The PPE protein Rv1168c of Mycobacterium tuberculosis augments transcription from HIV-1 LTR promoter

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Running Title: The Rv1168c PPE protein activates HIV-1 LTR transcription

Key Words: PPE Protein Rv1168c/Rv1196; HIV-1 LTR; Mycobacterium tuberculosis; TLR2-LRR domain; monocytes/macrophages; NF-κB.

Capsule

Background: Mycobacterium tuberculosis stimulates HIV-1 LTR transcription in co-infected individuals.

Results: A PPE protein of M. tuberculosis Rv1168c interacts with TLR2 to activate NF-kappa B leading to HIV-1 LTR trans-activation.

Conclusion: Mycobacterial components can directly activate HIV-1 LTR by modulating host signaling cascades.

Significance: This information may be helpful to develop therapeutics to control HIV-1 infection in co-infected patients.
Abstract

Cells of the monocyte/macrophage lineage are shown to play a role in the pathogenesis of human immuno deficiency virus (HIV). The incidence of HIV type 1 (HIV-1) infection is found to be accelerated in people infected with Mycobacterium tuberculosis, but the mechanism by which mycobacterial protein(s) induces HIV-1 LTR trans-activation is not clearly understood. We describe here that the M. tuberculosis PPE protein, Rv1168c (PPE17) can augment transcription from HIV-1 LTR in monocyte/macrophage cells. Rv1168c interacts specifically with Toll-like receptor-2 (TLR2) resulting in downstream activation of nuclear factor-kappaB (NF-κB) resulting in HIV-1 LTR trans-activation. Another PPE protein, Rv1196 (PPE18) was also found to interact with TLR2, but had no effect on HIV-1 LTR trans-activation because of its inability to activate NF-κB signaling pathway. In silico docking analyses and mutation experiments have revealed that the N-terminal domain of Rv1168c specifically interacts with the leucine rich repeats (LRR) 15~20 domain of TLR2 and this site of interaction is different from that of Rv1196 protein (LRR 11~15 domain), indicating that the site of interaction on TLR2 dictates the downstream signaling events leading to activation of NF-κB. This information may help in understanding the mechanism of pathogenesis of HIV-1 during M. tuberculosis co-infection.
Introduction

Approximately 33.3 million people are living with the human immune deficiency virus type 1 (HIV-1) World-wide and about 2.6 million people were newly infected in 2009 (1). In addition, there is a deadly syndemic interaction between the HIV and tuberculosis (TB) caused by Mycobacterium tuberculosis (3). It is widely accepted that HIV causes a depletion of CD4 T cells, which is likely to contribute to the susceptibility of co-infected persons to TB. HIV/TB co-infected persons have been shown to have a higher mortality rate than those without either infection alone, regardless of CD4 count (3). HIV/M. tuberculosis co-infection results in remarkably higher mortality (4,5). Due to the high incidences of both HIV and M. tuberculosis infection in several global pockets like sub-Saharan Africa and India, TB has emerged as the most common opportunistic infection in HIV-infected patients (6).

Cells of the monocyte-macrophage lineage play an important role in the transmission and pathogenesis of HIV (7,8) in addition to CD4 T cells. Infected monocytes can differentiate into macrophages and may form long-lived reservoir for the virus (9). On the other hand, the macrophages form a replicative niche for M. tuberculosis (10). The cells of the monocyte/macrophage lineage are not only the common target and a probable site of interaction for M. tuberculosis and HIV, but also a source of increased HIV production in co-infected patients (10). Incubation of HIV-infected PBMCs with pleural fluid from individuals with TB induced more replication compared to the pleural fluid obtained from healthy controls and was dependent on TNF-α and IL-6 indicating that an overt pro-inflammatory microenvironment produced by the activated monocytes/macrophones may increase HIV replication (11).}

It has been also shown that the live M. tuberculosis and its cell wall components can increase replication of HIV both in vitro and in vivo in monocyte/macrophage cells (12-16). The purified protein derivative (PPD), ManLAM and culture filtrate protein of M. tuberculosis (15,17) have been shown to enhance transcriptional activity of HIV-1 long terminal repeat (LTR), the sole promoter element of HIV (14,17). The expression of viral genes are regulated by several host transcription factors such as the Sp family, nuclear factor-kappaB (NF-κB) family, activator protein 1 (AP-1) proteins, nuclear factor of activated T cells (NFAT), and CCAAT enhancer binding proteins (C/EBP) family members by binding to the LTR that display different levels of sequence conservation (18). In addition, viral proteins such as Vpr and Tat also bind to the LTR and regulate transcription. Many of these host and viral proteins interact with each other leading to a complex transcriptional regulation of LTR (18). The NF-κB proteins are known to be one of the major modulators of the HIV-1 LTR in all cell types and is a potential pathway that can be targeted for anti-HIV-1 therapies (19). Activation of monocytes by LPS, IL-6 or TNF-α results in enhanced HIV replication, a process that well correlates with NF-κB activity (20-22). Induction of these pro-inflammatory cytokines during mycobacterial infection have been therefore postulated to be one of the important factors that drive hyper-transcription from LTR promoter (13,14,23,24). However, several studies have suggested that additional mycobacterial factors may also be responsible for increased transcription from LTR promoter (15,25,26). Interaction of monocytes/macrophages with various mycobacterial components may result in triggering of cascades of events leading to changes in the levels and activities of several cellular transcription factors in monocytes/macrophages and binding of these transcription factors to the specific LTR-regions, can alter the levels of HIV-1 LTR-driven gene expression (17,25-27). Although involvement of M. tuberculosis in the activation of HIV-1 LTR is documented, the molecular mechanisms involved in mycobacterial protein-induced HIV-1-LTR trans-activation in
monocytes/macrophages are not well understood.

One of the major distinctive features of *M. tuberculosis* genome is the presence of two glycine-rich gene families of proteins containing proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs near the N-terminus region with no known physiological function and account for about 10% of the total coding capacity of the *M. tuberculosis* genome (28). Many of these proteins are found to be differentially expressed in *M. tuberculosis* under different conditions (29,30). These proteins are proposed to be responsible for generating antigenic diversity and may also interfere with the host immune responses (31-35). Few recent studies indicate that some of these proteins can modulate the macrophage innate effector signaling pathways (36-38). Therefore, it is possible that some of these proteins may have the ability to modulate inflammatory-signaling and thereby regulate HIV-1 LTR transactivation.

We have reported earlier that one of the PPE proteins, Rv1168c (PPE17) is a highly immune dominant antigen detected during active TB infection (39). Interestingly, microarray and proteomic studies have also indicated up-regulation of Rv1168c under microaerophilic and anaerobic conditions, nutrient starvation, and also in the presence of palmitic acid that simulate the features of the phagosomal environment (28,40-43). Rv1168c is found to be over-expressed in macrophages infected with various clinical isolates of *M. tuberculosis* (44). Since some of the PPE family proteins are shown to be present in the cell surface (37,45), we speculated that Rv1168c may be exposed to the cell surface and could modulate the host immune responses by interacting with the monocyte/macrophage surface components. In the present study, we have examined whether Rv1168c interacts with monocytes/macrophages and modulates its signaling cascades that can eventually lead to a favorable environment for HIV-1 LTR transcription. Our data presented herein indicate that Rv1168c interacts specifically with the TLR2 leucine rich repeats (LRR) 15~20 domain and activates NF-κB signaling cascades that leads to HIV-1 LTR hyper-activation in a Tat-independent manner.

**EXPERIMENTAL PROCEDURES**

**Expression and purification of recombinant Rv1168c and Rv1196 PPE proteins**

The full-length Rv1168c, Rv1196, and Rv1168 truncated genes were cloned, expressed and the recombinant proteins were purified as described earlier by us (37,39). Briefly, the ORFs were cloned in-frame with 6X-histidine tags in pRSET vectors and the recombinants were transformed into BL21 (DE3) pLysE cells. Secondary cultures were inoculated with overnight grown primary cultures and allowed to grow till the absorbance at 600 nm reached about 0.5. The cultures were induced with isopropylthiogalactoside (IPTG) and the His-tagged recombinant proteins were purified using TALON resin (Clontech, Palo Alto, CA, USA).

The Rv1168cΔC and Rv1168cΔN mutants were cloned in frame in pRSET vector for affinity purification using Histidine tags. In order to remove LPS contamination, the recombinant proteins were treated with 10% (v/v) polymyxin B-agarose (Sigma-Aldrich, St. Louis, Mo; binding capacity 200 to 500 μg of LPS/ml) for 1 hour at 4°C as described earlier (37,46). Following incubation, the agarose beads were removed by centrifugation and the supernatant was filter sterilized and used in various experiments.

**Cell culture**

THP-1 cells were obtained from National Centre for Cell Science, Pune, India. BF-24 cells were
obtained through the AIDS Research Program, Division of AIDS, NIAID, NIH, from Dr. Barbara K. Felber and Dr. George N. Pavlakis. The cells were cultured in complete RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% FBS, Antibiotic-Antimycotic (1X, containing Penicillin G, Streptomycin, Amphotericin B), 2 mM L-Glutamine and 10 mM HEPES (All from GIBCO, Carlsbad, CA) and maintained at 37°C and 5% CO₂ in a humidified incubator. HEK293 cell line was maintained in Dulbecco Modified Eagle Medium (DMEM) high glucose medium (Hyclone) containing 10% FBS, Antibiotic-Antimycotic (1X), 2 mM L-Glutamine and 10 mM HEPES (all from GIBCO).

FITC labeling
FITC labelled rRv1168c (rRv1168c-FITC) or FITC labelled rRv1168ΔN (rRv1168ΔN-FITC) or FITC labelled rRv1168ΔC (rRv1168ΔC-FITC) was prepared by incubating the recombinant protein with FITC using a commercially available FITC Antibody Labeling Kit from Pierce Chemical Company (Rockford, IL) following the manufacturer’s protocol.

Biotinylation of rRv1168c
Biotinylation of recombinant protein was carried out using a commercially available biotinylation kit from Pierce Chemical Company (Rockford, IL) as described earlier (37). Briefly, rRv1168c was incubated with 5 fold molar excess of Sulfo NHS-biotin reagent (sulfosuccinimidyl-2-[biotinamido] ethyl-1, 3-dithiopropionate) for about 1 hour at room temperature. The non-reacted reagent was removed from the biotinylated protein sample by desalting using Amicon ultra centrifugal filter units. Biotinylation of the protein was confirmed by enzyme immunoassay (EIA) using streptavidin conjugated to HRP.

HIV-1 LTR-chloramphenicol acetyltransferase (CAT) constructs

Full-length HIV-1 LTR-CAT construct and the mutant construct, having mutations in both the NF-xB binding sites (pDKB-HIV-CAT) were kind gifts from Debashish Mitra, (NCCS, Pune, India) and AIDS Research Program, Division of AIDS, NIAID, NIH, USA.

Transient transfection
Transfection in THP-1 cells was carrying out using liposome-mediated transfection. DMRIE-C transfection reagent (Invitrogen, Carlsbad, CA) was used to carry out various transfections according to the manufacturer’s instructions. The THP-1 cells were co-transfected with HIV-1 LTR-CAT and pCMV-β-Gal (Clontech) constructs following manufacturer’s instruction. Briefly, 0.5 million cells were seeded in a 12 well plate in Opti-MEM (Invitrogen). One μg DNA and 3 μl DMRIE-C reagent where separately incubated in 50 μl of Opti-MEM each for 15 minutes. The two preparations were mixed slowly and incubated for 30 minutes with intermittent mixing. The complex was added to the cells drop-wise in a culture plate. Complete medium with 20% FBS was added after 5-6 hours post-transfection. The cells were further incubated with various concentrations of rRv1168c protein, 10 hours after transfection and CAT/β-Gal reporter assay was carried out 36 hours after treatment with the recombinant protein. The negative-control scrambled siRNA, TLR2 targeting siRNA (sense, 5'-GCCCUGACUCCUGCAACAtt 3'; the lowercase letters represent two deoxy bases that serve as overhangs for the cleavage by dicer) were purchased from Ambion Inc. (Austin, TX, USA) (35). The BF-24 cells were transfected with either the negative-control siRNA or with TLR2 specific siRNA using lipofectamine 2000 (Invitrogen). Depletion of TLR2 by siRNA was assessed by flow cytometry using TLR2-specific Ab from Imgenex (San Diego, CA, USA) 24 hours post-transfection.

Treatment of BF-24 cells with the recombinant Rv1168c/Rv1168ΔC protein
BF-24 cells were seeded as 1 million per well and either left untreated or treated with various concentrations of rRv1168c/rRv1168ΔC protein. Untreated cells were used to check for the basal level expression of HIV-1 LTR. After stimulation, cells were incubated for 36 hours and harvested to measure CAT activity. In some experiments, PMA-differentiated BF-24 macrophages (PMA was used at 10 ng/ml for overnight followed by a resting period for 24 hours) were treated with various concentrations of rRv1168c/rRv1168ΔC and CAT activity was measured after 36 hours.

**Western blotting for p50 and p65 nuclear factor kappa B (NF-κB) transcription factors**

The nuclear p50 and p65 transcription factors were detected by Western blotting. The nuclear extracts were prepared from NP-40-lysed cells as described earlier by us (47). Following separation on 10% SDS-PAGE and electrophoretic transfer of the nuclear extracts, nitrocellulose membranes were blocked using 5% fat free milk in PBS and then incubated with rabbit antibody to either p50 or p65 (Santa Cruz, Santa Cruz, CA) transcription factor. The membrane was next incubated with anti-rabbit-immunoglobulin G (IgG) coupled to horse radish peroxidase (HRP) (Sigma-Aldrich). Bound enzyme was detected by chemiluminescence following the manufacturer's protocol (GE Healthcare, Little Chalfont, UK). Equal loading of protein was confirmed by Ponceau S Red stain (Sigma-Aldrich).

**Estimation of TNF-α**

The TNF-α cytokine in various macrophage culture supernatants was quantified by two-site sandwich EIA as described earlier (48) (BD Biosciences Pharmingen, San Diego, CA). Standard curve for the cytokine was obtained using the recombinant standard protein provided in the kit.

**Electrophoretic mobility shift assay**

EMSA was performed as described earlier (49). Briefly, nuclear extracts (10 µg) were incubated for 30 minutes at room temperature with 1 ng of 32P-end-labelled NF-κB consensus binding sequence 5' AGTTGAGGGGACTTTCCCAGG-3' (50) in binding buffer (20 mM HEPES, 0.5 mM DTT, 1 mM MgCl2, 1 mM EDTA and 5% glycerol).
containing 2 μg of poly (dI-dC). The DNA-protein complex was resolved on 7% native gel in 1X TGE running buffer (25 mM Tris base, 190 mM Glycine, 1 mM EDTA, pH 8.3). The specificity of the binding was examined by competition with 100 fold excess of unlabelled probe. The gel was dried at 80°C for 1 hour and exposed to imaging plate (Fuji Film) overnight. Visualization of the radioactive bands was carried out using a STARION image scanner (Fuji Film FLA-9000).

**Competition assay**

The THP-1 cells were harvested and washed with staining buffer (1X PBS with 0.5% FBS) and incubated either with medium alone or with 2 fold or 15 fold excess recombinant Rv1196 protein for 30 minutes on ice followed by incubation with 10 μg/ml biotin labeled Rv1168c (Rv1168c-biotin) for another 30 minutes on ice. Cells were washed three times with staining buffer and incubated with streptavidin-FITC (Sigma-Aldrich) for 30 minutes on ice. Cells were washed and cell-bound fluorescence was analyzed with the BD FACS Vantage SE (Beckton Dickinson) using CellQuest data analysis software (Beckton Dickinson).

**Immunoprecipitation assay**

The full-length human TLR2 or TLR4 cDNA cloned in pcDNA3.1 plasmid vectors were kind gifts from Dr. Manikuntala Kundu, Bose Institute, Kolkata, India. The empty expression vector (pcDNA3.1) without any insert was used as negative control. The FLAG-tagged wild-type TLR2 as well as TLR2 ectodomain deletion mutants Mut3 LRR (10~15), and Mut4 (LRR 15~20) were all gifts from Dr. Carsten J. Kirschning (Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany). The plasmid constructs were transfected into HEK293 cells using the cationic lipid suspension lipo fectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. HEK293 cells transfected with TLR2 or TLR4 or pcDNA3.1 vector were washed with ice cold PBS and lysed with 500 μl of lysis buffer (1% NP-40, 20 mM Tris-Cl pH 7.4, 10% v/v glycerol, 150 mM NaCl, 20 mM NaF, protease inhibitor cocktail). After centrifugation at 12000 rpm for 15 minutes at 4°C, the supernatants from the cell lysates were incubated with TALON immobilized with rRv1168c at 4°C overnight on a rotating platform. The beads were washed extensively with lysis buffer and boiled in Laemmli sample buffer. The proteins were separated on 10% SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was washed and incubated with mouse anti-TLR2- or anti-TLR4 mAb followed by incubation with anti-mouse IgG-HRP conjugate (Sigma-Aldrich). The blot was developed by chemiluminescence following the manufacturer’s protocol (GE Healthcare). In a separate experiment, HEK293 cells were either left untransfected or transfected with the FLAG-tagged wild-type TLR2 or FLAG-tagged TLR2-Mut3 plasmid and after 24 hours, cell extracts were prepared and incubated with either TALON bound rRv1168c or TALON bound rRv1196. The pulled-down TLR2 was detected by Western blotting using anti-FLAG Ab.

**Mycobacterium smegmatis culture and transformation**

*M. smegmatis* mc2155 bacteria were grown in Middle brook 7H9 medium supplemented with 10% ADC (HiMedia, India), 0.5% Glycerol and 0.05% Tween 80 (7H9-ADCT). To prepare the competent cells, the culture was allowed to grow till mid log phase. The culture was centrifuged at 3000 rpm for 10 minutes and washed four times with 10% glycerol and resuspended in 1/100th of the culture volume in sterile deionized water, 100 μl aliquots of the cells were snap-frozen and stored at -80°C till further use. The Rv1168c and Rv1196 genes were cloned in pVV16 shuttle...
vector by excision of the respective genes from pRSET clones by restriction digestion. The digestion products corresponding to Rv1168c and Rv1196 were ligated into pVV16 vector. The N-terminal truncated mutant (Rv1168cΔN) was generated by cloning nucleotide sequence representing amino acids 175-346 from BAC contig Rv71(C2). The C-terminal truncated mutant (Rv1168cΔC) was generated by cloning nucleotide sequence representing amino acids 1-173 from BAC contig Rv71(C2) clone. All the clones were confirmed with restriction digestion and sequencing. The pVV16 backbone vector and different clones were transformed into the M. smegmatis mc²155. Prior to transformation, the cells were thawed on ice and 1 μg of DNA was added. The cells were incubated on ice for 10 minutes and transferred to pre-chilled 1 mm gap width cuvette. Electroporation was performed following standard procedure (51). One ml of Middle brook 7H9-ADCT was added immediately and the cells were allowed to grow at 37°C for 4 hours to allow the expression of antibiotic resistance genes. The transformants were selected on 7H9-ADCT agar plates containing 50 μg/ml kanamycin and 50 μg/ml hygromycin. Identification of the recombinant strains was performed by PCR as well as Western blotting using anti-Rv1168c/Rv1196 Ab (data not shown).

Infection of BF-24 cells

BF-24 cells were infected with M. smegmatis strains harboring either Rv1168c (M. smeg-Rv1168c) or Rv1196 (M. smeg-Rv1196) or Rv1168cΔN (M. smeg-Rv1168ΔN) or Rv1168cΔC (M. smeg-Rv1168ΔC) or the backbone vector (M. smeg-pVV16) at 10 multiplicities of infection (MOI). After 4 hours, cells were treated with gentamicin to a final concentration of 200 μg/ml to inhibit the growth of the extracellular bacteria. After 36 hours of infection, whole cell extracts were prepared and CAT expression was measured by ELISA.

Protein-protein docking studies

The N-terminal region (2-171 aa) of Rv1168c protein sequence showed nearly 35% sequence identity to B chain of PE/PPE protein complex (2G38) from M. tuberculosis when submitted to blastp. The B chain of 2G38 was selected as the template for homology modeling of Rv1168c protein N-terminal region. The sequence alignment for N-terminal Rv1168c and 2G38 chain B was carried out by Clustalw2 Software (ftp://ftp.ebi.ac.uk/pub/software/clustalw2/). The alignment was subsequently used for Homology modeling using Modeller9v1 version (http://salilab.org/modeller/) (52,53). To verify the generated Rv1168c models, the later were submitted to Structural Analysis and verification Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES/). Two of the well-known tools named PROCHECK (54) and VERIFY-3D (55) results of SAVES were considered for validating the secondary structure prediction. The model with best G-score of PROCHECK and with best VERIFY-3D profile was subjected to energy minimization. To minimize the energy of Rv1168c, the GROMOS96 43a1 force field (54) was applied with Simple Point Charge (SPC) water (56) using steepest descent algorithm. The energy minimized structure was used for the docking with human TLR2 structure. The crystal structure of TLR1-TLR2 heterodimer (Pdb-id: 2Z82) (57) was retrieved from Protein Data Bank. Before docking the missing hydrogen atoms were added to both the structures.

Docking studies were carried out using Hex 5.1 version software (58) by keeping TLR2 as the static molecule and Rv1168c protein as the mobile molecule. The docking was performed by choosing different initial orientations (by setting different α, β and γ angle values) of Rv1168c protein corresponding to TLR2. All the docking solutions that were obtained from HEX were further analyzed to identify the one which buries maximum surface area upon complex formation. We used a PSA (protein structure alignment) database (Sali and Blundell,
unpublished) to calculate the solvent accessible surface area of the molecules. HBOND program (59) was used to identify H-bonds at the molecular interface. The spdbv 3.7 version software (60) was used to identify the salt bridges and van Der Waals interactions.

**Statistical analysis**

Data were expressed as mean ± SD of three independent experiments performed with similar results. Student’s t test was used to determine statistical differences between the groups. p < 0.05 was considered to be significant.

**RESULTS**

The recombinant Rv1168c protein activates HIV-1 LTR-driven chloramphenicol acetyl transferase expression in THP-1 cells

To examine whether rRv1168c increases HIV-1 LTR transcription, human monocytic THP-1 cells were co-transfected with a chloramphenicol acetyl transferase (CAT) reporter gene driven by a full length HIV-1 LTR (HIV-1 LTR-CAT) and a β-Galactosidase reporter driven by a constitutive CMV promoter used as a control to determine the transfection efficiency. After 10 hours, cells were either left untreated or treated with various concentrations of purified recombinant Rv1168c protein (rRv1168c). Treatment of THP-1 cells with rRv1168c resulted in dose-dependent increase in the HIV-1 LTR-driven CAT expression, whereas rRv1168c had no effect on β-galactosidase expression (Fig. 1A). CAT was found to be maximally expressed when Rv1168c was used at 3 µg/ml final concentration. Therefore, all the subsequent experiments were carried out using 3 µg/ml rRv1168c protein. Since the transfection efficiency in THP-1 cells was low (about 30%, data not shown), we also used BF-24 cells (THP-1 cells containing a stably integrated HIV-1 LTR promoter sequence driving CAT reporter gene) to assess the effect of Rv1168c on HIV-1 LTR transcription (61). A dose-dependent increase in CAT expression was observed with increasing concentrations of rRv1168c in BF-24 cells also, underscoring a definitive role of Rv1168c in the activation of HIV-1 LTR promoter-driven transcription (Fig. 1B). In order to rule out possible LPS contamination in the recombinant protein preparation, the HIV-1 LTR-CAT-transfected THP-1 and BF-24 cells were also treated with 3 µg/ml of autoclaved rRv1168c as autoclaving does not have any effect on LPS activity but it denatures the protein (37). The denatured rRv1168c protein failed to increase HIV-1 LTR activity as indicated by near control levels of CAT gene expression (Fig. 1A and 1B). These observations confirm the specificity of the Rv1168c-mediated effect on the HIV-1 LTR activity. HIV-1 LTR-driven CAT gene expression was found to be increased by Rv1168c in PMA-differentiated THP-1 macrophages also (Fig. 1C) as well as in human monocyte-derived macrophages (data not shown).

Rv1168c-mediated activation of LTR is dependent on nuclear factor-kappa B (NF-κB) transcription factors

HIV-1 LTR is a well characterized transcription regulatory element (17,18,27). The LTR promoter activity is known to be critically dependent on the host transcription machineries. As NF-κB is one of the most important transcription factors responsible for HIV-1 LTR transcription (17,20-22,62,63), we first examined whether NF-κB is involved in the Rv1168c-mediated activation of HIV-1 LTR. Therefore, the THP-1 cells were transfected with HIV-1 LTR and after 10 hours post-transfection, cells were treated with 10 µM pyrrolidine dithiocarbamate (PDTC), a known inhibitor of NF-κB (64) followed by incubation with Rv1168c (0.3 µg/ml and 3 µg/ml). Rv1168c-mediated trans-activation of HIV-1 LTR was
found to be strongly inhibited by PDTC, suggesting a possible role of NF-κB in the activation of LTR by Rv1168c (Fig. 2A, compare bar 7 with bar 3 and bar 9 with bar 5).

The NF-κB transcription factor is known to be retained in cytoplasm by IκBα protein and its activity is regulated by its release from IκBα complex after phosphorylation and degradation of IκBα (65). We, therefore, co-transfected THP-1 cells with HIV-1 LTR-CAT and phosphorylation-defective IκBα (ΔIκBα) to sequester NF-κB in the cytoplasm and examined HIV-1 LTR trans-activation after treatment the cells with rRv1168c. The control group received the backbone vector (pRc/CMV) along with the HIV-1 LTR-CAT construct. After 10 hours post-transfection, all the groups were treated with 0.3 µg/ml and 3 µg/ml of rRv1168c. The CAT assay was performed 36 hours after treatment with the recombinant protein. The result shown in Fig. 2B indicates that Rv1168c-induced HIV-1 LTR activation is abrogated in the group transfected with ΔIκBα when compared with the control group that received pRc/CMV backbone vector alone (Fig. 2B, compare bar 5 with bar 2 and bar 6 with bar 3). These results further suggest an important role of NF-κB in the activation of HIV-1 LTR by Rv1168c. To further underscore the role of NF-κB in the Rv1168c-mediated activation of HIV-1 LTR, THP-1 cells were transfected with a HIV-1 LTR construct where the NF-κB binding sites were mutated (pDkB-HIV-CAT) (62) and followed by treatment with deoxy-oligonucleotide probe containing the HIV-1 LTR-specific NF-κB DNA-binding site was used for EMSA (data not shown).

Since p50 and p65 factors play important roles for the activation of HIV-1 LTR (63,66), we next examined the expression profile of the p50 and the p65 NF-κB in the nuclear extracts prepared from Rv1168c-treated THP-1 cells by Western blotting using anti-p50 and anti-p65 antibodies. Both p50 and p65 NF-κB levels were found to be significantly increased in THP-1 cells treated with rRv1168c protein (Fig. 3B). These data together with the EMSA observations suggest that Rv1168c increases the levels of nuclear NF-κB which plays a key role in the transcriptional activation of HIV-1 LTR by Rv1168c in THP-1 cells.

Interestingly, we observed that Rv1168c also increased production of TNF-α in BF-24 cells in a concentration-dependent manner (Fig. 4A). As TNF-α can activate HIV-1 LTR targeting the NF-κB (67), we next examined...
whether Rv1168c-mediated HIV-1 LTR transactivation in monocyte/macrophage cells was dependent on TNF-α cytokine induced by Rv1168c. The BF-24 cells were, therefore, treated with \{6,7-Dimethyl-3-(methyl-(2-(methyl-1-(3-trifluoromethylphenyl)-1H-indol-3-ylmethyl)-amino)-ethyl)-amino)methyl\} chromen-4-one, diHCl, a pharmacological inhibitor of TNF-α (Calbiochem, San Diego, CA, (68) that interferes with the functional trimer formation of TNF-α. The inhibitor was found to inhibit TNF-α induction in BF-24 cells treated with Rv1168c without affecting cell viability (Supplemental Fig. 1A and 1B). Interestingly, rRv1168c was found to activate HIV-1 LTR even in the presence of the specific TNF-α inhibitor (Fig. 4B). These data indicate that rRv1168c can still activate HIV-1 LTR even when TNF-α production by Rv1168c is inhibited.

\textbf{Rv1168c interacts with TLR2 to induce NF-κB-dependent activation of HIV-1 LTR}

Various studies have established that some of the PPE family proteins are localized in the cell surface (37,45,69,70). Interestingly, when we over-expressed Rv1168c in \textit{M. smegmatis} using pVV16 under the control of \textit{hsp60} promoter as described elsewhere (37,51), we found that Rv1168c was predominantly present in the insoluble cell wall fraction (Supplemental Fig. 2) but not in the culture filtrate (data not shown). It will be pertinent to mention here that \textit{M. smegmatis} is a non-pathogenic mycobacterium and its genome does not have most of the PE/PPE genes including Rv1168c (69). Again, a BLAST search of the Rv1168c sequence against the \textit{M. smegmatis} peptide database (http://blast.jcvi.org/cmr-blast/) failed to identify any significantly similar entry matching the Rv1168c protein sequence. Many PPE proteins are known to be surface localized (37,45,69,70) and since Rv1168c could be detected in the insoluble cell wall fraction (Supplemental Fig. 2), we hypothesized that Rv1168c is surface exposed on \textit{M. tuberculosis} and is therefore possibly available for interactions with some surface receptor(s) on the monocyte/macrophage cells to activate the downstream NF-κB signaling cascades that eventually drive the HIV-1 LTR transcription. To detect any interaction of Rv1168c with the surface receptors, we incubated titrating concentrations of FITC conjugated Rv1168c (Rv1168c-FITC) with THP-1 cells for 60 minutes on ice and measured the surface-bound florescence using flow cytometry. The result indicated that Rv1168c protein could strongly bind to the macrophage surface in concentration-dependent manner (Supplemental Fig. 3).

Various studies have indicated that TLR2 is the most predominant receptor recognized by the \textit{M. tuberculosis} components (71) and that the TLR2 could play an important role to modulate macrophage signaling cascades during \textit{M. tuberculosis} infection (37,72-74). TLR2-specific signaling is found to be essential in \textit{M. tuberculosis}-mediated activation of HIV-1 LTR both \textit{in vitro} and \textit{in vivo}. TLR2 deficient transgenic mice harboring HIV-1 pro-viral genome failed to transcribe genes under the control of the LTR promoter (75,76). Since the rRv1168c protein was found to bind strongly with THP-1 cells (Supplemental Fig. 3), we next investigated whether Rv1168c specifically recognizes the TLR2 and targets the TLR2-induced signaling to activate HIV-1 LTR. To assess the specificity of interactions with TLR2 receptors, we used HEK293 cells which do not express TLRs and therefore can be used to identify ligands for TLRs by over-expressing particular TLRs in these cells. So, the HEK293 cells were transiently transfected with either backbone vector pCDNA3.1 (used as control) or full-length TLR2 or full-length TLR4 plasmid construct. Surface expressions of the receptors were confirmed by flow cytometry (Supplemental Fig. 4) after 24 hours of transfection. Then we carried out pull-down assay using whole cell extracts prepared from these HEK293 cells transfected with either pCDNA3.1 or TLR2 or TLR4 over-expression plasmid. Total cellular extracts were incubated with rRv1168c immobilized on TALON beads. When the Western blots were probed with anti-
TLR2 or anti-TLR4 mAb, only TLR2 was detectable in the eluate (Fig. 5A). No bands were visible in the control group transfected with pcDNA3.1 or TLR4 or in the group containing only beads (Fig. 5A). These observations suggest that Rv1168c interacts specifically with the TLR2 receptors. Again, anti-TLR2 mAb but not anti-TLR4 mAb or isotype-matched (IgG2a) control Ab was able to inhibit binding of Rv1168c on THP-1 macrophages (Fig. 5B). These observations further confirmed that Rv1168c specifically interacts with the TLR2 receptors.

To investigate whether the interaction of Rv1168c with TLR2 is necessary for increased binding of NF-κB to LTR DNA and thereby resulting in up-regulation of LTR promoter activity, we next treated BF-24 cells with a TLR2 neutralizing mAb to block binding of Rv1168c with TLR2 and measured both the NF-κB DNA-binding activity by EMSA and HIV-1 LTR activity by estimating CAT expression levels by ELISA. The results shown in Figs. 5C and 5D indicate that blocking the binding of Rv1168c with TLR2 by pre-treating cells with anti-TLR2 mAb results in poorer NF-κB DNA-binding activity (Fig. 5C, compare lane 5 with lane 3) with concomitant inhibition of transcription of the CAT reporter gene from the LTR promoter (Fig. 5D, compare bar 3 with bar 2). To further corroborate the role of TLR2 in the activation of HIV-1 LTR by rRv1168c, silencing of the TLR2 was carried out by using TLR2-specific siRNAs and NF-κB activity and HIV-1 LTR promoter activation was measured in these cells. The BF-24 cells were transfected either with the negative control scrambled siRNA or with TLR2-specific siRNA and at 24 hours post-transfection, the cells were treated with rRv1168c for another 36 hours. Depletion of surface TLR2 expression by siRNA was confirmed by flow cytometry (Supplemental Fig. 5). Consistent with our previous observations with neutralizing antibody (Fig. 5C and 5D), we found that silencing of TLR2 expression on BF-24 cell surface resulted in strong diminishment of nuclear NF-κB activity when treated with rRv1168c (Fig. 5E, lane 5). However, in the negative control siRNA-transfected BF-24 cells significant amount of DNA-binding activity was detected (Fig. 5E, lane 3). Expectedly, the levels of NF-κB DNA-binding activities were well correlated with the LTR-driven CAT gene expression where the CAT expression level in the BF-24 cells transfected with TLR2-specific siRNA were almost reduced to the control levels (Fig. 5F, compare bar 9 with bars 1, 4 and 7). On the other hand, BF-24 cells with negative control siRNA had no significant deviation in the CAT expression levels when stimulated with rRv1168c (Fig. 5F, compare bar 6 with bar 3). These results suggest that Rv1168c mainly targets the TLR2 to induce NF-κB-dependent activation of HIV-1 LTR in BF-24 cells.

We have reported earlier that another PPE protein of M. tuberculosis, Rv1196 (known as PPE18) is surface exposed and predominantly binds to TLR2 and modulates macrophage signaling cascades (37). Interestingly, Rv1196 had no effect on HIV-1 LTR trans-activation (Fig. 6) which we found is likely due to its inherent ability to inhibit NF-κB activation (77).

Rv1168c activates HIV-1 LTR and requires the TLR2-signaling when presented as part of the whole mycobacterium

To check whether Rv1168c could also increase LTR activity when presented in the context of whole bacillus, we infected BF-24 cells with M. smegmatis over-expressing Rv1168c (M. smeg-Rv1168c) and HIV-1 LTR activity was measured at 36 hours post-infection. The control group was infected with M. smegmatis harboring the backbone vector alone (M. smeg-pVV16). Infection of BF-24 cells with M. smeg-Rv1168c as compared to the M. smeg-pVV16 resulted in significant enhancement of LTR-driven CAT expression (Fig. 7A, compare bar 3 with bar 1). In the earlier section, we observed that the recombinant Rv1196 protein was ineffective to trans-activate HIV-1 LTR (Fig. 6). It also failed
to increase the LTR activity when presented in the context of the whole bacillus corroborating the in vitro observed data using purified protein (Fig. 7A). Thus, Rv1168c but not Rv1196 when presented in the context of a heterologous M. smegmatis, can activate HIV-1 LTR transcription. Also, when Rv1168c was presented in the context of whole bacterium (M. smeg-Rv1168c), TLR2 receptor is found to be required to activate NF-κB (Fig. 7B) and HIV-1 LTR (Fig. 7C). Formation of NF-κB-DNA complex was abrogated when TLR2 expression in BF-24 cells was suppressed by using TLR2-specific siRNA (Fig. 7B, compare lane 6 with lane 4). Consequently, the M. smeg-Rv1168c-induced CAT expression level in the TLR2-siRNA transfected group was significantly reduced when compared to that of the group transfected with negative control siRNA or treated with medium alone (Fig. 7C, compare bar 9 with bar 6 and bar 3). These results cumulatively indicate that Rv1168c is capable to interact with TLR2 when presented in the context of the whole mycobacterium. The protein is also probably surface exposed and its interaction triggers the downstream NF-κB signaling events that drive increased transcription from the HIV-1 LTR promoter. On the other hand, another PPE protein Rv1196 is although found to be surface exposed and interacts with TLR2 (37), fails to activate HIV-1 LTR.

**Rv1168c specifically interacts with LRR 15~20 domain of TLR2**

In the present study, we have observed that although Rv1168c interacts with TLR2, in a way similar to Rv1196 (37), it triggers predominantly a pro-inflammatory-type signaling with increased NF-κB activity and TNF-α cytokine induction unlike Rv1196 which was found to inhibit pro-inflammatory cytokine production by interacting with the LRR 11~15 region (37). Therefore, we speculated that sites of interaction of these two proteins are different owing to their divergent cytokine response in THP-1 cells. A competition assay using recombinant Rv1196 protein revealed no significant inhibition of binding of Rv1168c protein on THP-1 cells (Fig. 8A) suggesting that probable sites of interaction of Rv1168c and Rv1196 are spatially separated. Also, in a pull-down assay carried out using mutant TLR2 (mut3) where LRR-11~15 is deleted, (Fig. 8B), Rv1168c showed interaction. These results suggest that Rv1168c binds to the TLR2 domain in a region other than LRR 11~15 to elicit a pro-inflammatory-type signaling.

We next generated Rv1168c protein either with deletions in the N-terminal region (Rv1168cΔN; with an intact C-terminal fragment containing 175-346 aa residues) or with deletions in the C-terminal region (Rv1168cΔC; with an intact N-terminal fragment containing 1-173 aa residues) to determine the domain of Rv1168c important for enhancement of LTR-driven transcription. We observed that the Rv1168cΔN did not bind to the THP-1 cells, whereas the C-terminal truncated Rv1168c protein (Rv1168cΔC) with an intact N-terminal domain could bind to these cells (Fig. 8C).

We used computational docking studies (as described in the materials and methods section) to understand how Rv1168c interacts with TLR2. Out of approximately 30 docking solutions obtained, the one with a maximum buried solvent accessible surface area was identified. In the top ranking docking score for the TLR2-Rv1168c complexes, Rv1168c was predicted to interact with the 15~20 LRR region of TLR2 (Fig. 8D). In order to confirm whether Rv1168c indeed binds to the TLR2-LRR 15~20 domain, HEK293 cells were transfected with wild-type or TLR2-Mut3 lacking LRR 11~15 domain or TLR2-Mut4 lacking LRR 15~20 domain (78) and binding of Rv1168c with these TLR2 mutants were compared by flow cytometry. The flow cytometry data indicate that Rv1168c was unable to bind to the HEK293 cells expressing the TLR2-Mut4 that specifically lack LRR 15~20 domain (Supplemental Fig. 6) whereas it could interact with HEK293 cells expressing either the full-length TLR2 or the
TLR2-Mut3 as also indicated earlier in our pull-down assay experiment in Fig. 8B. Therefore, it appears that TLR2 LRR 15-20 domain is important in triggering the downstream signaling events leading to activation of NF-κB.

Deletion of the N-terminal domain of Rv1168c failed to trans-activate HIV-1 LTR promoter in BF-24 cells when infected with M. smegmatis over-expressing the deletion mutant

We found that purified truncated Rv1168c protein with an intact N-terminal domain containing 1-173 aa residues (rRv1168cΔC) was able to sufficiently activate HIV-1 LTR driven transcription to the extent similar to that of the purified full-length protein (Fig. 9, bar 4 and bar 5). In line with our earlier observations, activation of HIV-1 LTR by rRv1168ΔC was strongly impaired when TLR2 expression in BF-24 cells was suppressed by using TLR2-specific siRNA (Fig. 9, compare bar 9 with bar 5). These experiments clearly indicate that the N-terminal domain of Rv1168c is essential for activation of HIV-1 LTR in BF-24 cells and this requires TLR2. This suggests that the region encompassing amino acids from 1-173 is crucial to activate the TLR2-triggered pro-inflammatory signaling and HIV-1 LTR trans-activation. The full length protein when presented in the context of the whole mycobacterium in M. smegmatis (M. smeg-Rv1168ΔN) and infected the BF-24 cells. We found that the sole C-terminal domain of Rv1168c when presented in the context of the whole bacteria failed to significantly activate NF-κB as compared to full length Rv1168c or Rv1168ΔC (Fig. 10A, compare lane 5 with lanes 4 and 6). These observations were also well correlated with the HIV-1 LTR-driven expression of CAT gene (Fig. 10B, compare bar 4 with bars 3 and 5) as well as in its ability to stimulate TNF-α production (Fig. 10C, compare bar 4 with bars 3 and 5). The N-terminal region of Rv1168c was found to be essential for activation of HIV-1 LTR also in PMA-differentiated BF-24 macrophages (Fig. 10D). These data indicate that the N-terminal region of Rv1168c is the functionally active domain and is required for elicitation of the pro-inflammatory signaling pathway and HIV-1 LTR trans-activation.

DISCUSSION

Cells of the monocyte/macrophage lineage are known to play an important role in the transmission and pathogenesis of HIV (79-82). Interestingly, HIV-1 replication was found to be increased in the lung regions infected with M. tuberculosis compared to regions infected with HIV-1 alone (83). Tuberculosis may develop in an HIV patient as a result of exposure to the mycobacteria or reactivation of latent TB due to decrease in immunity (84-86). In AIDS patients, once TB is established HIV-1 replication is enhanced (13-16) because of the indirect effects of host’s pro-inflammatory immune response against M. tuberculosis infection or due to the direct effects of mycobacterial components that modulate the signal transduction cascades of the macrophages (13,14,23). Interestingly, in many cases neutralizing antibodies against various pro-inflammatory cytokines did not abrogate HIV-1 transcription induced by M. tuberculosis (15,25,26). We also found that Rv1168c-mediated activation of HIV-1 LTR was not affected even when TNF-α production by
Rv1168c was inhibited using a pharmacological inhibitor suggesting that mycobacterial protein(s) can directly activate HIV-1 transcription by modulating macrophage innate-signaling cascades.

Recognition of pathogen associated molecular patterns by innate immune receptors like the TLR(s) could be an important event in the modulation of macrophage innate-signaling during mycobacterial infection (36,37,71). Importantly, the TLR2 is shown to interact with a number of mycobacterial components and modulates macrophage innate-signaling cascades (36,37,71). The TLR2 receptors are thought to play critical roles to enhance LTR-directed transcription and HIV-1 expression by the mycobacteria (75,76). Although several PPE proteins are found to be over-expressed during infection (37,45,69,70,87) and are shown to modulate macrophage signaling cascades (36,37,71), it is not clear whether such modulation in the macrophage innate-signaling cascades eventually can affect HIV-1 replication. The novelty of the present study is that we demonstrate direct role of a PPE protein Rv1168c inactivating HIV-1 LTR transcription in monocyte/macrophage cells that target the TLR2 receptor. We found that treatment of BF-24 (THP-1 cells stably expressing a HIV-1 LTR-CAT construct) with the anti-TLR2 Ab or depletion of TLR2 in BF-24 cells using TLR2-specific siRNA resulted in significant decrease in HIV-1 LTR trans-activation by rRv1168c. Interestingly, Rv1168c is found to be present in the insoluble cell wall fraction when over-expressed in M. smegmatis and infection with this strain (M. smeg-Rv1168c) significantly increased HIV-1 LTR activity in BF-24 cells indicating that Rv1168c can activate HIV-1 LTR when presented as part of the whole mycobacterium. This activation of the viral promoter was found to be independent of the Tat transactivator protein. That is known to be a viral protein necessary for the transcription initiation at HIV-1 LTR. Our results indicate a possible mechanism of mycobacterial protein(s)-induced activation of HIV-1 LTR at the initial stages of virus infection when Tat protein is unavailable or present in minute quantities.

Various groups have reported that the NF-κB transcription factors are activated downstream of the TLR2-induced signaling (36,88). The NF-κB factors interact with HIV-1 LTR DNA-binding sites and mediate LTR trans-activation (17,63). Binding of NF-κB transcription factors to HIV-1 LTR promoter is shown to be necessary for increased LTR activation and viral replication in monocytes/macrophages (89-91). In this study, we demonstrated that induction of HIV-1-LTR-driven transcription in THP-1 cells by Rv1168c was associated with the induction of NF-κB DNA-binding activity downstream of the TLR2 as inhibition of this signaling pathway by either treating cells with PDTC or over-expressing phosphorylation defective IκBα decreases transcription from the HIV-1 LTR promoter by more than 90%. A similar reduction of the Rv1168c-induced HIV-1 LTR trans-activation was observed in THP-1 cells transiently transfected with the HIV-1 LTR construct bearing mutations in the NF-κB binding sites. Pre-treatment of BF-24 cells with anti-TLR2 mAb or silencing of the TLR2 by TLR2-specific siRNA prevented rRv1168c-mediated activation of NF-κB in these cells which eventually leads to decreased HIV-1 LTR trans-activation. These results clearly indicate that the PPE protein, Rv1168c predominantly targets the innate TLR2-NF-κB signaling pathway to enhance LTR-mediated transcription. Deletion studies indicate that the N-terminal domain of rRv1168c (rRv1168ΔC) is the functional domain that specifically targets the TLR2-signaling pathway to activate HIV-1 LTR transcription in a similar way as with the full-length rRv1168c protein.

We have recently shown that the PPE protein, Rv1196 interacts with TLR2 and modulates innate-effector signaling in macrophages (37). However, in this study, we did not find enhancement of HIV-LTR transcription by Rv1196 presumably because of its inability to activate NF-κB signaling pathway (77).
contrast, interaction of Rv1168c with the TLR2 resulted in stronger activation of the NF-κB factors leading to an increased transcription from HIV-1 LTR. Furthermore, heterologous expression of Rv1168c in M. smegmatis induced HIV-1 LTR-driven CAT expression in the BF-24 cells while Rv1196 expressed in M. smegmatis failed to do so. This activation of NF-κB and HIV-1 LTR by Rv1168c when presented in the context of the whole bacterium (M. smeg-Rv1168c) to the BF-24 cells was also found to be TLR2-dependent as observed using recombinant Rv1168c because silencing of TLR2 gene expression by specific siRNA prevented M. smeg-Rv1168c-mediated activation of NF-κB and HIV-1 LTR in BF-24 cells.

When the N-terminal region of Rv1168c is deleted (with the intact C-terminal fragment containing 175-346 aa residues) and presented to BF-24 cells in the context of whole mycobacterium using M. smegmatis (M. smeg-Rv1168ΔN), there was no significant increase in the DNA-binding activity of NF-κB as compared to the control group that was infected with M. smegmatis harboring the vector alone. However, the C-terminal truncated protein of Rv1168 (with the intact N-terminus region containing 1-173 aa residues) when presented to BF-24 cells in the context of the whole bacterium using M. smegmatis (M. smeg-Rv1168ΔC) could trigger a strong NF-κB signaling similar to that of the full-length Rv1168c over-expressed in M. smegmatis (M. smeg-Rv1168c). Similarly, in BF-24 cells infected with M. smeg-Rv1168ΔN had very negligible increase in HIV-1 LTR transcription and TNF-α production. In contrast, infection of BF-24 cells with M. smeg-Rv1168ΔC resulted in stronger HIV-1 LTR activity and higher TNF-α production similar to those observed in BF-24 cells infected with M. smegmatis harboring full-length Rv1168c. The N-terminal region of Rv1168c was found to be essential for activation of HIV-1 LTR also in PMA-differentiated macrophages. These results clearly indicate that the N-terminal but not the C-terminal domain of Rv1168c is required for activation of NF-κB signaling and increased HIV-1 LTR transactivation in the context of the bacterial membrane.

The disparate activities of the Rv1168c and the Rv1196 protein could be due to their ability to recognize different stretches of the TLR2 ectodomain, resulting in differential modulation of post-receptor binding events that leads to activation of NF-κB transcription factors The Rv1196 protein is found to specifically interact with the LRR 11~15 domain of TLR2 (37) and triggers an anti-inflammatory signaling, while binding of Rv1168c with the TLR2 15~20 domain results in induction of pro-inflammatory signaling leading to activation of NF-κB and increased TNF-α production. It is possible that interaction of mycobacterial proteins at different sites on the TLR2-ectomain (LRR motifs) induces different structural plasticity which conveys disparate signaling cues downstream the cytoplasmic stem of the TLR2 receptor resulting in differential NF-κB activation. Although a link between the TLR2 signaling and enhanced LTR-driven transcription has been indicated earlier (75,76), the detailed mechanism by which this signaling can influence the LTR activity has not been addressed. Our data hint on the possible mechanisms on how the TLR2-LRR domain can influence HIV-1 LTR transcription in monocyte/macrophages by regulating the NF-κB signaling cascades positively or negatively and some of the mycobacterial PPE proteins can influence the LTR-driven transcription by modulating TLR2-NF-κB signaling. Although, we demonstrated a role of Rv1168c in transactivating HIV-1 LTR promoter through activation of NF-κB signaling cascades, however, regulation of this promoter by other signal-activated transcription factors cannot be ruled out. The TLR2-LRR specific pro-inflammatory signaling during opportunistic infections may influence HIV-1 LTR transactivation in a similar fashion (90). Though in the present study, we used THP-1 cell line as a model for cells of monocytes/macrophage lineage; our data indicate that site-specific interaction of mycobacterial protein(s) with the TLR2 ecto domain is crucial to dictate the
downstream signaling events that eventually lead to HIV-1 LTR hyper-activation.

Interestingly, only a small fraction (about 5%) of the total monocytes that are positive for CD16 (CD14+CD16+) are susceptible to HIV-1 infection and preferentially harbor the virus for long term (92). These CD14+CD16+ monocytes are also known to be more pro-inflammatory and play a greater role in infections than majority of the classical CD14+CD16− monocytes which may also get infected by HIV (93). In patients with HIV-1 infection, the CD14+16− monocyte population may increase to as high as 40% of the total circulating monocytes (94). However, it is unclear whether this expansion is due to the increased production of CD14+CD16+ monocytes in the bone marrow or due to the differentiation of CD14+CD16− to CD14+CD16+ monocytes (93). On the other hand, during M. tuberculosis infection, the peripheral CD14+CD16+ monocyte population can also be significantly expanded and in the tuberculous pleural fluid, the CD14+CD16− subset appears to be the main monocytes/macrophage population. Moreover, expression of CD16 in CD14+CD16− monocytes can be triggered by soluble factors found in this inflammatory milieu. (95).

Therefore, it may be possible that upon infection with M. tuberculosis, the already enriched HIV infected CD14+CD16+ monocytes probably expands even more and get recruited to the pleural region where they are exposed to M. tuberculosis antigens present in PPD (15), ManLAM (17) or Rv1168c (present study). These M. tuberculosis components activate the HIV-1 LTR through the production of TNF-α (14, 67) or even directly independent of TNF-α production e.g. Rv1168c. Although, the CD14+CD16+ monocytes/macrophages are infected at about 5 times less efficiently with M. tuberculosis than the CD14+CD16− monocytes/macrophages, both these subsets can produce significant amount of TNF-α upon M. tuberculosis infection (96). True to this proposition, we found that Rv1168c can stimulate production of significant amount of TNF-α in BF-24 cells and can also activate the HIV-1 LTR independent of TNF-α production. Since viral replication and multiplication are critically dependent on the LTR promoter transcription, it is likely that Rv1168c may play an augmenting role in trans-activating HIV-1 LTR promoter in M. tuberculosis/HIV-1 co-infected individuals.

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REFERENCES


availability on pathogenicity and gene expression in *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* **84**, 205-217


monocyte subset is more permissive to infection and preferentially harbors HIV-1 in vivo. J. Immunol. 178, 6581-6589


Footnotes

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2 The abbreviations used are

TB tuberculosis
CAT chloramphenicol acetyl transferase
LPS lipopolysaccharide
siRNA small inhibitory RNAs
NF-κB nuclear factor-kappaB
TLR Toll-like receptor
LTR long terminal repeat
TNF tumor necrosis factor-α
IL interleukin
LRR leucine rich repeats
PDTC Pyrrolidinedithiocarbamate
EIA Enzyme Immuno Assay
Figure legends

FIGURE 1. The rRv1168c protein activates HIV-1 LTR in THP-1 cells.

A. THP-1 cells were co-transfected with HIV-1 LTR-CAT and β-galactosidase (used as internal control of transfection efficiency) constructs and after 10 hours of transfection, cells were either left untreated or treated with different concentrations (0.5, 1.0, 3.0 and 5.0 μg/ml) of rRv1168c. After 36 hours, whole cell extracts were prepared and the CAT and the β-galactosidase activity in the cell extracts was measured by ELISA using the CAT and the β-galactosidase reporter assay kits respectively. B. BF-24 cells containing the stably integrated copy of HIV-1 LTR-CAT was treated with different concentrations (0.5, 1.0, 3.0 and 5.0 μg/ml) of recombinant Rv1168c protein. Whole cell extracts were prepared after 36 hours of treatment and ELISA was performed to measure the levels of CAT gene expression. C. Rv1168c increased CAT activity in PMA-differentiated BF-24 macrophages in dose-dependent manner. The results are shown as the mean ± SD of three independent experiments.

FIGURE 2. Activation of HIV-1 LTR by rRv1168c is mediated through NF-κB.

A. THP-1 cells were transfected with HIV-1 LTR-CAT and β-galactosidase (used as internal control of transfection efficiency) constructs. After 10 hours, cells were either left untreated or pre-treated with 10 μM PDTC for 1 hour and then treated with 0.3 and 3.0 μg/ml of rRv1168c. CAT and β-Gal expression levels were determined by ELISA after 36 hours of Rv1168c treatment. B. THP-1 cells were co-transfected with HIV-1 LTR-CAT and IκBα phosphorylation defective mutant (ΔIκBα) constructs. The control group was co-transfected with HIV-1 LTR-CAT and the backbone vector (pRc/CMV). Cells were then treated with 0.3 and 3.0 μg/ml of Rv1168c, 10 hours after transfection and after 36 hours whole cell extracts were prepared and the CAT and the β-Gal expression was measured by ELISA. C. THP-1 cells were transfected either with wild-type HIV-1 LTR-CAT construct or with pDKB-HIV-CAT construct (the clone carrying mutation within NF-κB binding sites of HIV-1 LTR-CAT). Next the cells were treated with 0.3 and 3 μg/ml of Rv1168c and CAT ELISA was performed after 36 hours of transfection. The results are shown as the mean ± SD of three independent experiments.

FIGURE 3. The rRv1168c protein activates NF-κB induction in THP-1 cells.

A. The THP-1 cells were treated with various concentrations of rRv1168c protein for 1 hour. Cells were harvested and nuclear extracts were prepared. The DNA-binding activity of NF-κB complex was measured by EMSA. B. Nuclear extracts prepared from Rv1168c-treated THP-1 cells were used to measure the levels of p50 and p65 NF-κB by Western blotting using anti-p50 and anti-p65 Ab respectively. Ponceau S staining was performed to check equal protein loading. The results are representative of three independent experiments.

FIGURE 4. The rRv1168c protein-mediated activation of HIV-1 LTR in BF-24 cells is independent of TNF-α.

A. BF-24 cells were treated with different concentration of Rv1168c and TNF-α secreted in the medium was measured by EIA. LPS (3 μg/ml) was used as positive control of TNF-α induction. B. BF-24 cells
were treated with 0.3 and 3 µg/ml rRv1168c protein in the absence or presence of 5 µM of the TNF-α inhibitor (6, 7-Dimethyl-3-((methyl-(2-(methyl(1-(3-trifluoromethyl-phenyl)-1H-indol-3-ylmethyl)-amino)-ethyl)-amino)-methyl)-chroomen-4-one, diHCl) dissolved in DMSO. The CAT expression was measured by ELISA. The results shown are mean ± SD of three independent experiments.

FIGURE 5. TLR2 plays an important role in the Rv1168c-mediated activation of NF-κB and HIV-1 LTR in BF-24 cells.

A. Lysates from HEK293 cells transfected with either the TLR2 over-expression plasmid or the TLR4 over-expression plasmid or the backbone vector (pcDNA3.1) were incubated with Rv1168c immobilized with TALON resin. The bound protein was eluted and loaded onto a denaturing 10% SDS-PAGE gel and immune blotted with anti-TLR2 Ab or anti-TLR4 Ab. The membrane was then incubated with antibody IgG-HRP conjugate and the blot was visualized by chemiluminesscence.

B. THP-1 cells were treated with 10 µg/ml of either anti-TLR2 monoclonal Ab or anti-TLR4 monoclonal Ab or IgG2a isotype control Ab and treated with biotin labeled Rv1168c (3 µg/ml) followed by incubation with streptavidin-FITC. The binding of Rv1168c was measured using FACS Vantage flow cytometer.

C. In another experiment, BF-24 cells were either left untreated or pre-treated with 10 µg/ml of either anti-TLR2 monoclonal Ab or IgG2a isotype control Ab for 1 hour and then incubated with 3 µg/ml of Rv1168c. Cells were harvested either after 1 hour to measure NF-κB induction in the nuclear extracts of various groups by EMSA or D. after 36 hours to measure CAT gene expression in the whole cell extracts by ELISA. The results are shown as the mean ± SD of three independent experiments.

F. In another experiment, BF-24 cells were treated with medium or transfected with either TLR2-specific siRNA or negative control scrambled plasmid and after 24 hours of transfection, the cells were either left untreated or treated with 3 µg/ml of Rv1168c. Protein extracts were prepared 1 hour post-treatment and EMSA was performed. Results shown are representative of three independent experiments.

FIGURE 6. Rv1196 does not activate HIV-1 LTR in BF-24 cells.

BF-24 cells were either left untreated or treated with various concentrations of native recombinant Rv1196 (0.3, 3 and 5 µg/ml) or autoclaved recombinant Rv1196 (3 µg/ml) protein. Bacterial LPS (5 µg/ml) was used as positive control. After 36 hours, cell extracts were prepared and CAT expression was measured by ELISA. The results are shown as the mean ± SD of at least three independent experiments.

FIGURE 7. Infection of BF-24 cells with M. smegmatis strains over-expressing Rv1168c resulted in increased HIV-1 LTR transcription and this involves the TLR2-triggered signaling.

A. BF-24 cells were infected with either M. smegmatis bacteria over-expressing Rv1168c (M. smeg-Rv1168) or M. smegmatis strains over-expressing Rv1196 (M. smeg-Rv1196) or M. smegmatis harboring the backbone vector (M. smeg-pVV16) at 1:10 MOIs. After 36 hours of infection, whole cell extracts were prepared and CAT expression was measured by ELISA. The results are shown as mean ± SD of three independent experiments.

B. In a separate experiment, BF-24 cells were transfected with either siRNA targeting the TLR2 or negative control scrambled plasmid and after 24 hours of transfection, the cells
were either left uninfected or infected with either M. smeg-pVV16 or M. smeg-Rv1168c. Cells were harvested either after 4 hours to measure the NF-κB-DNA binding activity by EMSA or C. cultured for another 36 hours and CAT expression in the whole cell extracts were measured by ELISA. The results are shown as mean ± SD of at least three independent experiments.

**FIGURE 8. Rv1168c interacts with 15~20 LRR region of TLR2, a region different from Rv1196.**

A. THP-1 cells were pre-treated with 5 or 15 fold excess concentrations of Rv1196, washed and then treated with biotinylated Rv1168c protein followed by incubation with streptavidin-FITC. The binding of Rv1268c was measured using FACS Vantage flow cytometer. B. HEK293 cells were either left untransfected or transfected with WT-TLR2 or TLR2-Mut3 plasmid and the cell extracts were incubated with either TALON bound rRv1168c (upper panel) or TALON bound rRv1196 (middle panel). The pulled-down TLR2 was detected by Western blotting using anti-FLAG Ab. As input controls, the levels of TLR2 in the cell extracts from WT-TLR2 and TLR2-Mut3 transfected macrophages were determined by immune blotting with anti-FLAG Ab (lower panel). The results are representative of three independent experiments. C. THP-1 cells were either left untreated or incubated with 10 µg/ml of either Rv1168AN-FITC or Rv1168AC-FITC for 1 hour on ice and the fluorescence was measured by flow cytometry. D. Molecular modelling of the interaction between the LRR domains of TLR2 (green) and the Rv1168c (57) by using random docking method of HEX-5.1v software. Most of the top ranking complexes showed that the N-terminal region of the Rv1168c protein interacts with the 15~20 LRR domain of TLR2 as shown in the circle. The two models depict two different orientations (top, front view) of the TLR2-Rv1168c complex, 90° with respect to each other.

**FIGURE 9. The N-terminal domain of Rv1168c is essential for activation of HIV-1 LTR in BF-24 cells and this requires TLR2.**

BF-24 cells were transfected with either TLR2-specific siRNA or negative control scrambled plasmid and after 24 hours of transfection, cells were treated with 0.3 µg/ml and 3.0 µg/ml of either full-length rRv1168c or rRv1168cΔC protein for 36 hours. Cells were then harvested and whole cell extracts were prepared to measure CAT expression by ELISA. Results are mean ± SD of at least three independent experiments.

**FIGURE 10. M. smegmatis bacteria harboring the N-terminal domain deletion mutant of Rv1168c (M. smeg-Rv1168cΔN) fails to trigger NF-κB activity and HIV-1 LTR-driven CAT gene transcription in BF-24 cells.**

BF-24 cells were either left uninfected or infected with M. smeg-pVV16 or M. smeg-Rv1168c or M. smeg-Rv1168cΔN or M. smeg-Rv1168cΔCat MOIs of 1:10. A. Cells were either harvested after 4 hours to measure the NF-κB activity by EMSA or cultured for another 36 hours either B. to measure CAT expression in the whole cell extracts by ELISA or C. to estimate TNF-α cytokine secreted in the culture supernatants by sandwich EIA. PMA-differentiated BF-24 macrophages were either left uninfected or infected with M. smeg-pVV16 or M. smeg-Rv1168c or M. smeg-Rv1168cΔN or M. smeg-Rv1168cΔC. and CAT expression was measured by ELISA after 36 hours. Results are mean ± SD of at least three independent experiments.
Figure 1
Figure 2

Panel A: Graph showing CAT pg/mg Lysate with different treatments.

Panel B: Graph showing CAT pg/mg Lysate with different treatments.

Panel C: Graph showing CAT pg/mg Lysate with different treatments.

Legend:
- CAT
- β-Gal
- rRv1168c (μg/ml)
- Medium
- 0.3
- 3.0
- pRc/CMV
- ΔIκBα
- pDkB-HIV-CATHIV-CAT

p < 0.001
Figure 3

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NF-κB complex

B

rRv1168c (μg/ml)

Medium

0.3 1 3

→ p65 NF-κB

→ p50 NF-κB

→ Loading control

→ Loading control
Figure 4
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
The PPE protein Rv1168c of Mycobacterium tuberculosis augments transcription from HIV-1 LTR promoter
Khalid Hussain Bhat, Chinta Krishna Chaytanya, Nazia Parveen, Raja Varman, Sudip Ghosh and Sangita Mukhopadhyay

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