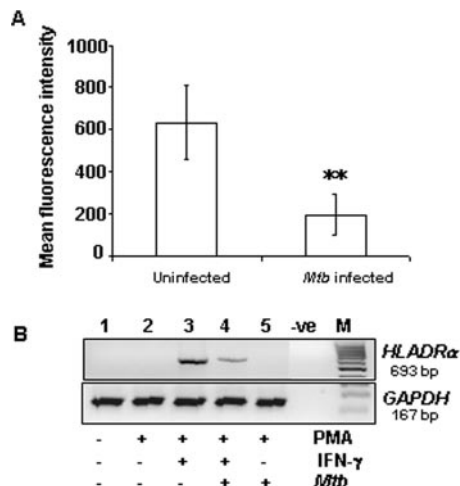


FIG. 7. *M. tuberculosis* (*Mtb*) down modulates IFN- γ -induced HLA-DR α expression. (A) pdTHP-1 cells were infected with live *M. tuberculosis* (MOI = 1:1) for 24 h. *M. tuberculosis*-infected and uninfected cells were stimulated with IFN- γ (100 U/ml) for 24 h at 37°C and stained for HLA-DR α . A total of 20,000 cells were acquired for analysis. Results from a histogram indicating the differences in the cumulative MFIs of HLA-DR α expression in uninfected and *M. tuberculosis*-infected cells are presented. **, $P < 0.01$. Error bars indicate standard deviations. (B) Semiquantitative RT-PCR was performed for HLA-DR α expression on RNA isolated from cells uninfected/infected/treated/untreated with IFN- γ . The result shown is representative of three independent experiments. -, absence of; +, presence of.

uninfected THP-1 cells with the PRE of the IFNGR1 promoter was assessed by EMSA. Figure 9B shows the competitive EMSA for Sp1 interaction. The presence of a 100-fold excess of unlabeled PRE (Fig. 9B, lane 3) and 2.5 μ g of anti-Sp1 Ab (lane 4) resulted in the disappearance of the major DNA-Sp1 complex seen (lane 2). Further, the interaction of equivalent amounts of nuclear extracts (1.5 μ g) derived from undifferentiated, differentiated, and infected THP-1 cells with PRE was carried out (Fig. 9C, panel i). Figure 9C, panel ii, depicts the relative intensity of the DNA-Sp1 complex detected. The intensity of DNA-Sp1 complex (Fig. 9C, panel i, lane 3) with a nuclear extract of pdTHP-1 cells was found to be more intense compared to that of a nuclear extract of undifferentiated cells (Fig. 9C, panel i, lane 2). Reduced intensity of DNA-Sp1 complex was observed when a nuclear extract of *M. tuberculosis*-infected pdTHP-1 cells was used (Fig. 9C, panel i, lane 4).

DISCUSSION

The ability of *M. tuberculosis* to persist within macrophages is known to be facilitated by various mycobacterium-induced evasion strategies. These include inhibition of the recognition of infected macrophages by T cells and macrophage-killing mechanisms (18). IFN- γ signals are mediated through its heterodimeric receptor (6), of which the IFN- γ R1 chain binds the ligand and determines signaling specificity (37). As infected human macrophages cannot be activated by IFN- γ to kill intracellular tubercle bacilli (61), we therefore examined the basal surface expression of IFN- γ R1 on PBMCs of PTB patients. The results showed that basal surface expression of IFN- γ R1 on circulating PBMCs and CD14⁺ cells was signif-

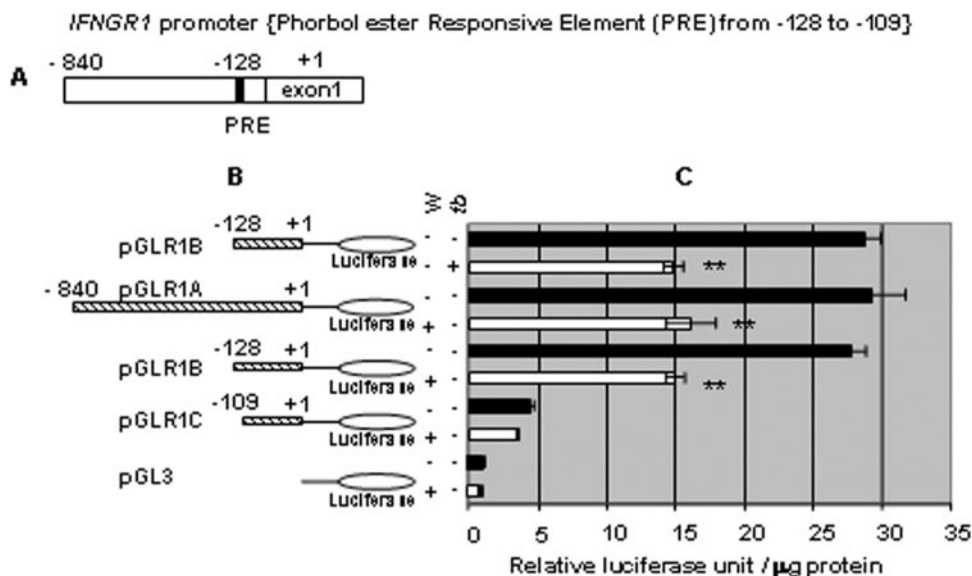


FIG. 8. *M. tuberculosis* infection decreases *IFNGR1* promoter activity in transiently transfected THP-1 cells. (A) Organization of the *IFNGR1* promoter. PRE (-128 to -109 bp) has been depicted. (B) The structure of each construct is shown; the 5' promoter fragment of each construct is shown as a shaded area. (C) The corresponding promoter activities are shown as the RLU per microgram of protein of lysate. Closed and open bars show the RLU of unstimulated and stimulated cells, respectively. Each construct was transfected into THP-1 cells. Transfected cells were cocultured with live *M. tuberculosis*/WCL and PMA differentiated, as described in Materials and Methods. The luciferase activity of cell lysates was estimated and normalized to the protein concentration of the lysate. The data represent mean \pm SD (error bars) of three independent experiments. The decrease in luciferase activity resulting from live *M. tuberculosis* and WCL was significant by one-way ANOVA with Tukey's test. **, $P < 0.05$.

icantly lower in PTB patients compared to that in healthy individuals. After ATT, the surface expression of IFN- γ R1 significantly increased in patient-derived PBMCs. However, Rugeles et al. (46) reported normal expression of IFN- γ R in Epstein-Barr virus-transformed B cells from four pediatric patients with extrapulmonary *M. tuberculosis* infections. Reduced expression of IFN- γ R1 on the surface of PBMCs derived from patients with visceral leishmaniasis has been reported (16). Further, sodium antimony gluconate-treated visceral leishmaniasis patients showed up regulation of IFN- γ R1 (16). The in vitro resurgence of IFN- γ R1 expression in sodium antimony gluconate-treated *Leishmania donovani*-infected pdTHP-1 cells was confirmed (16). Recently Ansari et al. (4) demonstrated that there is a restoration of IFN- γ R1 at both mRNA and protein levels in post-kala-azar dermal leishmaniasis patients upon treatment. This resulted in parasite clearance. The recovery of IFN- γ R1 expression in treated leishmaniasis/tuberculosis patients could be due to the absence of the pathogen/pathogen-derived inhibitory products in the milieu. Besides the absence of pathogen/pathogen-derived inhibitory constituents, an effect of cytokines and chemotherapeutic agents on the modulation of IFN- γ R1 expression could not be ruled out. Interleukin-1 β (IL-1 β) and tumor necrosis factor alpha have been reported to up regulate IFN- γ R1 expression in various cells (26, 54). Indole-3-carbinol and 5-fluorouracil, cancer chemotherapeutic agents, have been shown to induce the expression of IFN- γ R1 in tumor cells (10, 47).

The down modulation of IFN- γ R1 expression has been demonstrated in PMA-differentiated U937 cells following *L. donovani* infection (43), B lymphocytes following *Trypanosoma cruzi* infection (23), and murine macrophages harboring *M. avium*

(22). Further, *M. avium* infection was shown to inhibit the induction of IFN- γ -inducible genes in mouse macrophages by down regulating IFN- γ R, resulting in the reduced phosphorylation of IFN- γ R1, JAK1, JAK2, and STAT1 (22). IFN- γ R has also been shown to be required for the protective pulmonary inflammatory response to *Cryptococcus neoformans* in mice (11). Further up regulation of IFN- γ R1 in the J744 macrophage cell line has been shown to control the dissemination of virulent *Salmonella* (20). We observed a time-dependent down modulation of IFN- γ R1 surface protein and mRNA expression in human PBMCs exposed to live *M. tuberculosis*. However, *M. smegmatis* mc² infection does not modulate the expression of IFN- γ R1, indicating that IFN- γ R1 down modulation was seen exclusively with pathogenic *M. tuberculosis*. We further investigated the modulation of IFN- γ R1 expression by *M. tuberculosis*, using infected pdTHP-1 cells. pdTHP-1 cells have been extensively used by various groups to study the interaction of macrophages with *M. tuberculosis* (24, 63). Sakamoto et al. (48) have reported the increase in the expression of IFN- γ R1 in phorbol ester-differentiated THP-1 cells. This induction of IFN- γ R1 expression in pdTHP-1 cells was taken advantage of to examine the effect of *M. tuberculosis* on the modulation of IFN- γ R1. The infection of pdTHP-1 cells with *M. tuberculosis* leads to the down regulation of both cell surface and total expression of IFN- γ R1. This down regulation of IFN- γ R1 was accompanied by the inhibition of IFN- γ -induced HLA-DR α expression. The fall in expression of major histocompatibility complex class II molecules in *M. tuberculosis*-infected THP-1 cells and *M. avium*-infected mouse macrophages has been reported earlier (21, 22, 63). However, *M. tuberculosis* and *M. avium* infection do not inhibit STAT1 activation (24, 63). We

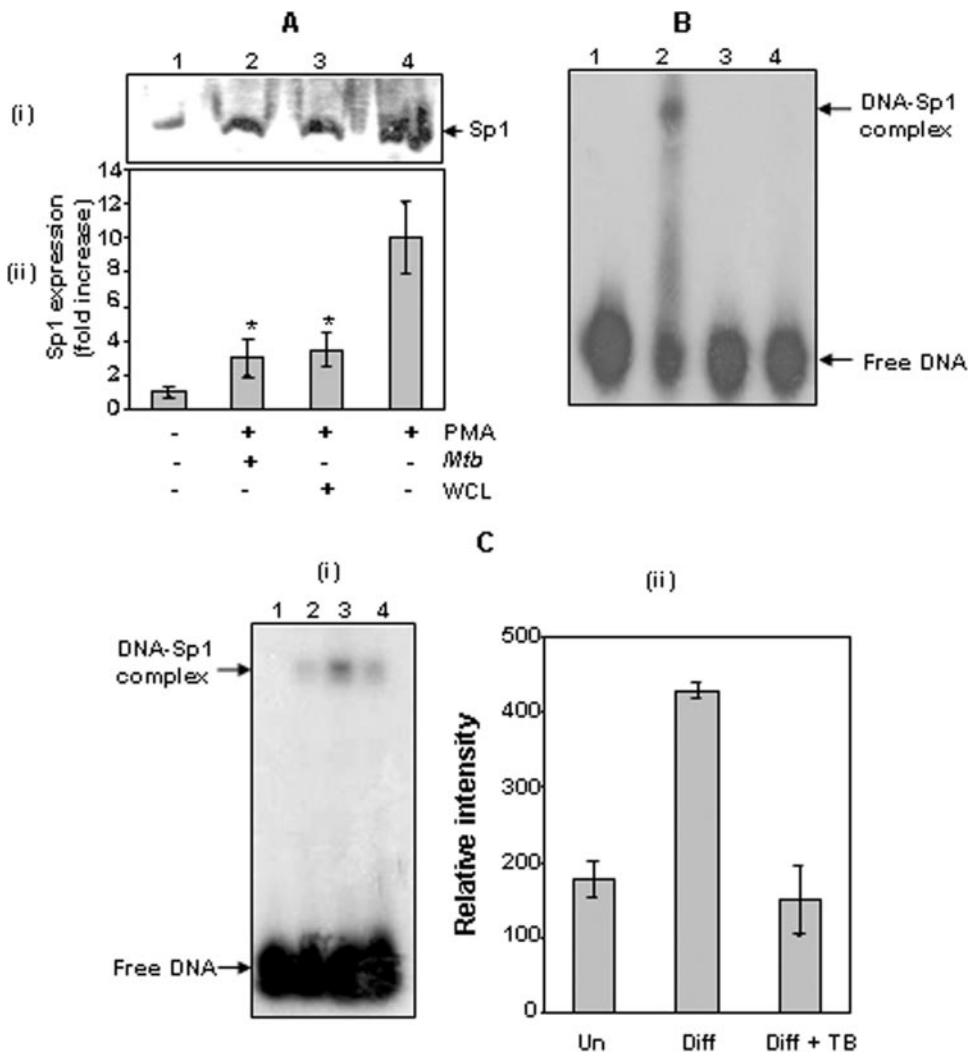


FIG. 9. *M. tuberculosis* (Mtb) inhibits Sp1 protein expression and its recruitment on the PRE of the *IFNGR1* promoter. (A) Nuclear extracts (20 μ g) prepared from undifferentiated, differentiated, and *M. tuberculosis*- and WCL-treated pdTHP-1 cells were analyzed for Sp1 protein expression by the immunoblot assay. Panel A shows a representative blot and composite densitometry data (mean percentage change observed in three independent experiments \pm SD). (B) The PRE (20 bp, from -128 to -109 bp) of the *IFNGR1* promoter was custom synthesized and labeled with γ - 32 P, and EMSA was performed. This panel represents the competitive EMSA. Nuclear extract (2.0 μ g) prepared from pdTHP-1 cells was incubated as such (lane 2), 100-fold excess of cold PRE (lane 3) and anti-Sp1 Ab (lane 4) for 30 min at 4°C prior to the addition of γ - 32 P-labeled PRE (30,000 cpm). Lane 1, no nuclear extract. (C, panel i) Representative autoradiogram of the EMSA carried out using γ - 32 P-labeled PRE (20,000 cpm) and 1.5 μ g of the nuclear extracts of undifferentiated (lane 2), pdTHP-1 (lane 3), and *M. tuberculosis*-infected pdTHP-1 cells (lane 4). (C, panel ii) Composite densitometry histograms (mean relative intensity of the DNA in DNA-Sp1 complex observed in four independent experiments \pm SD for different nuclear extracts used) are shown. Results are shown for undifferentiated THP-1 cells, differentiated pdTHP-1 cells (Diff), and *M. tuberculosis*-infected pdTHP-1 cells (Diff+TB). Error bars indicate standard deviations. -, absence of; +, presence of.

observed similar results of no effect of *M. tuberculosis* on STAT1 tyrosine phosphorylation (data not shown). Despite the down modulation of the IFN- γ R1 receptor, the details of the pathways associated with sustained STAT1 activation levels in *M. tuberculosis*-infected cells remain elusive.

This elusiveness could be due to the existence of multiple mechanisms for enhancing cytokine-induced STAT1 activation and function (60). STAT1 has been shown to participate in cross talk between signal-transduction pathways (40). Apart from IFN- γ -induced STAT1 activation, the dimerization of tyrosine-phosphorylated STAT1 monomers has been shown to be mediated by the interaction of type I interferon and IL-6

receptors in caveolar domains in the plasma membrane (33, 59). Tassiulas et al. (60) reported IFN- α -induced STAT1 activation in macrophages primed with low concentrations of IFN- γ . This has been shown to be Syk (spleen tyrosine kinase) dependent. Hence, the possibility of IFN- α - and IL-6-mediated STAT1 activation in the context of decreased IFN- γ -mediated STAT1 activation (due to lower IFN- γ R1 expression) during *M. tuberculosis* infection cannot be ruled out. This would result in STAT1 activation despite the restricted number of IFN- γ R1 receptors available for binding of IFN- γ . Further, IL-6 secreted by *M. tuberculosis*-infected macrophages has been shown to selectively inhibit the expression of IFN- γ -

responsive genes without inhibiting the activation or function of STAT1 (34). The existence of various STAT1 activation factors in addition to interferons has also been suggested (60). Prolactin (a hormone) has been shown to induce homodimerization and tyrosine phosphorylation of STAT1 (51). STAT1 activation has also been reported to occur during spontaneous in vitro differentiation of monocytes into macrophages, wherein it is induced by the adherence of monocytes to plastic surfaces (12). Further, fibronectin and laminin, host adhesion molecules present in the extracellular matrix (ECM), have been shown to enhance STAT1 activation by an unknown mechanism(s) (12). Various mycobacterial proteins, such as Ag85B (2), HbhA (32), MDP1 (5), malate synthase (25) of *M. tuberculosis*, FPA-A (52) of *M. avium*, and LBP (53) of *M. leprae*, have been shown to interact with ECM proteins, such as fibronectin, laminin, heparin, and hyaluronic acid. This interaction is responsible for the adherence of mycobacteria to the host cells (5) and their invasion (53). This result indicates that *M. tuberculosis* and other pathogenic mycobacteria could enhance STAT1 activation through their interaction with ECM proteins. Hence, the observation of no influence of *M. tuberculosis* and *M. avium* on STAT1 activation by us and other groups to date (24, 61, 63) could be due to the use of plastic adherent monocytes/macrophages in in vitro experiments and the enhancement of STAT1 activation through interaction with ECM proteins. Further, recently it has been shown that the up regulation of IFN- γ R expression due to *Chlamydia* infection does not enhance STAT1 signaling (55).

Despite optimal STAT1 activation in *M. tuberculosis* infection, the IFN- γ -mediated signal transduction pathway remains impaired, indicating the existence of an alternate pathway for the inhibition of IFN- γ -mediated signaling. PIASy, a member of the PIAS (protein inhibitor of activated STAT1) family, has been shown to function as a repressor of STAT1-dependent signaling (28). It binds to STAT1 and represses STAT1-mediated gene activation without blocking the ability of STAT1 to bind to DNA (28). The role of PIASy in *M. tuberculosis* infection is yet to be elucidated. Further, there exist numerous STAT1-independent pathways that play crucial roles in IFN- γ -mediated signaling (41). Whether the evasion of IFN- γ -dependent microbicidal effects by *M. tuberculosis* is due to the subversion of these STAT1-independent pathways remains unanswered.

It is possible that *M. tuberculosis* infection of THP-1 cells/PBMCs may interfere with the recycling of IFN- γ R1 or enhance its degradation by altering mRNA stability. Curry et al. (15) showed that *M. avium*-mediated down regulation of IFN- γ R1 expression in mouse macrophages was not due to reduced mRNA stability but to decreased levels of mRNA. This decrease in mRNA levels was shown to be related to alterations in the transcription rate. A similar scenario may exist in cells harboring *M. tuberculosis*. We found reduced mRNA levels of IFN- γ R1 in *M. tuberculosis*-infected pdTHP-1 cells. Further, the stimulation of transiently transfected THP-1 cells by live *M. tuberculosis* and WCL resulted in the decreased transcription of human *IFNGR1*. The degree of inhibition of *IFNGR1* promoter activity by live *M. tuberculosis* (~250 ng of protein) (14) was found to be equivalent to that observed by 500 μ g protein of WCL. Thus, integral bacilli showed a more potent effect on transcriptional inhibition of *IFNGR1* than did WCL.

The *IFNGR1* promoter is GC rich with no TATA box and is similar to the promoters of noninducible housekeeping genes (38). The Sp1 family of transcription factors have been shown to regulate the initiation of transcription in promoters devoid of the TATA box (3, 57). It was shown earlier that the expression of Sp1 increases in pdTHP-1 cells (27) and is related to the up regulation of IFN- γ R1 by binding to the PRE of the *IFNGR1* promoter (49). In the present study, we observed that *M. tuberculosis* down regulates not only the expression of Sp1 in pdTHP-1 cells but also its recruitment on the PRE of the *IFNGR1* promoter. Reduced DNA binding activity of Sp1 could be a result of posttranslational modification, as phosphorylation has been shown earlier to have a profound effect on DNA binding activity of Sp1 (39). This has not been investigated in the present study. Hence, it could be speculated that the down regulation of Sp1 resulted in the decrease of IFN- γ R1 expression in *M. tuberculosis*-infected pdTHP-1 cells. Little is known about the transcriptional regulation of human *IFNGR1*. Regulatory motifs present in mouse *IFNGR1* include multiple Sp1, Ap2, NF1, and CRE sites (42). Additionally, Sp1 is known to interact with several transcription factors, such as Oct-1 (58), nuclear factor Y (45), SREBP-2, ELF-1, etc. Thus, besides Sp1 modulation, the modulation of other factors which bind to the *IFNGR1* promoter and possibly to Sp1 could be important in the *M. tuberculosis*-induced down regulation of IFN- γ R1 expression.

Sp1 is a ubiquitous transcriptional factor which also has been shown to be involved in the regulation and expression of the human monocytic-specific gene CD14 (64). We observed that the expression of CD14 in pdTHP-1 cells remained unchanged following *M. tuberculosis* infection (MOI = 1:1) (data not shown), indicating that decreased Sp1 expression by *M. tuberculosis* had no effect on the expression of CD14. This may be because the level of Sp1 in infected pdTHP-1 cells was ~3-fold higher than that in undifferentiated THP-1 cells (Fig. 9A), which could be adequate to maintain the expression of CD14. Besides Sp1, other transcriptional factors, such as AP-1, have been shown to play critical roles in the expression of CD14 in the murine and rat systems (29, 31). Similar pathways for the regulation of CD14 expression may exist in human THP-1 cells. Further, increased DNA binding activity of AP-1 has been reported in *M. tuberculosis*-infected THP-1 cells (44). Santucci et al. (50) reported the MOI-dependent effect of live *M. tuberculosis* on the expression of CD14 in human monocytes.

In conclusion, we detected reduced IFN- γ R1 expression levels in untreated PTB patients. The IFN- γ R1 expression was restored in these individuals following ATT. The down modulation of IFN- γ R1 expression was observed in ex vivo experiments, wherein PBMCs were cocultured with *M. tuberculosis*. The down regulation of IFN- γ R1 expression by *M. tuberculosis* was confirmed by using PMA-differentiated THP-1 cells and was found to be related to the reduced transcription of the *IFNGR1* gene. This may be due to the decreased Sp1 expression and its recruitment on the PRE of the *IFNGR1* promoter. Therefore, the down regulation of IFN- γ R1 could be one of the probable mechanisms employed by *M. tuberculosis* to alter the responsiveness of an infected cell to IFN- γ , thereby inhibiting potential IFN- γ -mediated microbicidal functions of macrophages.

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